Nanotechnology in the Life Sciences

Hamed Barabadi
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Pharmaceutical Nanobiotechnology for Targeted Therapy

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Nano and biotechnology are two of the 21st century's most promising technologies. Nanotechnology is demarcated as the design, development, and application of materials and devices whose least functional make up is on a nanometer scale (1 to 100 nm). Meanwhile, biotechnology deals with metabolic and other physiological developments of biological subjects including microorganisms. These microbial processes have opened up new opportunities to explore novel applications, for example, the biosynthesis of metal nanomaterials, with the implication that these two technologies (i.e., thus nanobiotechnology) can play a vital role in developing and executing many valuable tools in the study of life. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale, to investigating whether we can directly control matters on/in the atomic scale level. This idea entails its application to diverse fields of science such as plant biology, organic chemistry, agriculture, the food industry, and more.

Nanobiotechnology offers a wide range of uses in medicine, agriculture, and the environment. Many diseases that do not have cures today may be cured by nanotechnology in the future. Use of nanotechnology in medical therapeutics needs adequate evaluation of its risk and safety factors. Scientists who are against the use of nanotechnology also agree that advancement in nanotechnology should continue because this field promises great benefits, but testing should be carried out to ensure its safety in people. It is possible that nanomedicine in the future will play a crucial role in the treatment of human and plant diseases, and also in the enhancement of normal human physiology and plant systems, respectively. If everything proceeds as expected, nanobiotechnology will, one day, become an inevitable part of our everyday life and will help save many lives.

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Pharmaceutical Nanobiotechnology for Targeted Therapy

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Preface

The field of nanotechnology for targeted therapy initiated more than a decade ago has grown fast, and interest in it is increasing. Given the importance of the field for targeted drug and gene delivery systems, there are a large number of laboratory investigations today researching nanobiomaterials for diagnostic and therapeutic applications. Because of the ability of scientists to load nanoparticles with any agent, interest continues to grow, and technology in this arena is rapidly evolving. These emerging nanobiomaterials-based medicines can overcome the disadvantages of traditional medicines by target-oriented and site-specific delivery of precise medicines (immunotherapeutic agents, chemotherapeutic agents, diagnostic agents, and so on).

Pharmaceutical Nanobiotechnology for Targeted Therapy presents an updated overview of recent advancements in the field of pharmaceutical nanobiotechnology and nano-based drug and gene delivery systems. This comprehensive knowledge will allow researchers to discover innovative nanobiomaterials for targeted therapeutics. The chapters deal with various emerging nanobiomaterials for targeted therapeutic delivery systems and the writing is in a style that is easily disseminated and in a manner that can be readily adopted as sources for new and further studies.

This book should be useful for researchers and professionals from academia and industry working in the field of nanotechnology and nanobiotechnology, as well as in the field of pharmaceutical nanotechnology. It should also be useful to those interested in a range of disciplines from material science, chemistry, molecular biology, polymer chemistry, and many more interdisciplinary areas.

Tehran, Iran — Hamed Barabadi
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About the Editors

Hamed Barabadi, PharmD, PhD works as an assistant professor in the Department of Pharmaceutical Biotechnology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. He received a PhD degree from Shahid Beheshti University of Medical Sciences, Tehran, Iran, in 2019. He graduated as a Doctor of Pharmacy (PharmD) from Mazandaran University of Medical Sciences, Sari, Iran, in 2014. He has to his credit a number of research papers and book chapters. He has received many awards, such as IET—Nanobiotechnology Premium Awards two times continuously in the years 2019 and 2020. Dr. Barabadi has been featured on the World's Top 2% scientists list, according to a Stanford University study 2020. He is the guest editor/editor for various reputed indexed journals such as *Current Nanomedicine, Nanoscience and Nanotechnology-Asia*, *Frontiers in Pharmacology*, *MDPI International Journal of Molecular Sciences*, and a few other prestigious journals. His research interests lie in the area of pharmaceutical nanobiotechnology, ranging from green synthesis, characterization, and optimization of nanobiomaterials to their pharmaceutical potential evaluations, such as anticancer, antimicrobial, and antioxidant. Moreover, he has collaborated actively with researchers in several other disciplines of pharmaceutical sciences, particularly the nanoformulation of drugs for drug delivery systems and nanomedicine.

Ebrahim Mostafavi is currently at Stanford Cardiovascular Institute, Stanford School of Medicine. His research interests revolve around the use of cardiac iPS cells to design and develop in vitro models (organoids, 3D bioprinted constructs, and vascular grafts) for cardiovascular disease modeling and drug screening, as well as CRISPR/Cas gene-editing for cardiovascular diseases. During the course of his PhD, Dr. Mostafavi received training at both Northeastern University and Harvard Medical School on engineering and development of (nano)biomaterials, nanocarriers, and 3D in vitro models (hydrogels, 3D bioprinted constructs, and nanoporous scaffolds) to create biologically complex tissues and organs for tissue engineering, regenerative/translational medicine, cancer therapy, biosensing, and infectious diseases. He also obtained both master's and bachelor's degrees in nanoscience and materials science and engineering, respectively. Dr. Mostafavi currently serves as associate editor-in-chief of the *International Journal of Nanomedicine* (Q1, IF=7.033) at Dove Medical Press/Taylor & Francis, and an associate editor of OpenNano (Q1, CS=21.9); Springer Environmental Chemistry Letters (Q1, IF=13.615), *Frontiers in Nanotechnology-Biomedical Nanotechnology*, as well as academic editor of *The Innovation* from Cell Press publisher. He is also on the editorial board of 25+ prestigious biomedical, medicine, and materials science journals. His scholarly work comprises >140 publications with an H-index of 27 (i10-index= 60) including papers published in The Lancet family journals. So far, he has contributed to writing more than 35 book chapters and edited several books.

Muthupandian Saravanan has more than 21 years of teaching and research experience and is presently working as a professor in the AMR and Nanotherapeutics Lab, Department of Pharmacology, Saveetha University, SIMATS, Chennai, India. Since January 2021, he has graduated in microbiology from Madurai Kamaraj University and obtained a doctorate with a specialization in medical microbiology and nanomedicine from Sathyabama University, India. Thereafter, he worked as a postdoctoral researcher (2011–2012), focusing his research on nanobiomaterials and their biomedical

applications. Prior to his postdoc, he worked as an assistant professor (SG) in the Department of Biotechnology at SRM University for 6 years, from 2005 to 2011. After his postdoctoral research, he worked as an associate professor, under the United Nation Development Program, Department of Medical Microbiology and Immunology, School of Medicine, Mekelle University, Ethiopia, till 2021. His research specialization is Development of Novel Biomaterials for Emerging and Re-emerging Infection in particular Antimicrobial Resistance (AMR) & Cancer. He has published more than 180 research papers including high-impact journal *The Lancet* and *Nature* in peer-reviewed Journal with more than *20,000 citations and h-index of 55 and i10 index of 125*. He has published *5 edited books and 15 book chapters*. Prof. Dr. Saravanan has participated in more than 75 national and international conferences and is reviewer of more than 100 peer-reviewed journals. He is guest editor for various reputed PubMed and Scopus indexed journals, in particular. He is an associate editor of *Frontiers in Pharmacology, Frontiers in Oncology*, and *MDPI Functional Biomaterials*. Prof. Dr. Saravanan has received many fellowships and awards, notably top 2% scientist listed by Stanford University in 2021; IET—Nanobiotechnology Premium Award two times continuously in the years 2019 and 2020; the international fellowship "Advanced Course on Diagnostics," sponsored by LSH&TM & Fondation Mérieux, in France, 2013; international fellowship "Pertussis: Biology, epidemiology, and prevention" meeting sponsored by Fondation Mérieux & WHO in France 2014; International Union of Microbiological Societies (IUMS) travel grant in 2015 to Canada; and international fellowship "Advanced Course on Antibiotics" (AdCAb) sponsored by Institut Pasteur and Fondation Mérieux France, 2016 .

Outer Membrane Vesicles (OMVs) as a Platform for Vaccination and Targeted Drug Delivery

Amal A. Al-Dossary, Adaugo C. Isichei, Songqi Zhang, Jiahe Li, Abdelhamid Errachid, and Abdelhamid Elaissari

Abbreviations

APCs	Antigen-presenting cells
ClyA	Cytolysin A
COVID-19	Coronavirus disease 2019 pandemic
DCs	Dendritic cells
DLS	Dynamic light scattering
DOX	Doxorubicin
ETEC	Enterotoxigenic *E. coli*
HEK293T	Human epithelial cells
HER2	Human epidermal growth factor 2
IM	Inner membrane
KSP	Kinesin spindle protein
LPS	Lipopolysaccharides
MALS	Multiangle light scattering
MR	Mannose receptor
NOVC OMVs	*Vibrio cholerae*-derived OMVs
NPs	Nanoparticles
NSCLC	Non-small-cell lung cancer
NTA	Nanoparticle tracking analysis
OM	Outer membrane

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OmpA	Outer membrane protein A
OMPs	Outer membrane proteins
OMVs	Outer membrane vesicles
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed death 1
PD-L1	Programmed death 1 ligand 1
PGN	Peptidoglycan
PLs	Phospholipids
PRRs	Pattern recognition receptors
PTE	Phosphotriesterase
PTX	Paclitaxel
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
TAM	Tumor-associated macrophages
TLR	Toll-like receptor
TME	Tumor microenvironment

1 What Are OMVs

Outer membrane vesicles (OMVs) are nanosized vesicular structures generated through a "budding out" of the bacterial outer membrane surface. OMVs are spherical vesicles with a diameter ranging between 10 and 300 nm [1] and possess similar characteristics to the cell from which they are derived [2] (Fig. 1). OMVs were initially reported by Chatterjee and Das [3] to be an artifact of bacterial growth from observation of *Vibrio cholerae*.

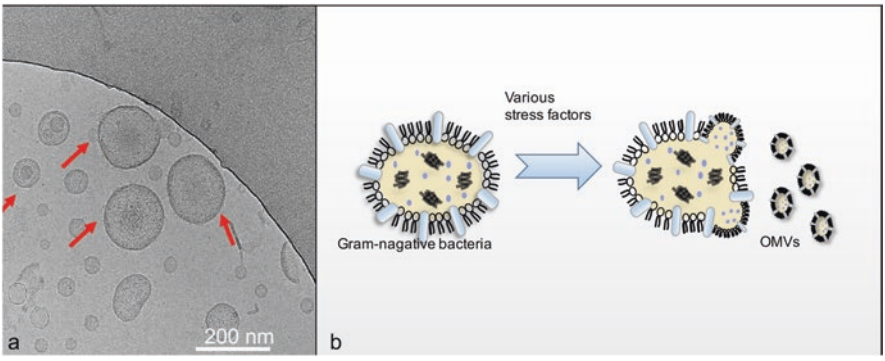

Fig. 1 (**a**) Cryo-TEM micrograph of OMVs. OMVs are spherical in shape and range from 10 to 300 nm in size. (**b**) A schematic illustrating the biogenesis of OMVs production in flagellin-deficient *S.* Typhimurium. Scale bars = 200 nm. (Adapted with permission from [112])

Today, they are well studied and known to be a common phenomenon observed in almost all Gram-negative bacteria purposefully secreted to serve as a mediator of bacterial communication and homeostasis and also harnessed for their potential in biotechnological applications. OMVs contain cargo derived from their parent bacterium such as phospholipids (PLs), membrane proteins, periplasmic proteins, nucleic acids (DNA and RNA), ions, metabolites and signaling molecules, and pathogen-associated molecular patterns (PAMPs). Notably, PAMPs such as lipopolysaccharides (LPS) and peptidoglycans (PGN) can activate the host innate immune response via activation of the pattern recognition receptors (PRRs). Their cargo transportation ability makes them ideal for bacterial communication to distant sites, transfer of virulence factors, and maintenance of bacterial communities [4]. Due to their small size, diverse cargo, transportation ability, and immune-stimulatory properties, they are pliable and gaining popularity in the scientific community. They are currently genetically engineered by protein expression for use in diverse biotechnological applications such as adjuvants, vaccine development, drug delivery, fluorescence tracking, cancer immune therapy, and antibacterial therapy. For this chapter, we focus on vaccine development and target drug delivery (Fig. 2).

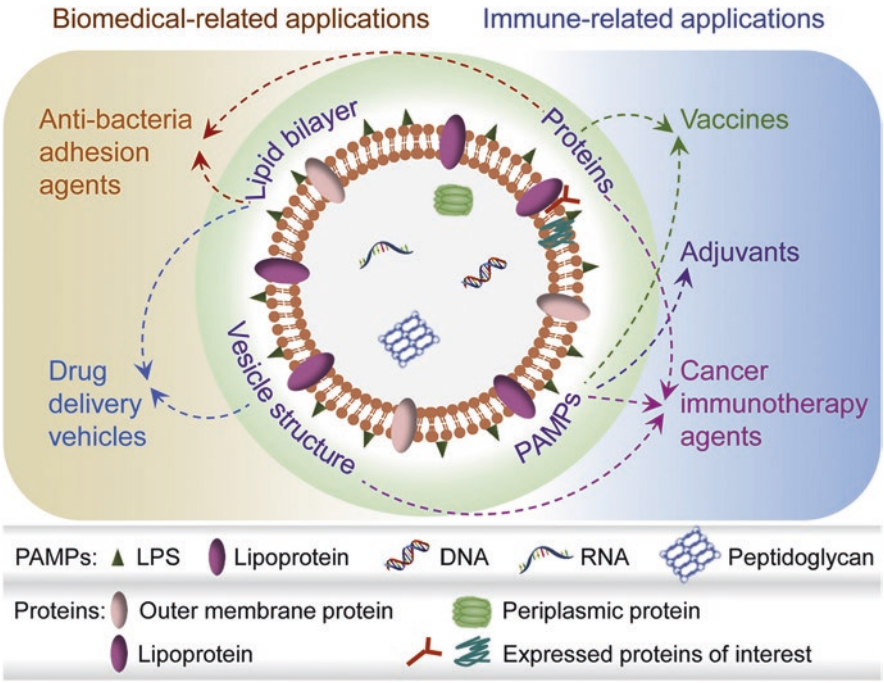

Fig. 2 Schematic representation of the structure composition of OMVs and their potential in biomedical applications. OMVs packed with a variety of PAMPs, including LPS, lipoprotein, DNA, RNA, and peptidoglycans. (Adapted with permission from [84]. Copyright (2020) Elsevier)

1.1 Biogenesis of OMVs

Despite over 60 years of studying OMVs [3, 5], there is no unified mechanism for their formation. Several models have been developed over the years and it has been extensively reviewed by Avila-Calderón et al. [6]. However, three key mechanisms exist elucidating the role of lipoproteins, LPS, flagellin, and PGN, respectively, on the biogenesis of OMVs, but more than one molecule may be involved in the biogenesis of OMVs. Overall, OMVs' biogenesis seems to be a collective set of multifactorial mechanisms that influence their production and cargo selection.

Mashburn and Whiteley showed that the accumulation of the outer membrane with LPS, PLs, and other curvature-inducing molecules causes curvature and OMV production [7]. Conditions of stress also lead to the production of OMVs such as a change in temperature in *Serratia marcescens* [8] and *E. coli* [9]. Kadurugamuwa and Beveridge showed the presence of antibiotics caused *Pseudomonas aeruginosa* to produce OMVs [10].

A recent study described a highly conserved model for the biogenesis OMVs in Gram-negative bacteria. Briefly, Roier et al. [11] have identified genes that are involved in the biogenesis of OMVs. Their result showed that the deletion of the outer membrane lipoprotein *VacJ* and/or the inner membrane permease *Yrb* genes significantly increases the production of OMVs by *H. influenzae*, where VacJ and Yrb proteins play a role in maintaining the lipid asymmetry in the Gram-negative bacteria outer membrane by reverse trafficking of PLs from the outer membrane to the inner membrane. The authors conclude that the distribution of VacJ and Yrb proteins causes the accumulation of PLs in the outer leaflet of the OM, which initiated the outward bulging of the OM [1]. Thus, the accumulation of PLs in the outer leaflet of the OM induces a positive and negative curvature which leads to the budding of the OM [2] and eventually, pinching of the OM (Fig. 3) [11].

1.2 Structure, Function, and Composition of OMVs

1.2.1 Structure and Compositions of OMVs

OMVs are produced by all Gram-negative bacteria in both planktonic culture and the native host environment [12]. OMVs belong to a type of lipid bilayer nanostructure of spherical structures, and the compositions of OMVs include proteins, lipids, and nucleic acids, which were analyzed by high-speed ($\geq$40,000× *g*) density gradient centrifugation and mass spectroscopy [13].

I. Proteins

The protein content of OMVs has been well studied by immunoblotting, mass spectrometry, and other functional experiments. To date, over 3500 diverse proteins have been identified in OMVs that originate from the membranes of host bacteria

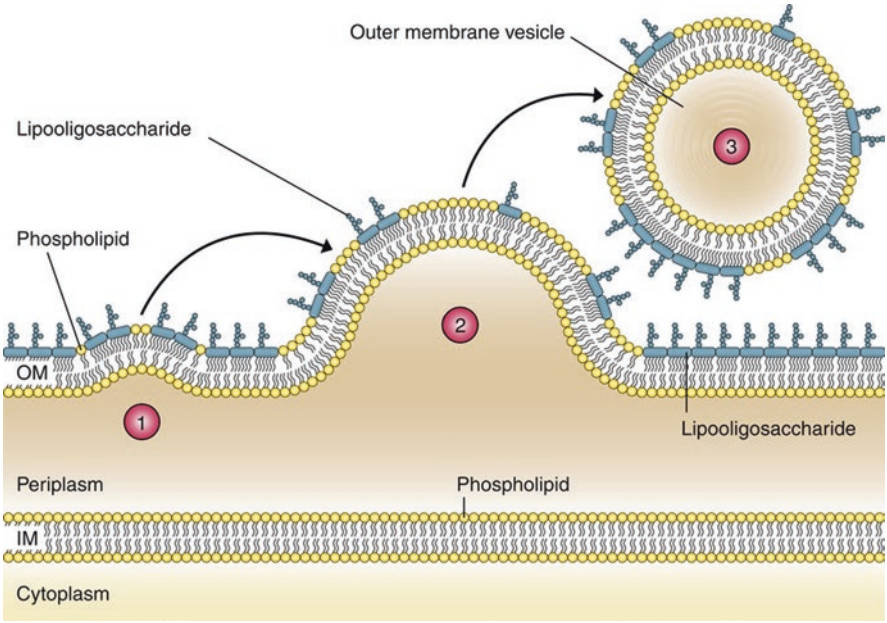

Fig. 3 Schematic representation of the biogenesis of OMVs. (Adapted with permission from [11]. Copyright (2022) Springer Nature)

such as OM proteins and periplasmic proteins. OM proteins are related to transport systems, adhesins, enzymes (e.g., phospholipases and proteases), and flagellum or pilus proteins. Of note, periplasmic proteins that are associated with the inner surface of OM display increased incorporation within OMVs, in comparison to proteins firmly bound to the inner membrane. However, certain cytoplasmic and inner membrane proteins were also identified in OMVs.

II. Lipids

Lipids are a crucial structural component of OMVs. According to Chowdhury and Jagannadham's paper, OMVs are primarily composed of phosphatidylglycerol and phosphatidylethanolamine [14]. In addition, a high extent of saturated fatty acids builds a rigid structure for OMVs [15]. Although virulence factors, such as LPS, are present in the Gram-negative bacteria, only a small percentage of parent LPS are found in the OMVs [16]. According to the investigation of Kadurugamuwa and Beveridge, they found that charged B-band types of LPS in OMVs are enrichment in *P. aeruginosa* [10]. However, in *Porphyromonas gingivalis*, they are high components of A-band LPS of OMVs [17].

III. Nucleic Acids

OMVs also contain nucleic acids, including DNA, RNA, plasmid, phage, and chromosomal DNA, in several studies. In addition, surface-associated and luminal DNA are both present in OMVs [18]. The DNA fragments can be circular and linear

[19]. However, Zhou et al. discovered that some strains' OMVs didn't contain DNA. Zhou et al. found the presence of OMVs without DNA in *Porphyromonas gingivalis* strain 33,277 and *P. gingivalis* strains W50 and A7436 [20].

1.2.2 Functions of OMVs

OMVs exhibit diverse functions in the interactions between bacteria and the host. For example, Lee et al. reported that OMVs provided a long-distance delivery system for several specific components, such as adhesion molecules and virulence factors [13], and certain toxins transported by OMVs have been demonstrated to modulate the physiology of host cells [1]. Additionally, OMVs have been shown to be responsible for promoting homeostasis, formatting biofilm, and inducing the SOS response to repair DNA.

Furthermore, Mashburn-Warren and Whiteley found that OMVs signal between cells through encapsulated small molecules to control gene expression and confer favorable mutations in bacteria [21, 22]. Of note, Li et al. reported that OMVs could be utilized by bacteria for horizontal gene transfer [23]. In addition, OMVs-based communication is also associated with antibacterial activity because the small size and endogenous membrane structure of OMVs facilitate the entry of antibacterial molecules into the bacteria [24].

The production of OMVs can be elevated in response to stress conditions [25]. For instance, OMVs can help remove misfolded proteins when bacteria are subject to physical or chemical stresses [26]. Additionally, OMVs play a role in scavenging or neutralizing antibiotics when bacteria are exposed to antibiotics [27]. Of note, OMVs are also responsible for nutrient acquisition by carrying metal ions, degradative enzymes, and target-specific receptors [2, 28]. For example, in *Myxococcus xanthus*, their OMVs are equipped with alkaline phosphatase to liberate phosphate which can provide nutrition to promote the improvement of a multicellular community [29]. Pulido et al. demonstrated that *Acinetobacter baumannii* OMVs are deficient in LPS synthesis and that immunization with these OMVs elicits protective immunity against infection with *A. baumannii* [30]. Interestingly, this strain of bacteria is resistant to antibiotics and is difficult to treat. Furthermore, Martora et al. confirmed the secretion of OMVs by *Klebsiella pneumoniae*, an important pathogen of nosocomial infections due to resistance to antibiotics, and they showed the presence of outer membrane proteins (OMPs) and porins (OmpA and OmpC), which are important innate immune-activating ligands that play a role in the activation of host innate immune response pathways. The authors concluded the involvement of OMVs in the pathogenesis of *Klebsiella pneumoniae* could be future targets for novel therapy and potential vaccine against *Klebsiella pneumoniae* [31]. However, as existing studies of OMVs suggest more functions than we have previously thought, it warrants further investigation into the roles of OMVs for both fundamental and translational studies.

1.3 Isolation and Purification of OMVs

To accelerate OMV-based therapeutic applications, it is critical to report a detailed description method of isolation and purification of OMVs from pathogenic species. The majority of techniques for isolation and purification of OMVs were described in the literature with very similar procedures, starting with low-speed centrifugation followed by subsequential filtration of the supernatants through the use of both 0.22 and 0.45 μm filters, and this primarily depends on the size of the vesicles [32]. After filtration, the filtrate was subjected to several steps including concentration and purification which have been listed in Table 1. Hence, ultracentrifugation alone is not sufficient for the purification of OMVs. Thus, OMVs need to be purified by density gradient ultracentrifugation which will be resulted in the purest fraction of OMVs [32–34].

1.4 Characterization of OMVs

In OMV vaccine development, it is essentially important to check the consistency of production, size, quality, and stability [41]. For the last two decades, researchers have been interested in understanding the proteinaceous cargos of OMVs from different species of bacteria; however, the characteristics and functions of the OMVs are still lacking. Particle size, appearance, structure, and stability need to be reported for the complete characterization of OMV particles.

Mostly, the Gram-negative OMVs are spherical nanoparticles consisting of a single lipid bilayer encapsulating a proteinaceous lumen [42]. Their lipid bilayer is composed of an inner membrane (IM) and outer membrane (OM), separated by a thin layer of peptidoglycan [43, 44] (Fig. 4). OMVs carry nucleic acids (DNA, RNA), LPS, PLs, peptidoglycan hydrolases, periplasmic content, as well as proteins localized to the cytoplasm, IM, periplasm, and OM, and other insoluble components

Table 1 Isolation methods of OMVs

Method	Advantage	Disadvantage	Reference(s)
Differential centrifugation	Low technical requirements; ease of execution	Laborious, low purity, generally needs to be combined with density gradient centrifugation for further purification	[36]
Size-exclusion chromatography	Rapid isolation process; high purity	High cost; unsuitable for large-scale production	[37]
Hydrostatic filtration dialysis	Low cost; suitable for large-scale production	Limited data on the purity of the isolated OMVs	[38, 39]
Affinity purification	Fast; specific isolation of targeted OMV populations	Only available for OMVs carrying exposed tags; low recovery rate	[40]

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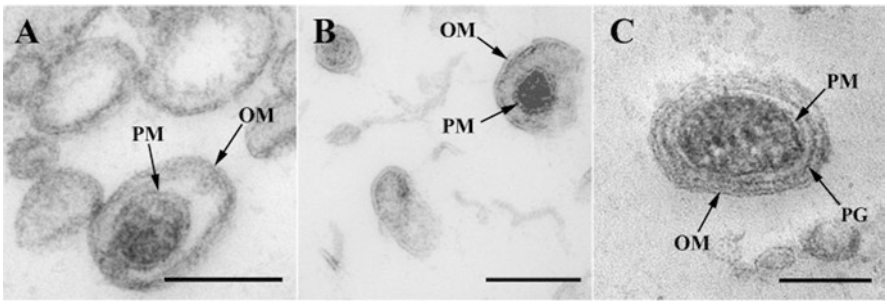

Fig. 4 Observation and characterization of OMV by cryo-TEM from three strains of Gram-negative bacteria. (**a**) OMV from *Neisseria gonorrhoeae.* (**b**) OMV from *Pseudomonas aeruginosa.* (**c**) OMV from *Acinetobacter baumannii.* All images showing spherical bilayered membrane vesicles surrounded with outer membrane (OM) and inner membrane which correspond to the plasma membrane (PM), separated by thin layer of peptidoglycan (PG). Bar = 100 nm. (Adapted with permission from [44]. Copyright (2015) Pérez-Cruz et al.)

that are delivered to the environment to accomplish several functions. OMVs are membrane-bound proteins, including protein channels, signaling molecules, transporters, antigens, and receptors [1, 30, 45–48]. The OMV's cargos depend on the species of bacteria from which they originate, the mechanism of biogenesis, and stress conditions [49–51]. Numerous studies have demonstrated that the composition of OMSs differs from that of the bacterial outer membrane by the enrichment or exclusion of specific OMPs and LPS modifications [10, 52].

There are multiple techniques to determine the OMV particle size including dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and multi-angle light scattering (MALS) coupled with high-performance liquid chromatography-size exclusion chromatography (SEC) [41].

1.5 Mechanisms of OMVs' Entry Into Cells

OMVs can transfer a diverse range of cargo, including DNA, RNA, microRNA, protein, and lipids, to proximal host cells. A recent study demonstrated the presence of DNA on the external and internal surfaces of OMVs [53]. Moreover, they showed that most of the DNA is present externally, and the authors concluded that external and internal OMV-associated DNA plays a vital role in pathogen-host communication and may modulate host cell responses and highlighted their importance for their use as vaccines.

OMVs can interact with many host cell types including mucosal epithelial cells and with immune cell populations in the host's submucosa, where they can directly interact with immune cells, including macrophages, neutrophils, and dendritic cells (DCs), where the latter suggests the involvement of OMVs as a modulator for immune response. Moreover, OMVs can interact with cells that are distal to the site of OMV entry [12].

Consistently, they can enter host cells and deliver their DNA, LPS, peptidoglycan, and protein cargos [12, 54]. Bielig et al. reported that *Vibrio cholerae*-derived OMVs (NOVC OMVs) deliver PGN to host cells and had intrinsic inflammatory potential. NOVC OMVs were internalized by human epithelial cells (HEK293T) and induced inflammatory responses via activation of two cytosolic-expressed members of the nucleotide-binding domain-containing proteins (NOD1, NOD2) [55].

Notably, OMVs express several physiologically relevant PAMPs on their surface that can be recognized by host PRRs and that may facilitate their entry into host cells.

OMVs enter host epithelial cells by the use of multiple endocytic mechanisms including lipid raft-dependent and lipid raft-independent endocytosis, micropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and dynamin-dependent entry [12, 56, 57]. Moreover, OMVs present a range of surface-exposed antigens such as Toll-like receptor (TLR) activating components (flagellin, triacylated lipoproteins, acylated lipoproteins) which allow them to be internalized by the host cells via activation of TLR. Kaparakis-Liaskos and Ferrero concluded that OMVs use multiple routes of entry into host cells and this may be regulated by the size and membrane composition of OMVs [12].

The most commonly reported mechanism of OMVs' entry into host cells involves lipid rafts. Kesty et al. were the first group who identified the ability of OMVs to enter non-phagocytic host's epithelial cells via lipid raft fusion. They have demonstrated that enterotoxigenic *E. coli*-derived OMVs were internalized by host cells via cholesterol-rich lipid rafts in a temperature-dependent manner [58]. Following endocytosis into host cells, OMVs traffic into early endosomes and trigger signaling cascades where they interact with intracellular PRRs. In addition, OMVs are degraded by autophagy, and their degradation results in the generation of a pro-inflammatory response which is mediated by the production of cytokines and chemokines. This ultimately results in the recruitment and activation of DCs to facilitate the development of T cell immunity [58].

2 Cargo Loading and Surface Modification Using OMVs

OMVs have become well appreciated in recent times as nanodrug delivery systems because they could be utilized as delivery agents by encapsulating drugs and delivering them to target tissues. They have several unique properties desirable for development of drug delivery systems including biodegradable, biocompatible, nontoxic, easy to synthesize, cost-effective, prolonged circulation, evading the immune system, and selective targeting. The lipid bilayer of OMVs acts as a barrier to protect the loading materials from decomposition in the circulation system and prolong the half-life. Ligands and proteins can be functionalized on OMVs' surface to achieve targeted delivery [59]. There are two strategies through which cargos are loaded into OMVs: passive and active loading approaches (Fig. 5).

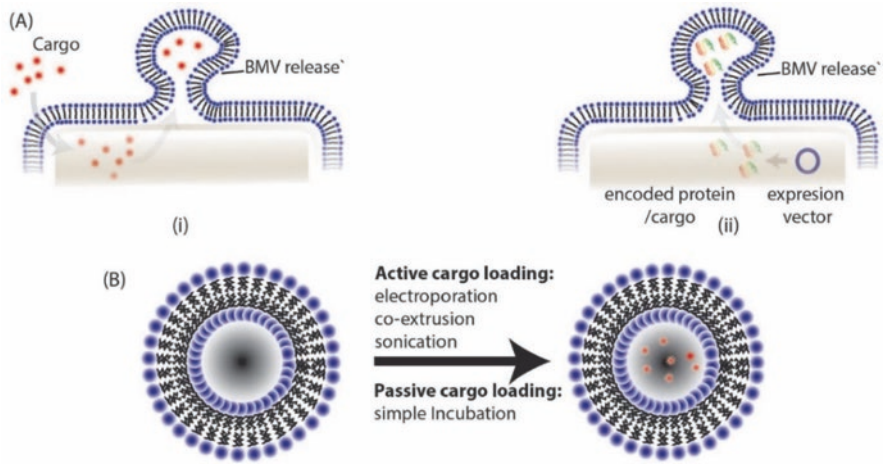

Fig. 5 Strategies for cargo loading into OMVs involve (A) (i) loading a cargo directly into parental bacteria cell to produce cargo-loaded OMVs, or (ii) by transformation of parental bacteria cell using expression vector that contains desired gene which encoded protein / cargo or (B) via various active and passive cargo loading methods post OMVs isolation. (Adapted with permission from [113]. Copyright (2021) Pharmaceutics)

2.1 Active Cargo Loading

Active loading is commonly used for loading small molecules or hydrophilic drugs into the OMVs' lumen. OMVs can be functionalized via different physical methods including electroporation, extrusion, and sonication [60, 61]. However, only a few studies have addressed the mechanism behind specific cargo loading into OMVs. Take electroporation methods as an example.

2.1.1 Electroporation

The electroporation technique involves the application of short high-voltage pulses to the OMV membrane to create a transient state of permeability which allows the entry of drugs and different cargos. The phospholipid membrane then recovers its structure once this process is completed. Ayed et al. showed for the first time the successful encapsulating of metallic gold (Au) nanoparticles into bacteria-derived OMVs using electroporation. In this study, small gold nanoparticles (AuNPs) were loaded into OMVs derived from *Pseudomonas aeruginosa* by applying an optimal voltage of 470 V and 1 pulse yielding an encapsulation efficiency of ~35% and further validated the integrity of the vesicles after electroporation. They concluded that their approach could be adapted to include other nanomaterials or drugs for biomedical applications [60].

In another encapsulation approach, wherein OMVs derived from genetically engineered *E. coli* BL21 (ΔmsbB) were used for the delivery of nucleic acid drugs and genetic medicine with less endotoxicity to triple-negative brest cancer model

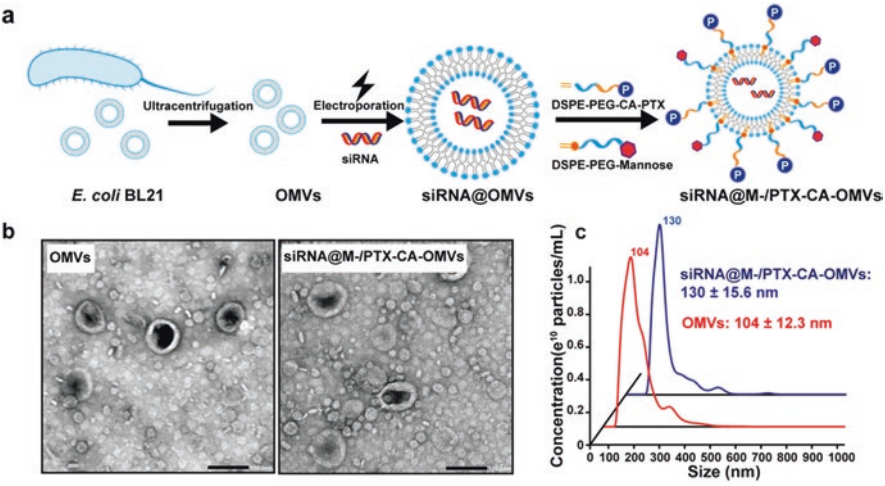

Fig. 6 (**a**) Schematic representation of cargo loading into OMVs via electroporation technique. (**b**) TEM images of empty OMVs and siRNA@M-/PTX-CA-OMVs displayed a bowl-like bilayer structure (scale bar = 100 nm). (**c**) Nanoparticle tracking analysis (NTA) demonstrated that the average size of empty OMVs and siRNA@M-/PTX-CA-OMVs was (104 ± 12.3 nm) (130 ± 15.6). (Adapted with permission from [66]. Copyright (2022) American Chemical Society)

(66). They designed a pH-sensitive drug delivery system coloaded with therapeutic siRNA (Redd1 siRNA) and paclitaxel (PTX, a microtubule-stabilizing drug) (siRNA@M-/PTX-CA-OMVs) that sequentially targets different cells in the tumor microenvironment (TME) and regulates the tumor metabolic and suppresses tumor growth. The loading of Redd1 siRNA, in this case, was achieved through the electroporation technique leading to a high loading efficiency of siRNA in OMVs. The surface of OMVs was modified with mannose to target M2 macrophages via the mannose receptor (MR, $CD206^{+}$). Upon the arrival of siRNA@M-/PTX-CA-OMVs to the tumor site where the pH is 6.8, this triggers the release of PTX and the rest of the system is taken up by M2 macrophages to increase their level of glycolysis. Their system showed a promising potential for tumor-associated macrophage (TAM) repolarization, tumor suppression, tumor immune activation, and TME remolding in the triple-negative breast cancer model (Fig. 6).

2.2 Passive Cargo Loading

2.2.1 Simple Incubation

Passive loading is to load the drugs of interest OMVs that have been isolated via simple incubation with cargo. Passive diffusion is suitable for small-molecule drugs that are positively charged and hydrophobic, which interact with the negatively charged membranes and subsequently are retained in or cross the membranes. Kuerban et al. reported the successful encapsulating of doxorubicin (DOX) into

OMVs. OMVs were obtained from *Klebsiella pneumonia* to drive chemotherapeutic agents to non-small-cell lung cancer (NSCLC) A549 cells. OMVs were isolated from attenuated *Klebsiella pneumonia* and loaded with DOX (DOX-OMVs) through a gentle co-incubation approach and further found that DOX-OMVs nanocarriers exhibited promising positive immune responses with no obvious cytotoxicity in the NSCLC mouse model. The efficacy of their approach was characterized by the accumulation of OMVs and DOX inside tumor cells [62].

3 OMVs in Vaccination

3.1 OMVs as Modulators of the Innate Immune Response

Naturally derived bacterial OMVs contain a large number of PAMPs which can induce the activation of antigen-presenting cells of the host tissues, including the maturation of DCs and their production of cytokines, and drive the activation of the innate immune response and qualified as immunoadjuvants [12, 63]. Moreover, OMVs can function as vaccine carriers. In fact, OMVs are safe as immunotherapeutic agents and have been approved by the European Commission to be used as a meningococcal B vaccine against gonorrhea disease. Both OMV-based vaccines (MeNZB and Bexsero) induced anti-gonococcal antibodies which recognize gonococcal proteins [64, 65]. Furthermore, OMVs can be genetically engineered and modified to express any chosen antigen and can be manipulated to reduce their endotoxicity. Intravenous injection of OMVs produced by *E. coli* BL21 mutant showed a reduction in systemic cytokine release and low endotoxicity compared to wild-type OMVs [66]. Moreover, their system siRNA@M-/PTX-CA-OMVs induced a favorable synergistic antitumor effect at the tumor site. Upon reaching the tumor site where the pH is 6.8, this causes the release of PTX from the complex due to the pH-sensitive linker inserted in the OMVs, and this causes a direct tumor killing and the rest of the system was taken up by M2 macrophages and downregulates and suppresses Redd1 and increases the macrophage glycolysis and blocks tumor metabolic microenvironment and inhibits tumor growth and metastasis (Fig. 7).

In a previous report, Li et al. developed a genetically engineered OMVs carrying on their surface the ectodomain of programmed death 1 (PD-1) which functions as immune suppressive on active T cells and has been used to normalize the immune system. Briefly, their data shows a strong accumulation of OMV-PD1 at the tumor site which increased the infiltration of immune cells and activated the immune response. Simultaneously, PD1 ectodomain binds to its ligand, immune checkpoint programmed death 1 ligand 1 (PD-L1) on the surface of tumor cells and antigen-presenting cells (APCs) facilitating their internalization and reduction, thereby protecting $CD8^+$ T cells from the PD1/PD-L1 immune inhibitory axis and leading to tumor growth reduction both in mouse melanoma and colorectal cancer models (Fig. 8) [67].

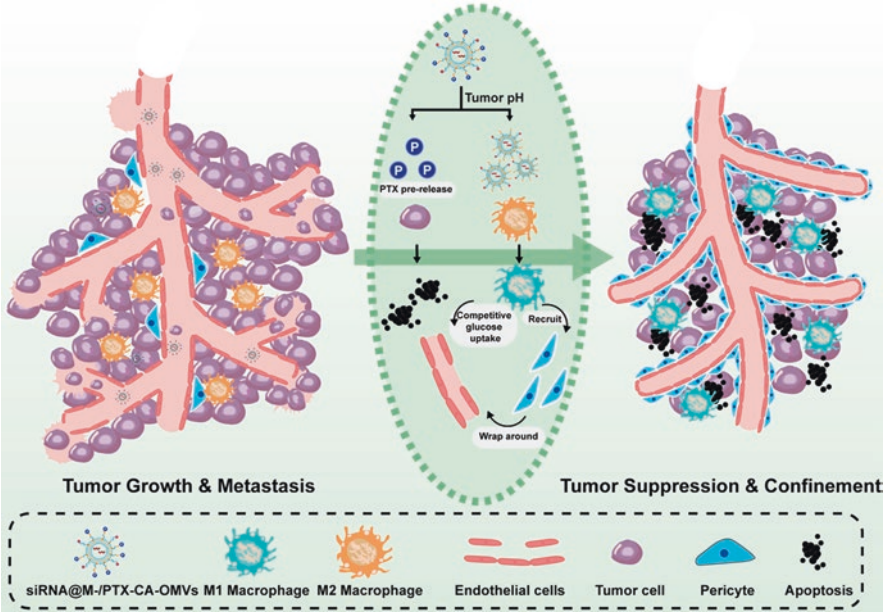

Fig. 7 Schematic representation of the process of drug release from siRNA@M-/PTX-CA-OMVs and the interaction of their components with different cell types in TEM. Firstly, PTX is released at 6.8 pH. Secondly, the rest of the system will be taken up by macrophages which induce suppression of tumor. (Adapted with permission from [66]. Copyright (2022) American Chemical Society)

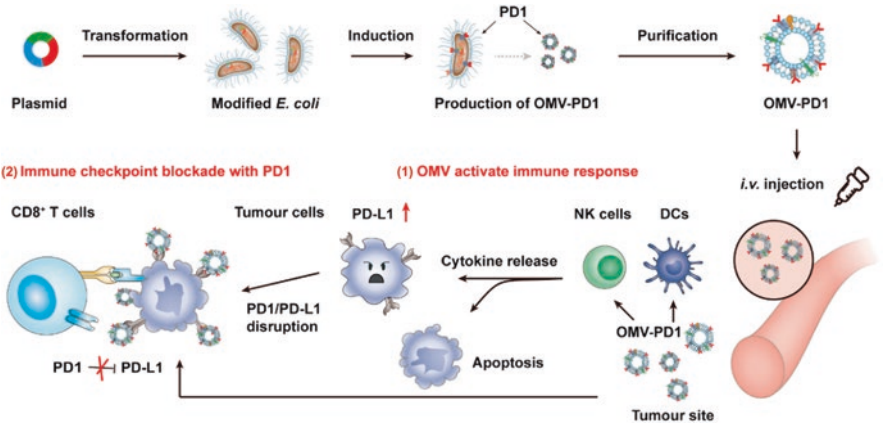

Fig. 8 Schematic representation of the production and purification of OMV-PD1 from engineered *E. coli* and their use as effective antitumor agents. The injection of OMV-PD1 causes (1) activation of the immune response and (2) immune checkpoint blockade with PDI. (Adapted with permission from [67]. Copyright (2022) American Chemical Society)

3.2 Bioengineered OMVs for Vaccination

Over the past decades, OMVs have been used as vaccine delivery vehicles by incorporating antigens of interest. Engineering efforts of OMVs for antigen delivery largely center around genetic fusion between antigens and anchor proteins such as cytolysin A (ClyA). Furthermore, some researchers revealed that engineered OMVs with some fusion proteins would dramatically enhance OMVs' functions [68].

3.2.1 Recombinant OMVs Based on ClyA Fusion Protein

OMVs can present antigens on the outer layer of the vesicles through proteins, like a ClyA fusion protein. The ClyA protein is a transmembrane protein with a molecular weight of 34 kDa found in OMVs [69]. By fusing green fluorescent protein (GFP) with ClyA protein, Cheng et al. successfully expressed GFP on the surface of OMVs [70] by producing OMVs in *E. coli*. Of note, while no additional adjuvant was applied in their immunization protocol, the GFP-OMVs elicited GFP-specific antibodies, suggesting the effect of self-adjuvanticity. In addition, Kim et al. demonstrated that only the C terminal fusion of ClyA could produce desired functions of derived OMVs, while the N terminal fusion of ClyA is often problematic [68]. To further prove the translational potential of recombinant OMVs, in a murine sepsis model, Omp22 antigens from *Acinetobacter baumannii* were fused into *E. coli* DH5α-derived OMVs for treating *Acinetobacter baumannii* infection. The results showed that active immunization with Omp22-OMVs increased the survival rate of mice [71]. Meanwhile, Rosenthal et al. demonstrated that recombinant OMVs could produce novel antiviral vaccines [72].

3.2.2 Recombinant OMVs Based on Other Carrier Fusion Proteins

Currently, other carrier fusion proteins are also applied for OMV-mediated vaccine delivery (Table 2). Salverda et al. expressed the lipoprotein outer surface protein A (OspA) in *Borrelia burgdorferi* on the surface of OMVs through fusion with fHbp in *Neisseria meningitidis* for Lyme's disease [73], and their experimental results proved that OMVs in Neisseria can generate OspA-specific antibodies for *Borrelia* vaccines.

Additionally, Basto et al. explored the outer membrane protein I (OprI) from *E. coli* fusion can infuse A104R antigen into OMVs in *Pseudomonas aeruginosa* to defend against African swine fever [74]. Also, researchers infused outer membrane protein A (OmpA) protein to locate antigens of *S. pyogenes*, such as SpyCEP, streptolysin O, and Spy0269, in *E. coli* OMVs' lumen to treat *Streptococcus* disease. And experiment results proved that Slo-OMVs and SpyCEP-OMVs could help mice against *S. pyogenes* infection [75]. However, according to several analyses, lumen-fused OMVs just evoked minor specific antibody production and even

Table 2 Common antigens for application of OMVs' vaccine

OMVs' source	Fusion protein	Antigen	Diseases application	Reference
Scherichia coli	ClyA	Omp22	*Acinetobacter baumannii*	[71]
Escherichia coli	ClyA	M2e	Influenza A	[77]
Salmonella Typhimurium	fHbp	PspA or Ply	Pneumococcal disease	[78]
Neisseria meningitidis	fHbp	OspA	Lyme's disease	[73]
Salmonella Typhimurium	fHbp	MOMP fragments	Chlamydia	[79]
Salmonella Typhimurium/ *Escherichia coli*	fHbp	ESAT6, Ag85B fragments, and Rv2660c	Tuberculosis	[79]

decreased protection [76]. Therefore, we can conclude that bioengineered OMVs which focus on the surface of OMVs would produce higher antibodies and could practice protection abilities. However, we need more research to clarify the mechanism of the different results between lumen and surface of OMVs.

In summary, while we have discussed the potential of using OMVs as a novel platform for vaccines, there remain many limitations for clinic applications. As a result, innovative approaches must be developed in order to scale up the manufacturing of OMVs with high purity.

4 OMVs in Drug Delivery

Synthetic nanocarriers have been studied for many decades and optimized, as drug carriers however are unable to effectively replicate the trafficking pathways seen by OMVs. Cytotoxicity is another disadvantage of liposomes and other synthetic nanocarriers. OMVs possess many attributes that make them beneficial for use in drug delivery and have gained attention in the scientific community due to the aforementioned properties such as the diversity of proteins on its surface, small size, and pliability to enable them to carry diverse cargos. Target drug delivery can also be achieved by modification of the outer membrane surface composition as well as genetic manipulations [80].

The effective delivery of enzymes has posed a problem for scientists, but OMVs' native ability to carry enzymes in their cargo and deliver enzymes without degradation is another advantage of OMVs in drug delivery. Alves et al. successfully packaged phosphotriesterase (PTE) into OMVs by conjugating it to outer membrane protein A. The study showed that enzyme activity was unchanged [81]. Koeppen et al. also showed that OMVs protect nucleic acid cargo from degradation [51]. Several studies have also shown that genes encapsulated in OMVs were protected from DNAse digestion [18, 82]. Alves and colleagues went on to further show that the protein cargo of OMVs remained protected in OMVs at different stability

testing parameters such as varying temperature conditions and freeze-drying [81], making OMVs an effective vehicle for delivery of drugs in vivo. Furthermore, the protection of cargo in storage would enhance the drug formulation development and improve the efficacy of the drug as lower quantities would need to be delivered [83].

4.1 Cancer Therapy

The application of OMVs in cancer therapy is just in its infancy. However, OMVs have gained great attention in the biotechnology fields as a novel cancer-targeting nanocarrier. OMVs have the added advantage of being non-replicating but possessing immunostimulatory molecules that enable recognition and uptake by immune cells, thereby inducing an immune response. Their nano-size allows for accumulation at the tumor site to induce local immunity through enhanced permeation and retention effect [84].

Gujrati et al. reported for the first time the use of bioengineered OMVs as targeted drug delivery vehicles for tumor cells. In their study, they have co-deliver anti-HER2 and therapeutic siRNA targeting kinesin spindle protein (KSP). OMVs were generated from ClyA-affibody-overexpressing *E. coli* to express human epidermal growth factor 2 (HER2) affibody on their outer membrane surface ($Affi_{EGFR}$-OMVs) [59]. In addition, Sepahdar and colleagues showed that bioengineered $Affi_{EGFR}$-OMVs were more internalized into triple-negative breast cancer cells expressing EGF receptors [85].

Kim et al. were the first to successfully utilize OMVs' capability to elicit an immune response to develop OMVs for use as cancer immunotherapeutic agents. In their study, OMVs were engineered to reduce the endotoxic effects of LPS by using modified *E. coli* with an inactivated gene for lipid A acyltransferase (msbB), which is the lipid component of LPS. Mice were subcutaneously transplanted with CT26 murine colon adenocarcinoma and treated with varying amounts of W3110ΔmsbB OMVs derived from *E. coli* [86]. Their OMVs accumulated mostly in the tumor cells displaying high specificity and also induced an immune response. The study showed that OMV treatments significantly reduced tumors in dose-dependent manner and with IFN-γ antibody determined to be the major mechanism of antitumor response. Immune memory was also established as cured mice rejected a second and third challenge of tumor cells. Whereas IFN-Y-deficient mice were unable to reject the tumor cells after OMV treatment, OMVs' immunomodulatory effects have also been studied by several scientists [62, 67, 87, 88].

OMV can elicit an antitumor immune response, to completely eradicate tumors and prevent recurrence and metastasis. Combination therapy would be required to enhance the immune therapeutic effects of OMVs [67]. Chen et al. combined OMVs and polymeric nanoparticles to boost the efficacy of cancer immunotherapy and prevent cancer metastasis. In their study, they collected OMVs from the culture of attenuated *Salmonella* and fused OMVs with DSPE-PEG-RGD through extrusion to generate OMV-DSPE-PEG-RGD (OR). Then, they coated OR with pluronic

F127 micelles (ORFT). They demonstrated that the functionalized OMV-coated polymeric micelles hold great potential as an immunotherapeutic and anti-metastatic delivery system.

Kuerban et al. went a step further to harness the immune stimulation function of OMV in combination with their role as effective nanocarriers for the delivery of chemotherapeutics [62]. OMVs derived from *Klebsiella pneumonia* were loaded with DOX (DOX-OMV) and treated against NSCLC cells A549. Their results showed that DOX-OMV had better uptake than DOX alone and DOX liposomal formulation. Although DOX is an FDA-approved drug for the treatment of neoplastic diseases including NSCLC, its clinical application has been limited due to its side effects of cardiac toxicity such as cardiac myopathy [89]. Kuerban and colleagues successfully showed that OMV-DOX enhanced the effects of DOX without any obvious toxicity in vivo [62]. OMVs have also been used for the delivery of nucleic acids for cancer therapy [59]. Gujrati and colleagues bioengineered *E. coli* to generate OMVs displaying anti-HER2 affibody as a targeting ligand for the delivery of small interfering RNA (siRNA) targeting kinesin spindle protein (KSP) which resulted in tumor regression and tolerability in animal models.

4.2 Antibacterial Therapy

One of the special properties of OMVs is their ability to be utilized to kill other bacteria to reduce competition in bacterial flora. Many NPs have been developed as antibacterial carriers; however, one impediment remains which is the tissue of bacterial resistance that inhibits the drugs from penetrating infected cells or the bacterial membrane. OMVs being derived from bacteria possess the characteristic of the bacterial membrane and therefore are advantageous over other synthetic NPs in terms of easier delivery of their cargo to Gram-negative bacterial cells because they are recognized as biocompatible. Studies showed that *Shigella flexneri* exposed to gentamycin produces OMVs containing gentamycin. Interestingly, when these OMVs were delivered to Henle cells infected by *Shigella*, it caused a reduction in the growth of the pathogen [90]. Kudurugamuwa and colleagues also observed that OMVs play a role in microbiota interactions and have an inherent bacteriolytic effect on other bacteria. The group also demonstrated that OMVs from *Pseudomonas aeruginosa* contain virulence factors, hydrolytic enzymes, DNA, and endotoxin, and the amount of OMVs produced increased in the presence of gentamycin [10, 91]. Li et al. observed the lytic effects of OMVs from several strains of bacteria and noted that they contained peptidoglycan hydrolases, which serve to dissolve the peptidoglycan layer of dissimilar cells to increase the nutrient load [48]. Goes and colleagues explored the use of OMVs derived from myxobacterial strains *Cystobacter velatus* Cbv34 and *Cystobacter ferrugineus* Cbfe23 as natural antibacterials for the treatment of intracellular infections caused by *Staphylococcus aureus* [92]. Myxobacteria are found in soil and are known to produce potent antimicrobial compounds [93–95] that are nonpathogenic to humans. Their results showed that

the OMVs displayed selective uptake by infected cells and displayed a bacteriostatic effect against *S. aureus* intercellular infection versus bacteriomimetic liposomes which were rapidly taken up by all cells. The OMVs from Cbv34 also showed storage stability at different storage conditions and maintained its dose-dependent antibacterial effect, which is an important criterion for successful formulation development for clinical use. Interestingly, scientists have combined NPs and OMVs for active targeting of bacteria-infected cells. Gao et al. developed an actively targeted nanoparticle coated with OMV from *E. coli* and showed that in vitro NP@OMV had higher uptake in *S. aureus*-infected macrophages compared to NP coated with polyethylene glycol or NPs coated with EVs derived from *S. aureus* (NP@EV) [96]. However, when loaded with antibiotics, the NP@EV showed significantly higher uptake than the OMV, and this could be because the EVs used were secreted from *S. aureus* and would therefore have higher biocompatibility and uptake in *S. aureus*-infected cells compared to the OMVs which were obtained from *E. coli*. These results suggest high selectivity of bacterial OMVs for infected cells and could be harnessed for the development of nano-biotic sepsis management [97].

Free antibiotics have poor accumulation at infection sites [98] therefore requiring high doses to achieve an effective bactericidal concentration. Long-term use of high doses leads to adverse events [99], and over time, prolonged use of antibiotics leads to drug resistance by bacteria [100]. It is therefore important to develop antibacterial drug delivery platforms that can deliver effective doses to target sites. Huang and colleagues secreted OMVs from *Acinetobacter baumannii* loaded with high concentrations of antibiotics via the efflux pump method. These OMVs penetrated and killed enterotoxigenic *E. coli* (ETEC) infection in mice model of intestinal infection. They demonstrated that quinolone-loaded OMVs effectively reduced bacterial infection versus the free antibiotic of the same concentration. For example, levofloxacin at a low dose of 1.4×10^{11} particles/kg effectively reduced the CFU in ETEC-infected mice model of intestinal infection compared to the same dose of free levofloxacin [101]. Their study showed that OMVs have good biocompatibility and high specificity. OMVs are therefore a novel way to effectively deliver antibiotics without the risk of adverse effects associated with antibiotics.

5 Future Perspective of Utilizing OMV for Vaccination and Drug Delivery

OMVs are spontaneously shed from Gram-negative bacteria during the exponential phase both in vitro and in vivo [102]. OMVs typically range from approximately 10 to 300 nm in diameter which possesses the optimal size to be internalized by host cells [28]. OMVs' components are typically representative of the outer surface of parental cells. OMVs consist of a lipid bilayer and contain biological active products, such as nucleic acids, toxins, proteins, and lipids. OMVs are associated with a variety of biological functions including pathogenesis, biofilm formation, antibiotic

resistance transfer, modulation of host immune response, interspecies communication, DNA transfer, and virulence factor delivery into the cytosol of the host cell [10, 21, 46, 58, 103, 104]. Interestingly, some specific proteins have been shown to be enriched or excluded from OMVs suggesting a specific sorting mechanism(s) for these proteins [69, 105, 106].

The composition of OMVs and their mechanism of cargo delivery hold great promise as cargo delivery vehicles. In 2013 a meningococcal group B vaccine (4CMenB), Bexsero®, was approved in Europe and Australia, which is formulated with vesicles from the *Neisseria meningitidis* New Zealand strain and contains a bacterial OMV component [107].

Nanovaccines have advantages compared to conventional vaccines against infectious diseases. They can induce a protective immune response via an efficient display of specific antigens and lymph node accumulation. During the ongoing coronavirus disease 2019 pandemic (COVID-19), lipid nanoparticle vaccination has proven the effectiveness of nanovaccines in defense against pathogens. OMVs are becoming increasingly popular and have become a highly effective vaccine platform due to several attractive features, including, but not limited to, the intrinsic adjuvanticity [46, 70], the ease of their surface decoration with heterologous antigens [108–110], the simplicity of their production [111], and the ability of the vesicles to accumulate in lymph nodes.

Thus, nano-sized and lipid membrane vesicles of Gram-negative bacteria are an ideal platform for broad applications in vaccine designs and drug delivery. However, there is also a pressing need for further investigation and creation of safer and more effective OMV-based platforms for human health.

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CRISPR/Cas9 Nano-delivery Approaches for Targeted Gene Therapy

Eden Mariam Jacob, Ankita Borah, and D. Sakthi Kumar

1 Introduction

Human genome alterations contribute to several well-identified genetic diseases out of which only a few hundreds have found efficient treatment in the modern day of healthcare technologies. In recent years, progress in the development of groundbreaking genome-editing techniques like programmable sequence-specific endonucleases that include zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), and meganucleases have been observed exponentially [1]. Gene editing is a genetic engineering technique where DNA modification in living cells is carried out by insertion, removal, and modifying target DNA sequences precisely. ZFNs and TALENs are restriction enzyme-based gene-editing tools where the DNA-binding domain binds to the target DNA sequence, which is then cleaved by the restriction-endonuclease domain. A wide array of scientific disciplines such as agriculture, synthetic biology, and medicine has greatly benefitted from these breakthrough gene-editing tools. Although ZFNs and TALENs experienced their fair share of success, lately they have been deterred in areas like extensive design strategy because of their laborious, time-consuming, and expensive procedures, making it difficult for researchers to use them in the long run. This desire to seek a reliable and efficient gene-editing tool propelled scientists to look in a different direction, which ultimately made them utilize the unprecedented features of the bacterial immune system used to fight viral infections. The discovery of a protective immune system mechanism that existed in the prokaryotes called the

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clustered regularly interspaced short palindromic repeat (CRISPR) was made in 1987 in the IAP gene of *Escherichia coli* (*E. coli*) [2]. Bacteria employ CRISPR/Cas9 system as an adaptive immune defense mechanism to fight against phage infections and plasmid transfer. Due to its unique mechanism of selectively targeting and manipulating the target genomic site directed by a short stretch of RNA known as guide RNA (gRNA), scientists tried to repurpose the CRISPR/Cas9Cas9 machinery and realized that it could be a potentially powerful tool in the realm of gene-editing machinery harnessed for beneficial purposes in microbes, plants, and humans. It was in the year 2012 when Jennifer A. Doudna and Emmanuelle Charpentier discovered that this extraordinary bacterial genome-editing machinery could be used to manipulate any DNA sequences by providing the correct template that ultimately earned them the Nobel Prize in Chemistry in 2020 [3]. Doudna, Charpentier, and co-workers simplified the CRISPR/Cas9 machinery in such a way that the Cas9 nuclease protein requires only a template sequence within the CRISPR RNA (crRNA) and a conserved protospacer adjacent motif (PAM) upstream of the crRNA binding site for target recognition along with the gRNA that comprises crRNA and trans-activating crRNA (tracrRNA) [3]. This simplified CRISPR/Cas9 machinery offers a user-friendly platform and flexibility while conducting genome-editing experiments eliminating the requirement for additional proteins to recognize the target sequence. CRISPR/Cas9 possess advantages like curing a variety of genetic disorders including cancer, cardiovascular diseases, Duchenne muscular dystrophy (DMD), sickle cell anemia, cystic fibrosis, etc. by repairing/activating/deactivating genes involved in the development and progression of such disorders and also promising to elucidate multiple gene functions simultaneously involved in large sets of mutations responsible for disease pathological process [4]. It is also essential that the cons associated with such great technological inventions must be approached and resolved in the right direction to unleash the full potential, which is also the case with CRISPR/Cas9 system. CRISPR/Cas9 system has certain obstacles in terms of inducing off-target mutations at homology sequences and its safe and effective delivery to the target sites that are paramount before its clinical applications. In this chapter, we discuss CRISPR/Cas9 biology and its mechanism and present delivery approaches, nanotechnology-mediated delivery strategies, and clinical applications that have become one of the revolutionary biomedical discoveries in the twenty-first century.

2 Features of CRISPR/Cas9 Gene-Editing Machinery

Ishino et al. in 1987 discovered an odd pattern of DNA sequence in the IAP (isoenzyme of alkaline phosphatase) gene of *E. coli* that is composed of 30 base pairs of recurring DNA repeats [2]. These recurring patterns of DNA repeats are often referred to as palindromic sequences and are commonly present in the microbial domains of the prokaryotic class that denoted the name CRISPR. CRISPR and Cas9 genes together play a crucial role in the immune intervention process of the

bacterial system where they also possess the ability to incorporate newer spacer sequences to develop phage resistance. These spacer sequences are homologous to the target sequences assisting the CRISPR/Cas9 to specifically cleave the target DNA sequence upstream of the protospacer adjacent motif (PAM). This RNA-guided adaptive immune system that helps bacteria and archaea in its acquired immunity process has evolved in a sophisticated manner, and currently, the CRISPR systems are categorized into six types based on their CRISPR/Cas loci each having a unique group of Cas proteins together with crRNA for CRISPR interference [5]. In addition to their classification based on their Cas9 protein structure and function, they are grouped into class I and II systems [6]. Class I systems comprise types (I, III, IV) having a multi-subunit Cas9 protein complex, while class II systems include types (II, V, VI) that possess a single Cas9 protein [7, 8]. Among the different types of CRISPR/Cas9 systems, type II acquired immense popularity over the years due to its relatively simple structure of a single DNA endonuclease Cas9 protein recognizing its target dsDNA and cleaving the strands with high precision, thus making it an extensively studied system for gene-editing purposes [9].

The three essential components of this efficient and cutting-edge gene-editing tool are Cas9 endonuclease protein, crRNA, and tracrRNA [4]. The Cas9 protein was first isolated from *Streptococcus pyogenes* (SpyCas9) and was also reported to have structural variants and orthologues across different bacterial species. Cas9 extracted from *S. pyogenes* (SpyCas9) [3, 10] and *Staphylococcus aureus* (SaCas9) [11] are the popular types of Cas9 proteins widely used for genome-editing research purposes. Cas9 is a large multifunctional and multi-domain DNA endonuclease protein having 1368 amino acids that snip the target DNA 3bp upstream of the PAM sequence like molecular scissors [12]. The crystal structure of Cas9 protein reveals the presence of two regions called the recognition (REC) lobe and one nuclease (NUC) lobe (Fig. 1a) [13]. The REC lobe is a long α-helix Cas9-specific functional domain that has a REC1 domain and REC2 domain binding to the guide RNA [13], while the NUC lobe has three components: RuvC, HNH, and PAM-interacting (PI) domains [12]. The HNH and RuvC domains are known to function in target DNA binding and cleavage of both the complementary and noncomplementary strands, respectively, whereas PI domain is responsible for conferring PAM specificity on the noncomplementary strand and initiating binding to the target DNA sequence [13]. Besides these crucial components, Cas9 also has an arginine-rich motif called the binding helix (BH) domain playing a central role in bridging the lobes of REC and NUC, thus assisting in the direct and indirect interaction with the target DNA and single-guide RNA (sgRNA) [14].

The CRISPR-RNA (crRNA) and trans-activating RNA (tracrRNA) together comprise the single-guide RNA (sgRNA) molecules that recruit Cas9 to its assembly for guiding it to the target DNA, and ultimately cleaving it via complementary base-pair formation. The crRNA is 18–20 nucleotides long and has an extra 12 nucleotides repeat region assisting in target DNA recognition and consequently pairing with the DNA sequence. The tracrRNA has 14 nucleotides anti-repeat region with three stem-loops (loops 1, 2, and 3) and serves as a binding scaffold for Cas9 nuclease [12]. The tracrRNA base pairs with the crRNA repeat sequence forming a

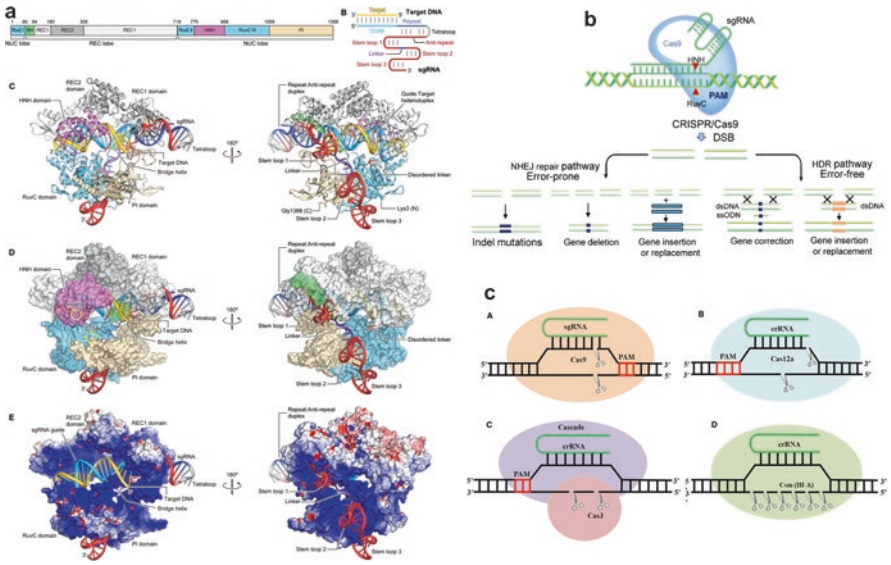

Fig. 1 (**a**) Overall structure of the Cas9-sgRNA-DNA ternary complex; (A) Domain organization of *S. pyogenes* Cas9. BH, bridge helix; (B) Schematic representation of the sgRNA/target DNA complex; (C) Ribbon representation of the Cas9-sgRNA-DNA complex. Disordered linkers are shown as red dotted lines; (D) Surface representation of the Cas9-sgRNA-DNA complex. The active sites of the RuvC (D10A) and HNH (H840A) domains are indicated by dashed yellow circles; (E) Electrostatic surface potential of Cas9. The HNH domain is omitted for clarity. Molecular graphic images were prepared using CueMol. (Adapted from [13]) (**b**) The potential applications of CRISPR-Cas systems in genome editing. CRISPR-Cas systems mediated genome modification depending on the two main double-stranded break (DSB) repair pathways. Indel mutation and gene deletion are outcomes of the dominant nonhomologous end joining (NHEJ) repair pathway. Gene insertion, correction, and replacement, using a DNA donor template, are outcomes of the homology-directed repair (HDR) pathway. (Adapted from [20]) (**c**) Schematic of the mechanisms of different types of CRISPR systems. (A) The working principle of type II Cas9. In the presence of the PAM sequence (NGG), the targeting effect of sgRNA is used to guide Cas9 protein to cleave both the complementary and noncomplementary strands, forming a blunt-ended nick; (B) The working principle of type V Cas12a protein. In the presence of the PAM sequence (NTTT), the targeting effect of crRNA is used to guide Cas12a protein to cleave both the complementary and noncomplementary strands, forming a sticky-ended nick; (C) The working principle of type I Cas systems. In the presence of the PAM sequence, the targeting effect of crRNA is used to guide the Cas3 protein to cleave the noncomplementary strand to form a large gap; (D) The working principle of type III Cas systems. In the absence of a PAM sequence, the targeting effect of sgRNA is used to guide Csm protein to cleave the noncomplementary strand to form short nucleic acid fragments. The green transverse U represents sgRNA or crRNA, the nucleotide sequences marked in red represent the PAM sequence, and scissors represent the cleavage site of nucleases. (Adapted from [6])

dual-RNA hybrid structure followed by guiding to the Cas9 protein to recognize the target DNA sequence forming a ternary complex of Cas9-sgRNA-target DNA [3, 9]. This is followed by DNA cleavage on the target complementary 20 nucleotides sequence adjacent to the PAM motif via Watson-Crick base-pairing interactions

[15] using one-metal-ion/two-metal-ion catalytic mechanism [16] by the HNH and RuvC endonuclease domains, respectively [13]. The guide RNA in the bacterial kingdom cleaves the viral DNA; however, in terms of its application in the genetic engineering, crRNA and tracrRNA could be customized and combined as a result of which any target sequence could be edited to achieve the desired outcome.

3 Mechanism of CRISPR/Cas9 System and Advantages

The accuracy at which the CRISPR/Cas9 system cleaves their target DNA sequences is a natural marvel to wonder in the bacterial kingdom, which is adapted and refined for its gene-editing applications in biomedical research. The CRISPR/Cas9-mediated genome engineering mechanism is divided into three fundamental steps: recognition, cleavage, and repair [17]. The synthetic and customizable sgRNA (crRNA and tracrRNA) directs a Cas9 endonuclease to its target DNA sequence in the genome through its 20 nucleotides long 5′ crRNA component of the sgRNA complementary base pairing. The nuclease activity of Cas9 remains curtailed in the absence of sgRNA. Once the recognition is made on the DNA sequence, Cas9 induces double-stranded breaks (DSBs) at three base pairs upstream of the conserved PAM site having the common sequence 5′-NGG-3′, but the size varies across different bacterial species [4, 18]. The prepositioning of the PAM recognition sites assists Cas9 protein to interrogate the potential target DNA sequences. Cas9 after having found its target genomic DNA within the appropriate PAM triggers the unwinding of the local DNA to form the RNA-DNA hybrid and continues searching for additional target sequences. The base pairing of gRNA and target DNA facilitates conformational changes of Cas9 reaching an active state that finally leads to the complete annealing of the gRNA and target DNA and is cut by the Cas9 protein's HNH domain. Followed by the target strand cleavage by the HNH due to its conformational changes also triggers the concerted catalytic cleavage of the nontarget strand by the RuvC domain. During this time, the Cas9 remains tightly bound to the cleaved target DNA, which will be later displaced by cellular factors for recycling. The cleavage results in the formation of blunt-ended double-stranded breaks (DSBs) to be repaired by the host machinery culminating in the final step of the CRISPR/Cas9 mechanism [4]. The human body utilizes two major pathways for the repair of DSBs, namely, nonhomologous end joining (NHEJ) and homology-directed repair (HDR) depending on the length of the 3′ single-stranded DNA (ssDNA) overhang formed due to the DNA resection [19, 20] (Fig. 1b). In the case of NHEJ, it does not rely on the creation of a 3′ ssDNA tail but on the other cofactors like 53BP1 and RIF1-shieldin that protect the broken DNA and repair the DNA via an enzymatic process. NHEJ is one of the predominant DNA-repair mechanisms that occur throughout the cell-cycle phases, causing direct ligation of the two strands, which is often associated with an error-prone process. It often leads to insertion/deletion of DNA strands generating random frameshift mutations or premature stop codons (nonsense mutation) [19, 21]. Contrary to the NHEJ, HDR

occurs during the S/G2 phase and requires an exogenous DNA template or end processed to form the 3′ ssDNA tail ensuring the accurate insertion of DNA into the target site. HDR mechanism is a more feasible and precise approach to repairing the DSBs in CRISPR/Cas9 mechanism by inserting the correct gene sequence with the assistance of a homologous sequence DNA template at the predicted DSB site [22, 23]. Both of these mechanisms could be employed by scientists for genome-editing purposes depending on the requirements of whether to correct, disrupt/delete, and insert a target gene and accomplish the desired goal. Figure 1c depicts the mechanisms of different CRISPR systems especially types I, II, III, and V [6].

Ever since the advent of CRISPR/Cas9 as a groundbreaking gene-editing tool became popular in the realm of biomedical applications especially to treat genetic disorders with great efficiency, it has rendered other existing genome-editing approaches like ZFNs and TALENs obsolete. The rising popularity of CRISPR/Cas9 over other techniques is attributed to its simplicity, high efficiency, and cost-effective nature allowing researchers to design and construct appropriate guide RNA sequences against the target DNA site using bioinformatics tools and optimization protocols thus leaving no room for error. CRISPR/Cas9 additionally enables to perform multiple loci editing with the help of one Cas9 protein and disparately designed sequence-specific guide RNAs aided by versatile programmability. The resounding success of this promising tool is often hampered by certain setbacks, which require retrospection and further studies before its implementation in gene therapy applications, which is discussed later in this chapter.

4 Delivery Approaches of CRISPR/Cas9 System

The effective and safe delivery of the CRISPR/Cas9 complex into the cells, tissues, and organs is one of the pertinent questions and a primary prerequisite that needs to be addressed to potentially exploit its advantages in therapeutic applications. To date, the desired way of safely delivering the Cas9 protein and sgRNA has remained a challenging aspect due to the large molecular weight (160 kDa) and hydrodynamic diameter (~7 nm) of Cas9 protein [24] and the negative charge of sgRNA owing to its PO^{3-} groups of the bases [25]. Keeping these caveats in mind, one must analyze to find tangible solutions on how to efficiently deliver the critical components of the CRISPR/Cas9 system into the cellular space to achieve the desired gene effects. The use of delivery vehicles including viral and nonviral to load the CRISPR cargo in different formats is one of the sought-after strategies that has been utilized for a long time ensuring that the cargo components reach their targeted destination effectively (Fig. 2a) [26]. The commonly used viral delivery systems for CRISPR/Cas9 include adenovirus, adeno-associated virus (AAV), and lentivirus, which are widely employed for the treatment of genetic disorders [25, 27]. Having said that viral delivery systems are also criticized for several of the associated side effects despite their superiority in terms of high transfection efficiency. Viral vectors accompany issues like immunotoxicity, risk of host genome integration, limited

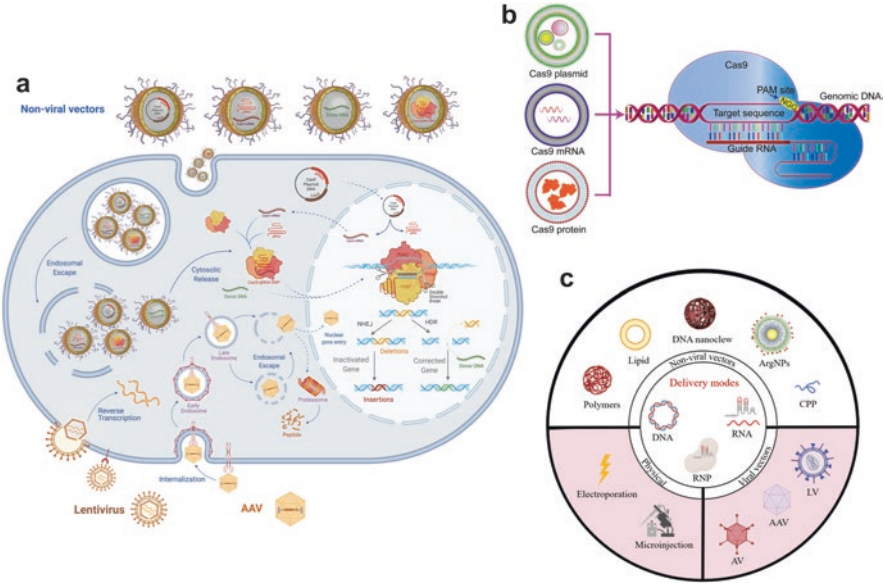

Fig. 2 (**a**) CRISPR/Cas9 genome editing through viral or nonviral delivery. Representative depiction of mechanisms and strategies involved in CRISPR/Cas9 delivery with both viral and nonviral vectors. AAV and lentivirus both bind to cell surface receptors prior to cellular infection. Following cellular internalization, AAVs have the capacity to escape the endosomes and transport across the nuclear membrane prior to uncoating, though the capsid degradation mediated by proteasome can also occur in the cytoplasm. Following lentiviral cell membrane fusion is uncoating and release of its RNA contents, which then undergo reverse transcription to form complementary DNA. Nonviral vectors offer the advantage of carrying various forms of CRISPR/Cas9 cargoes including plasmid DNA, RNA, donor DNA, and RNP. Cellular entry of nonviral vectors is accomplished via endocytosis which requires the NP to escape these endosomes in order to carry out its intended genome editing. Following endosomal escape and cytosolic release, the cargo carried by a nonviral NP must travel to varying sites, such as the nucleus for transcription and/or cytoplasm for translation. Once necessary transcription and translation steps have taken place with nucleic acid delivery approaches, a RNP is formed and can translocate across the nuclear membrane for targeted genome editing. RNPs work to perform targeted DSBs by PAM- and sgRNA-mediated recognition of a specific sequence of chromosomal DNA. Once this recognition occurs, the Cas9 nuclease can perform a DSB utilizing its two nuclease domains the HNH and RuvC which cleave complementary and noncomplementary DNA strands, respectively. Following a DSB, there are multiple fates for genome editing such as, but not limited to, NHEJ and HDR. NHEJ is utilized for genomic disruption or deletion, while HDR is utilized for gene correction, but requires the administration of an exogenous donor DNA template. AAV, adeno-associated virus; NP, nanoparticle; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; RNP, ribonucleoprotein complexes; PAM, protospacer adjacent motif; DSB, double-stranded break; NHEJ, nonhomologous end joining; HDR, homology-directed repair. (Adapted from [26]) (**b**) Representative genome editing by three forms of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9. Cas9 plasmid DNA, RNA, or protein delivery via nanoparticles to be used for precise genome editing. PAM, protospacer adjacent motif. (Adapted from [31]) (**c**) Representative schematic showing different vectors including viral vectors, physical transduction methods, and nonviral vectors for the delivery of CRISPR/Cas 9 components. (Adapted from [35])

packaging capacity, and tumorigenicity [28], which could be overcome by the use of nonviral delivery vehicles to a certain extent. Nonviral vectors include physical and chemical methods, which are most suitable for the ex vivo-based gene-editing technique [29]. Electroporation, hydrodynamic injection, and microinjection are some of the well-known physical methods utilized for CRISPR/Cas9 delivery directly into the cells, whereas the use of lipid nanoparticles and polymeric nanoparticles has gained popularity as the chemical method for CRISPR/Cas9 delivery [30] due to their biocompatible nature, low immunogenic risk, and flexible loading capacity.

CRISPR/Cas9 systems are delivered via these carriers in three different formats. The first format relies on the delivery of a plasmid DNA encoding both the Cas9 protein and the sgRNA (Fig. 2b) [31]. The second format posits the delivery of a Cas9 mRNA (Fig. 2b) and sgRNA where the host cellular machinery translates the Cas9 mRNA molecule and finally the delivery of Cas9 and sgRNA as a ribonucleoprotein complex [32] (Fig. 2b). Now each of these formats has a fascinating outlook to make CRISPR/Cas9 delivery a success story, nevertheless burdened with few shortcomings. For example, plasmid DNA may often result in the persistent expression of the CRISPR/Cas9 elements that might lead to consequential off-target effects such as undesired genetic mutations. mRNA and protein delivery compared to plasmid DNA offer safety in circumventing off-target effects and effective gene-editing outcomes, however limited by their short half-life property [25]. In this section, we will discuss in detail the pros and cons of the different types of CRISPR/Cas9 delivery vehicles and how each of them has presented to be the front-runner to be potentially translated into a clinical setting for genome-editing purposes.

4.1 Viral-Mediated Delivery

Viruses are the natural vectors that possess the ability of tropism and high transduction efficiency, which can be exploited to deliver the components of the CRISPR/Cas9 machinery. The delivery of the foreign genetic material via viral carrier is accomplished through fundamental steps of infection and replication [33]. The infection stage involves the recognition and entering of the specific cells via cell surface receptors followed by internalization where the viral genome releases its genetic material in the cytoplasm for RNA-based viruses or the nucleus for DNA-based viruses. Once the nucleic acid is released, it undergoes replication to produce copies of the virus, which then exits the cells to repeat the infection-replication cycle in the neighboring cells [34]. Currently, the widely used viral vectors for CRISPR/Cas9-mediated genome editing include adeno-associated virus (AAV), lentivirus (LV), and adenovirus (AdV) (Fig. 2c) [35].

Adeno-associated virus (AAV) is a single-stranded DNA virus belonging to the genus *Dependovirus* and *Parvoviridae* family and is a popular choice for the CRISPR/Cas9 delivery [36]. The reasons for using AAV as the first choice for genome-editing purposes in recent times are multifarious. To begin with, the safety

profile of these vectors is well-established through numerous clinical trials in gene augmentation therapies [37]. AAV has multiple serotypes that allow them to transduce multiple cell types without provoking acute innate or adaptive immune responses imparting low immunogenicity contrary to other viruses [36]. The advantage of having multiple serotypes manifests to develop vectors for tissue-targeted gene-editing strategies. In addition to that, it does not contribute to tumorigenesis because after transduction it largely remains episomal and integrates only to the mitochondrial DNA hotspots and specific location of chromosome 19 called AAVS1, where both the regions are considered to be safe [38, 39]. Furthermore, AAVs impart persistent transgene expression not only in dividing cells [40] but also exist long term in nondividing cells either as exogenous DNA or with some modifications [41]. Finally, AAVs are nontoxic or mildly toxic even at high doses in reported animal studies [37]. Naturally, AAVs have a single-stranded DNA genome of 4.7 kb in length encapsulated by an icosahedral protein capsid, though the recombinant versions used for gene-editing applications are composed of double-stranded DNA devoid of any genes that may express viral replication proteins freeing up space for the transgenes or CRISPR components [42, 43]. A few examples of clinically successful AAV-mediated gene therapy are Zolgensma®, Glybera®, and Luxturna® [44]. There are four different methods of dual AAV created to meet the objective of packaging large foreign genes: overlapping, fragmented, trans-splicing, and hybrid [45]. The overlapping AAV system features two transgenes having a specified overlap flanked by an inverted terminal repeat (ITR) sequences on both the ends to mediate AAV genome recombination [46]. In the case of a fragmented dual AAV system, transgenes are truncated at unspecified points to be packaged as incomplete fragments inside separate AAVs, which then translates into a complete target protein once the DNA strands with the overlapping regions undergo homologous recombination (HR) [47]. Hybrid AAV systems comprise an overlapping region and splice donor/acceptor sites and express the final transgene product after a combination of HR processes aided by the overlapping region and trans-splicing approaches by the splicing sites [48]. The trans-splicing dual AAV vectors are the only systems that do not feature an overlapping region; instead, the transgene fragments are separated by ITR, which facilitates 5′ end to 3′ end joining. The ITR is then removed by splicing mechanisms to create the complete transgene product [49]. The use of dual AAV systems for gene therapy research is the current favorite with researchers due to their expanded size capacity to incorporate all the essential elements of the CRISPR/Cas9 system and other elements like reporters, DNA templates, etc. to meet the gene-editing objectives. Dual AAV systems were harnessed by Bak et al. [50], to serve as donor DNA templates in separate AAV vectors when co-transduced to cells (primary human T-cells and $CD34^+$ hematopoietic stem cells and progenitor cells) to facilitate large transgene cassette integration via consecutive HR. This methodology is a single-step procedure allowing the integration of transgenes of larger size in the genomes of primary cells via HR-mediated genome editing. The progress in the development of AAV-mediated CRISPR/Cas9 delivery ever since its first report in gene editing has been unparalleled, and several evidence

of its applications in a myriad of disease models like blood-related disorders [51], muscular disorders [52], and metabolic diseases have been demonstrated [53].

Lentivirus (LV) is a single-stranded RNA spherical virus that belongs to the *Retroviridae* family transducing both dividing and nondividing cells, thus having a broad tropism. Being a retrovirus, the replication of lentiviral genomic materials occurs via reverse transcription in the host cells. Once the virion infects the cell, it is endocytosed via the host cellular receptors, and the virion releases its core components like integrase and reverses transcriptase enzymes. The reverse transcriptase enzyme transcribes the cDNA from the viral RNA utilizing the host nucleotides, followed by nuclear transportation of the cDNA and integration into the host genome [54]. LV vectors have a cargo-carrying capacity of 8 kb [55] making provision for the simultaneous delivery of Cas9 protein and sgRNA and reported to elicit low immunogenicity as the vital genes are removed from the viral genome before its application [56]. The safety profile in the current generation of the LV vectors is ensured by splitting the essential genes across three different plasmids to prevent any production of viable virions inside the host cell [57]. Another advantage of using LV vectors is they can be pseudotyped with other viral proteins to tune their cellular tropism [57]. The genome integration ability of LV can be leveraged to some extent in case stable long-term transgene expression is required to construct gene libraries to study disease mechanisms [58, 59]. However, this feature is not feasible for ensuing therapeutic applications as the integration of transgenes at undesired locations such as protooncogenic sites will result in the likelihood of developing random mutations, which will eventually give rise to tumorigenesis. Therefore, non-integrating LV vectors are designed and developed in a way to eliminate the activities of the integrase coding region while [60] still keeping the functions of reverse transcriptase and nuclear transport activity of the pre-integrating complex intact [61], thus finding a niche for CRISPR/Cas9 delivery purposes.

Studies related to the usage of LV delivery vectors for CRISPR elements in diverse therapeutic applications, for example, cancer [62], HIV [63], eye-related disorders [61], etc., have been encouraging so far. In another study conducted on HIV-related infection, Hou et al. used the LV-mediated CRISPR/Cas9 gene-editing technique proposed to disrupt the CXCR4 gene, which is responsible for the entry of HIV into the CD4+ human T-cells [64]. The results of this study elucidated that Cas9-mediated ablation of CXCR4 conferred resistance to the CD4+ T-cells against HIV-1 infection, without eliciting any off-target effects or affecting the cellular proliferation [64]. Other viral-related infections caused by the retrovirus porcine reproductive and respiratory syndrome virus (PRRSV) were also studied to disrupt the viral RNA via LV-mediated CRISPR/Cas 13b system [65]. The authors strategized the design of a single vector system to incorporate double crRNAs against two PRRSV genes (ORF5 and ORF7) and also the Cas 13b protein. The uniform delivery of the CRISPR components to the transgenic MARC-145 cells resulted in the stable knockdown of the target genes concurrently to abrogate viral infections [65]. Additionally, LV-mediated CRISPR/Cas9 also finds their role in constructing gene libraries and genome screening that has been exploited in numerous independent studies, for example, creating genome screens to identify genes involved in T-cell

activation [66], investigating various host factors involved in the replication of influenza virus [67] and norovirus causing gastroenteritis [68], and finally detecting novel drug targets conferring sorafenib resistance in hepatocellular carcinoma [69].

Adenovirus (AdV) from the *Adenoviridae* family is a non-enveloped virus with an icosahedral nucleocapsid that possesses a linear double-stranded DNA genome. AdVs are responsible for causing a range of illnesses including cold-like symptoms, sore throat, fever, and bronchitis, to name a few [45]. The AdV genome is approximately 34–43 kb long flanked by two ITRs, and following infection into the host cells, it largely remains extrachromosomal, thus limiting potential off-target effects associated with the host genome integration [45, 70]. Due to its episomal nature similar to the AAVs, it can be used for transduction purposes in both dividing and nondividing cells, thereafter been optimized and developed over the years to serve as a CRISPR/Cas9 delivery vector. The first-generation AdV vectors even though devoid of the viral gene E1 were still causing acute and chronic immune responses, which could be inferred from the viral capsid and viral gene expression respectively accompanied by severe toxicities [71]. The second- and third-generation AdV vectors were designed to delete the viral genes E2 and E4, thus mitigating chronic immune responses [72, 73]. The current generation of AdV vectors is devoid of any viral genes, possesses ITRs and encapsidation ψ, and has a packaging capacity of 35 kb sufficient enough to deliver all the components of the CRISPR system at one go [74, 75]. The state-of-the-art AdV vectors still carry the potential to provoke acute immune responses in humans due to constant exposure to the virus, which might generate neutralizing antibodies. This preexisting immunity might be a bottleneck in the clinical efficacy of gene augmentation therapies while using these AdV vectors. Even with these caveats, the use of AdV vectors in CRISPR/Cas9 delivery has been studied extensively in the past and recently in diverse areas such as treating diseases, establishing disease models, and as a tool for drug discovery [76].

Wang et al. successfully attempted to model nonalcoholic steatohepatitis (NASH), a condition in which excessive fat buildup in the liver is reported, by targeting the Pten gene involved in NASH and as a negative regulator in the PI3K-Akt pathway active in the liver [77]. AdV vector was employed to deliver the Sp-Cas9 system targeting the Pten gene in mouse liver, demonstrating features of NASH and a substantial increase in hepatomegaly after 4 months of injection. Both humoral and cellular immune responses were also reported against Sp-Cas9, which provides an idea to understand immune responses against Cas9 and model human liver disease in experimental animal models [77]. In another example of disease modeling using AdV-mediated CRISPR delivery involving cancer, Maddalo et al. tried to generate chromosomal rearrangements in a mouse model leading to gene fusions that play a central role in cancer pathogenesis [78]. The authors used an AdV-based CRISPR/Cas9 delivery system in somatic cells to induce genetic fusion products of the genes echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) via chromosomal rearrangements causing non-small cell lung cancer (NSCLC) in humans and also develop resistance against ALK inhibitors [78]. For the treatment of diseases using AdV vectors, promising results have come

out so far in the direction of reducing plasma cholesterol levels in the mouse liver by targeting PCSK9 [79], improving the quality of life in Duchenne muscular dystrophy (DMD) patients by restoring muscle function in in vivo *mdx* mice [80], and also generating HIV-resisting CD4+ T-cells [81].

4.2 Physical Transduction

Physical methods of transduction for CRISPR/Cas9 delivery do not include the use of external vectors, instead employ electrical energy, mechanical forces, and thermal energy to directly introduce the cargo into the target cells. There are no limitations in cargo size and also the internalization of the cargo does not depend on endocytosis but rather on the diffusion of the cargo owing to a compromised cell membrane. The widely adopted techniques for the physical method of CRISPR/Cas9 delivery include microinjection, electroporation (Fig. 2c), and hydrodynamic injection. Unlike viral vectors, these techniques are not accompanied by drawbacks like off-target effects, genome integration, and limited packaging size.

Microinjection is the "gold standard" technique that involves the mechanical transfection of the CRISPR components using a micro-sized capillary needle directly into the cells [57, 82]. The common cargo choice for microinjection delivery is nucleic acids, which are introduced to the cells by three different modes: (i) directly as DNA to the nucleus, in vitro transcribed mRNA to the (ii) cytoplasm, and (iii) nucleus [57]. For CRISPR cargo delivery, plasmid DNA and mRNA encoding the Cas9 protein and sgRNA or the Cas9-sgRNA complex with the help of a microscopic needle of 0.5–5.0 μM diameter can be directly injected into the target site inside the cell cytoplasm or nucleus circumventing cellular barriers like extracellular matrices and other cytoplasmic contents, finally allowing to have a high delivery efficiency and improved transgene expression, thus minimizing off-target effects [57]. Using microinjection-mediated delivery in CRISPR gene editing overcomes the limited packaging size encountered with viral delivery and cargo of any molecular weight and quantities could be delivered simply. The technique is more suitable for in vitro and ex vivo transfection due to its precise delivery to the target site which often precludes the use in in vivo experimentation and is also accompanied by arduous procedures [82].

Another well-established method of gene-editing tools that has stood the test of time is electroporation utilizing an electrical field to transfer genes of interest into cells [83]. Short pulses of high voltage electrical currents when administered to cells result in the transient opening of the cellular membranes in the form of nano-sized pores that allows the transfer of the cargo inside the cell cytoplasm [57]. Parameters to be considered for the effective cargo delivery via electroporation method include the amount of voltage, duration of the electrical pulse, type of cells, type of target molecules, and the buffer solution to ensure reduced cell death and reverse cellular permeabilization [82]. Often electroporation has shown to pose cellular health risks in terms of pH and ionic imbalance, heat shock to the cells, and

buffer solutions affecting cell viability [82]. Despite all these concerning issues, it still finds a place to be a sought-after choice for scientists to conduct gene editing due to its simple and efficient nature making it a commercially viable option. To ensure the cell health and other setbacks encountered during the conventional electroporation process such as bubble formation and nonuniform distribution of electric field, manufacturers have now started coming up with next-generation solutions to confront such issues like developing improved buffer solutions to minimize cell death [84] contrary to regular phosphate buffer saline and incorporating nanotechnology like nanostraws [85] to enhance the local electric field preventing bubble formation, respectively. For CRISPR/Cas9 delivery to a myriad of cell types, electroporation can deliver all three types of cargo with RNP as a more frequently opted cargo due to its relatively smaller size and easier delivery. This approach to effectively target T-cells and perform efficient gene editing in primary T-cells was conducted by Seki et al., using an optimized Cas9/RNP cargo delivery via electroporation [86]. The study demonstrated that a single transfection of Cas9/RNP in both human and mouse T-cells resulted in the knockout of the target gene measured by the reduced protein expression and did not require the prior T-cell activation, allowing to perform studies on genes that are involved in the activation and differentiation of T-cells [86]. As a proof of concept, the group performed to create knockouts of cell surface receptors such as CXCR4, CTLA4, PD-1, transcription factors, and cytokines that will be relevant for developing effective immunotherapies using this optimized approach [86]. Nucleofection is a specialized electroporation technique developed to deliver cargoes to the nucleus directly without compromising the nuclear envelope or disturbing the cells in the cell division state [57]. Few examples of this technique have been applied to generate resistance against HIV infection by introducing CCR5Δ32 mutation to human cells [87], developing cancer models [88], and also curing herpesvirus infections by CRISPR-mediated destruction of viral genomes [89]. Shortcomings currently accompanying electroporation include the inability to transfect certain cell types with the risk of damaging the mammalian cells due to repeated exposure to electrical currents and also not feasible for in vivo studies.

The hydrodynamic injection is another method of physical transduction initially developed to deliver naked DNA in vivo but also found its application in siRNA delivery and other molecules [90–92]. Hydrodynamic delivery involves injecting large volumes of a solution containing the gene cargo solution rapidly into the blood circulation of an animal via the tail vein [54, 57]. The rapid pushing of the gene solution creates a hydrodynamic pressure resulting in the cell membrane permeabilization transiently enabling gene transfer into parenchymal and endothelial cells [54]. The technique is quite advantageous for liver-specific delivery of gene-editing cargo. Once the gene solution enters the bloodstream via the inferior vena cava to finally reach the heart, the excess gene solution in the heart flows into the hepatic vein in a retrograde manner, ultimately finding its destination in other tissues like kidneys, muscles, and lungs [93]. Furthermore, to achieve more tissue-specific delivery, the gene solution could be injected directly into the blood vessels supplying the organ of interest [93, 94]. Gene delivery by hydrodynamic injection is

advantageous to other physical transduction techniques due to its applicability in animals, simplicity, and without the requirement of a sophisticated instrument. CRISPR/Cas9-mediated genome editing in the human metabolic disease model could be undertaken effectively by hydrodynamic injection as evidentially through several studies, for example, hereditary tyrosinemia caused by Fah mutation that encodes fumarylacetoacetate hydrolase. Separate groups have previously attempted to rectify the Fah-mutated gene in hereditary tyrosinemia through different approaches [95, 96] in Fah-deficient mice thus restoring the mutation to therapeutically cure the disease. Similarly, many infectious diseases caused by the hepatitis B virus (HBV) have been conducted independently to target the virus via hydrodynamic delivery of CRISPR/Cas9 components [97]. Zhen et al. investigated targeting the surface antigen HBsAg-encoding region of HBV via CRISPR/Cas9 gene-editing approach [98]. Their results demonstrated the inhibition of HBV replication and antigen expression by downregulation in the HBV DNA and HBsAg levels from the qPCR, ELISA, and immunohistochemical analysis, respectively [98]. In addition to its application in therapeutic purposes, hydrodynamic delivery of CRISPR/Cas9 components was also studied to create cancer disease models [99, 100] and assist in exploring the molecular basis of cancer pathogenesis. Despite the success in the application of hydrodynamic delivery of CRISPR/Cas9 components, it still struggles to pave its way to clinical translation due to the requirement of large volumes of gene solution for human administration, which is not clinically feasible [54]. Moreover, there have been adverse side effects such as an increase in intravascular pressure [101] and hemodynamic changes [102] observed upon injection that might lead to trauma and other associated long-term morphological complications in organs.

4.3 Nonviral Transduction

As discussed in the earlier sections, the use of viral carriers and physical transduction methods for CRISPR delivery is reputable in innumerable ways and has seen considerable success in a plethora of experimental studies to date. Nonetheless, the side effects that accompany these delivery methods, for instance, gene integration, sustained Cas9 expression triggering off-target effects, in the long run, cellular damage due to external forces involved in physical transduction methods, etc. precincts their likelihood of translating into clinics. Since CRISPR/Cas9 has demonstrated immense possibilities as a resourceful tool for gene-editing purposes, the designing of a suitable vector for the safe and effective delivery of the CRISPR components would propel their applications in the clinic. Nonviral delivery methods including the use of nanoparticles have attracted attention in the past few decades as a practical solution for a myriad of biomedical applications owing to their facile preparation methods, safety, and biocompatibility profile, among others. Henceforth, the idea of implementing these nanoscale carriers for the delivery of CRISPR/Cas9 cargo could evade all the shortcomings arising from viral carriers and physical

transduction methods and accomplish the goal of targeted and effective genome editing. Nanoparticles such as liposomes, lipid nanoparticles, polymeric nanoparticles, and cell-penetrating peptides (CPPs) (Fig. 2c) are some noteworthy examples of nonviral carriers utilized for CRISPR delivery in recent years. These carriers bear low immunogenicity risks, high cargo-loading capacity, flexibility in designing parameters, easy scalability, and cost-effectiveness and also protect the CRISPR/Cas9 cargo from the physiological environment increasing their half-life.

Lipid nanoparticles have been in use as a delivery vehicle for a variety of molecules including nucleic acids for multiple decades. In delivering CRISPR/Cas9 via lipid nanoparticles, two strategies are generally followed: Cas9 and sgRNA delivery in the form of plasmid or mRNA and Cas9-sgRNA RNP complexes [57]. The anionic nature of nucleic acids makes them impermeable to cellular internalization; hence, encapsulating them inside cationic lipid-based vectors eases their delivery inside the cells without raising any immunogenic and toxicity concerns. A few considerations that must be addressed while using lipid nanoparticles for CRISPR/Cas9 delivery are an endosomal escape to avoid lysosomal degradation and the ability to translocate to the nucleus to ensure high efficacies. Liposomes are another class of lipid-based vectors characterized by an aqueous core surrounded by one or two concentric layers of hydrophobic lipid bilayers. Based on their physical characteristics liposomes used for the delivery of CRISPR/Cas9 components are divided into four categories: stable nucleic acid lipid particles, lipopolyplexes, lipoplexes, and membrane/core nanoparticles [103], and parameters concerning liposomal fusogenicity, surface charge, PEGylation degree, size, type of cargo, and target location will finally affect the delivery efficiency [104]. Lipid nanoparticles differ from liposomal vectors structurally where it lacks the continuous hydrophobic bilayer and the inner aqueous pool [105]. The common feature between lipid nanoparticles and liposomes is that they have natural phospholipid composition, cationic/ionizable lipids, cholesterol moieties, and PEG-lipids, and over the years, developments are made to replace the permanently charged lipids (DOTAP and DOTMA) with ionizable cationic lipids (ALC-0315) improving the transfection efficiency and minimizing toxicity for the delivery of nucleic acids [26]. Lipofectamine is one of the commercially available common cationic liposomal formulations widely used for gene delivery. Due to its cationic nature, it complexes readily with the anionic nucleic acids, followed by endocytosis which takes place because of the complex formation with the negatively charged cell membrane [57].

Polymeric nanoparticles are another category of nonviral carriers popular with drug delivery researchers for their unparalleled strengths in terms of their biocompatibility, biodegradability, and controlled drug release features. Frequently used polymers for gene and protein delivery include poly-lactic-co-glycolic-acid (PLGA), polyethyleneimine (PEI), and poly (β-amino ester) (PBAE), to name a few [26]. PEI and chitosan are cationic polymers commonly utilized for the delivery of different nucleic acid types like mRNA and plasmid DNA, hence a suitable carrier choice for CRISPR/Cas9 cargo delivery. Polymeric nanoparticles like lipid-based vectors traverse the cellular membrane and are endocytosed while protecting the cargo from nucleases [31]. Polymers offer more flexibility in terms of their design

functionality and chemical composition that helps scientists to continuously evolve the design parameters and generate multifunctional polymers like amphiphilic block polymers [106] and stimuli-responsive polymers [107] to achieve target specificity while delivering CRISPR/Cas9 cargo for effective genome editing.

Cell-penetrating peptides (CPPs) are polycationic, amphipathic/nonpolar short stretches of amino acids facilitating the uptake of different proteins into a variety of cell types. CPPs are an interesting choice for the delivery of CRISPR/Cas9 RNP complexes where they can be attached covalently to the Cas9 protein followed by complexation with the sgRNA ready for delivery into the cells. CPPs could be used for in vitro and ex vivo CRISPR/Cas9 delivery purposes requiring an extensive optimization process to ensure efficiency in the transfection [57]. Peptide nanoparticles have garnered interest in recent years as an additional carrier option for CRISPR/Cas9 delivery in addition to the existing list of nonviral vectors. Peptides not only could be used as the core backbone material but also find their application as a surface-modifying molecule [26]. Peptide nanoparticles could be formed by the self-assembly process [108] providing protection to the cargo, assisting in endosomal escape, and achieving intracellular targeting [109]. Several preclinical studies [110–112] have been conducted to demonstrate the ability of peptide nanoparticle-mediated delivery of CRISPR/Cas9 payload, though there's a long road before peptide nanoparticles to reach clinical translation in CRISPR therapeutics.

5 Nanoparticles in CRISPR/Cas9 Delivery

The concept of "genome editing" is not new in the pharmaceutical field and focuses on the betterment of human society through research findings. This permits the exactly designated change of genomic groupings of living cells and the entire creatures, filling in as a useful asset in organic fields, and the adjustment interaction for hereditary problem treatment. The initial phase during the time spent exact genome altering at the objective site by the nuclease actuating a twofold abandoned break. CRISPR-based genome-altering innovation has opened a succeeding possible stage for the restorative viewpoint of genomic altering [113]. Although accessibility of CRISPR system technology is new to the genetic engineering field, technically versatile insusceptible components are utilized by numerous microbes to safeguard themselves from unfamiliar nucleic acids, for example, infections or plasmids. Because of expanded flexibility, straightforwardness, and viability, the CRISPR/Cas9 frameworks have incredible potential for RNA-directed exact genome altering in different cell types. A vital challenge includes providing a protective, proficient, and clinically appropriate delivery of a CRISPR-associated protein and a single-guide (sg) RNA. The key credits incorporate (i) a transient, non-coordinating Cas9 articulation build to restrict potential off-target occasions, foundational harmfulness to typical cells/tissue, surprising spillage, insusceptible reactions, and reconciliation occasions into the genome, (ii) ingenious conveyance capacitating the vehicle of the little to enormous Cas9 protein (or its encoding mRNA) including numerous

sgRNAs, (iii) controlled dose in pertinence to the degree of altering, and (iv) versatility of the definition empowering gross-scale assembling to address different infectious diseases [114]. A typical conceivable translational way to deal with the application of CRISPR-based innovation is by using viral vectors, for example, an adeno-related virus. However, such vectors may induce off-target impacts and the risk of immunogenicity in vivo upon long-standing exposure. The inefficiency of clinically relevant delivery systems hinders the advancement of CRISPR/Cas9 from proof-of-principle to in vivo clinical treatment explicitly, and the clinical relevance of the delivery system along with the capability to target the site of delivery and minimizing immune system stimulation are top priorities [115]. Nonviral conveyance frameworks, for example, nanoparticles (NPs)-based conveyance techniques, can resolve these issues. The beauty of NPs delivery systems in gene therapeutics is arising as a result of its ability in specific targeting, tuneable sizing of the particles, the power of customization, minimized activation of the immune response, and reduced exposure to nucleases [116].

5.1 Polymeric-Based CRISPR/Cas9 Delivery System

Systemic and sustained release polymeric gene systems guarantee efficient uptake, resistance to nuclease degradation, strong biocompatibility, strong biodegradability, and controlled dose-dependent administration of the gene to the targeted site of delivery [117]. The bioavailability of the therapy can be amplified using biodegradable NPs by postponing the untimely release of the biomaterial from the body [118]. Cellular uptake of polymeric NPs can be done by conjugating with superficial cell-infiltrating peptides, and for delivery to the nucleus, the single peptide can be localized inside the nucleus [119]. Research depicts that compared to lipids, polymer-based NPs convey CRISPR genome-altering material with prevalent altering viability and target specificity [120]. Polymeric transporters are normally intended to cross layers and safeguard the payloads from immunological reactions and corruption pathways and can have explicit capacities for various purposes in vivo and in vitro. They impeccably encapsulate various cargos followed by explicit in vivo focusing of receptors in applicable cell films and delivering triggers of explicit intracellular microenvironments.

Poly-lactic-co-glycolic acid (PLGA) copolymer has been long employed in vaccine and drug delivery. A recent study conducted has successfully developed polyethylene glycol (PEG)-PLGA-based lipid-assisted NPs (CLAN) encapsulating CRISPR/Cas9 expressing guide (g) RNA targeting the overhanging fusion region of the breakpoint cluster region (BCR) and the Abelson murine leukemia viral oncogene homolog (ABL) (pCas9/gBCR-ABL) usually disrupted in chronic myeloid leukemia (CML). Through intravenous infusion, CLANs stacked with pCas9/gBCR-ABL proficiently took out the BCR-ABL combination quality of CML cells subsequently working on the endurance of a CML mouse model while saving the BCR and ABL qualities in ordinary cells. This experiment superbly exhibits the

immeasurable potential of combining the CRISPR/Cas9 system with nanocarrier strategy for targeted therapies [121]. Another study displayed the capability of CLAN encapsulated with CRISPR/Cas9 plasmid that is macrophage-specific promoting the expression of Cas9 in macrophages and their precursor monocytes both in vitro and in vivo. The human CD68 advertiser, equipped for driving explicit gene articulation in monocytes and macrophages, was utilized for the macrophage-explicit gene-altering technique. The CRISPR/Cas9 was encoded with gRNA to disrupt the netrin (Ntn)-1 gene for enhancing symptoms of type 2 diabetes. The Ntn1 gene present in the normal cells was not hindered because of the particular articulation of Cas9 by the CD68 promoter [106].

Hyperbranched cationic poly (β-amino esters) (PBAEs) have recently procured interest as an equipped quality conveyance specialist for exceptionally negatively charged nucleic acids. Rui et al. [122] designed and approved a progression of carboxylated branched PBAE encapsulating a wide range of protein types into self-assembled NPs. The polymer terminating with C5 was found to be outperforming the other ligands in the degree of cellular intake and endosomal interruption. The C5 polymer NPs induced CRISPR/Cas9 ribonuclease protein (RNP) delivery inducing the gene alteration in engineered orthotopic mouse brain tumors in vivo [122]. Branched polyethyleneimine (PEI) 25 kDa was utilized to convey CRISPR/Cas9 encoding with single gRNA in Neuro2a cells for conveying genome-altering materials for genetic control. Specialists accept that PEI isoform-interceded gene therapy in the cochlea by delivering Cas9 straightforwardly to the internal ear of infected animal models can be contemplated to safeguard genetic disorders [123]. Nonetheless, delivery of an enormous bulk of plasmid vector would build the cytotoxicity brought about by BPEI-25K, restricting its effectiveness. Therefore, coating the carrier with PEG can modify the purpose of CRISPR/Cas9 gene therapy. This can improve efficiency, can minimize cytotoxicity, and can be implied to target specific tissues such as tumors [124].

PEGylation of chitosan nanocomplexes was found to be suitable as carriers for delivering CRISPR/Cas9 genome-editing systems in vitro in a mucus model and HEK293 cell lines, attaining optimal transfectant efficiency at N/P (amine/phosphatase) ratios of 5 at pH 6.5 and 6.8. The PEGylated chitosan not only improved the mucus-penetration capability of nanocomplexes but also secured CRISPR/Cas9 in the setup of DNA against DNase I digestion and nebulization [125]. Poly (beta-amino ester) NPs being biodegradable were designed to co-deliver CRISPR gene-editing complexes with Cas9 and short gRNA as plasmid DNA. This CRISPR-stop reporter system was to enable gene knockout mediating cleavage of iRFP fluorescent reporter can be hushed by indels after 1-cut alters, as well as gene deletion involving a ReNL reporter upstream expression stopper using 2-cut edits for gain-of-function ReNL expression. The expression cassette was duplicated into a piggyBac transposon framework which can be utilized in generating stably articulating cell lines for further exploration of gene-editing efficacy in vitro, terminating culturing of primary cells from the Ai9 mouse. These results present the numerous possibilities of designing and screening cutting-edge nonviral conveyance frameworks committed to CRISPR/Cas9 gene altering [126].

5.2 Lipid-Based CRISPR/Cas9 Delivery System

Probably the earliest modes of gene therapy include lipid-mediated gene transfer. 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) [127] and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [128] are widely used commercial cationic lipids in this delivery systems. The cationic lipids have three areas: a cationic head section, a hydrophobic tail, and a linker between these two areas. Apart from the CRISPR/Cas9 delivery, the lipid layer protects enzymatic degradation, RNases, and immunological reactions [129]. Once the nanoparticle is encased within an endosome after passing through the surface of the cell, it tends to be coordinated into the lysosomal pathway by the cell, causing the corruption of all lysosome constituents. Escaping the endosome is a crucial factor. Likewise, if the Cas9/sgRNA complex escapes the endosome, it should translocate to the nucleus, which can likewise be a possible weak spot. Other variables incorporate the size of the freight, the objective cell type, and the kinds of lipids that are proper or valuable in the framework [57]. The two types of lipid-based vectors included liposomes and solid lipid NPs (LNPs).

Liposomes are irrefutably the most broadly investigated transporters for CRISPR/Cas9 conveyance. Liposomes have high encapsulation efficiency, carry complex cargo, minimize unintended immune activation, and have meticulous control over drug delivery. Multiple parameters are entangled in determining the gene delivery efficiency. They include the size, surface charge, PEGylation, ligands targeted, type of CRISPR/Cas9, and site of delivery [104]. Anionic liposomes and neutral liposomes are less efficient in encapsulating CRISPR/Cas9 as they have passive loading capacity, poor circulation time, and inadequate tumor penetration. Although PEGylation of these liposomes is the next best choice, it is a tedious operation, and PEGylated anionic liposomes would display similar to unPEGylated neutral liposomes. Therefore, these liposomes do not serve as potential applicants for a gene-editing conveyance framework. The pH-sensitive cationic liposomes with stable nucleic acid lipid particle are PEGylated and have gene slicing capabilities in vivo. They have high encapsulation capacity for CRISPR/Cas9, neutral surface charge with generally lengthy flow time, and effective cancer infiltration, guaranteeing maximum cancer aggregation [130].

Lipoplex is a type of liposomal CRISPR/Cas9 that can be formed only with cationic liposomes. In a lipoplex, lipids from various bilayers and CRISPR/Cas9 are inserted between nearby lipid bilayers. Synthesizing lipoplex involves the simple method of blending cationic liposomes and CRISPR/Cas9 at known proportions. For example, PEGylated lipoplexes were developed for the conveyance of CRISPR/Cas9 to knockout human papillomavirus E6 and E7 oncogenes in cervical cancer. Two variants of Cas9, wild-type (WT) and highly specific FokI-dCas9, were used to study the editing efficiency. This delivery method conferred the foundational delivery of the CRISPR/Cas9 system by minimizing cytotoxicity, proper targeting of the organ, and internalization of the cargo by the cells [131]. A light-sensitive liposome incorporating verteporfin into the lipid bilayer for the delivery of the CRISPR/Cas9

gene-editing system. Verteporfin can lead to damage to the liposomal structure for the delivery of CRISPR when coming in contact with light by reacting with the available oxygen, releasing singlet oxygen that oxidizes unsaturated lipids. Liposomal delivery induced the reduction in eGFP fluorescence intensity and tumor necrosis factor α-induced protein (TNFAIP) 3 gene in HEK293 cells. In vivo analysis using zebrafish also confirmed the deficiency of green fluorescent signal over individual slow-twitch muscle fibers demonstrating the diminishing of eGFP expression; however, the control group deprived of eGFP sgRNA displayed no defect of the green fluorescent signal [132]. To lay out a framework for the concurrent conveyance of protein and nucleic acids, Chen et al. [133] designed and synthesized liposome-templated hydrogel NPs (LHNPs) for the efficient delivery of CRISPR/Cas9 gene-altering system in a mouse flank tumor model. The LHNPs were also engineered through an autocatalytic brain tumor-targeting (ABTT) mechanism designated for the conveyance of CRISPR/Cas9 to cancers in the cerebrum.

By far most of LNPs presently utilized in clinical applications bring about bioaccumulation in the liver and possible dangers as they comprise a nondegradable ionizable lipid. To address this issue, Finn et al. [114] designed LNPs (LNP-INT01) to be biodegradable utilizing labile ester linkages. In contrast to the commercially used DLin-MC3-DMA with no apparent corruption over 24 h, LNP-INT01 was found to be cleared with a T1/2 of ∼6 h from the liver. These LNPs were designed to deliver CRISPR/Cas9 components (LNP-INT01), for targeting the mouse transthyretin (TTR) gene, the homolog of objective for corrective gene alteration in the treatment of amyloidosis in humans. Through the aid of LNP-INT01, in vivo editing resulted in >97% knocked down of the mouse TTR protein. This delivery system was found to provide clinically pertinent levels of CRISPR/Cas9 genome editing sustainable over 52 weeks following a single dose. The powerful and solid knockdown in a solitary organization in vivo editing was achieved due to the chemical modification pattern in sgRNA. The designed lipid NPs can fill in as a clinically feasible treatment for liver-based hereditary illnesses as they are synthetically prepared, scalable, and nonviral. Zhang et al. [134] designed cationic lipid nanoparticles modified with polyethylene glycol phospholipid (PLNP) encapsulating Cas9/single-guide RNA (sgRNA) plasmid (DNA) to frame a core-shell structure (PLNP/DNA) focusing on barriers that are usually met with the current commercial lipid systems like Lipofectamine 2000. These barriers are probably due to the enormous quantity of Cas9/sgRNA-fused plasmid (>10,000 bp) and mediocre encapsulation of anionic plasmid causing repelling from the like-charged cell membrane. The PLNP/DNA system was capable of mediating interceding 47.4% effective transfection of Cas9/sgPLK-1 plasmid in A375 cells in vitro (Fig. 3) [134]. LNP is also designed to convey Cas9 mRNA alongside single-guide RNA that designated genome editing of *SERPINC1* encoding antithrombin (AT) in the mouse liver. Improvement in thrombin generation was observed through LNP-mediated inhibition of AT. Bleeding-related phenotypes were recuperated in both hemophilia A and B mice. No dynamic off-targets, liver-incited poisonousness, and significant autoimmune enemies of Cas9 are recognized. The LNP-interceded CRISPR/Cas9 conveyance fills in as a protected and capable methodology exerting durable therapeutic effects with a possibility of limited dose requirement [135].

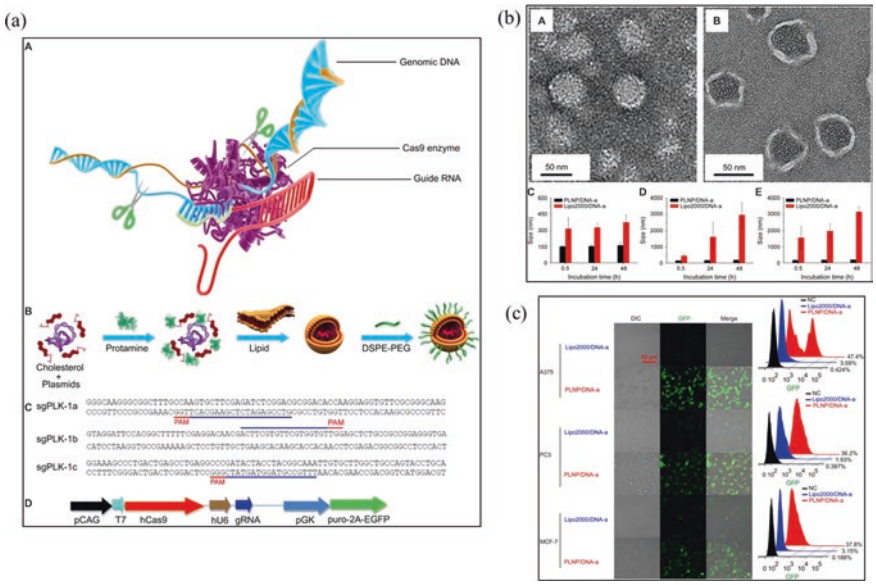

Fig. 3 (**a**) Schematic illustration of the strategy for delivering CRISPR/Cas9 system targeting tumors. (A) Schematic illustration of the CRISPR/Cas9 system; (B) The packaging and encapsulation processes of the Cas9-sgRNA plasmid by chondroitin sulfate, protamine, 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), dioleoylphosphatidylethanolamine (DOPE), and DSPE-PEG; (C) The targeting sites of different single-guide RNAs (sgRNAs) on human Polo-like kinase 1 (PLK-1) locus indicated by blue lines with corresponding protospacer adjacent motifs (PAMs); (D) The map of the Cas9/sgPLK-1-fused plasmid. pCAG, chicken β-actin promoter with cytomegalovirus enhancer; T7, T7 RNA polymerase promoter; T7 promoter can drive transcription of the downstream coding of hCas9 in the presence of T7 RNA polymerase. EGFP, enhanced green fluorescent protein; gRNA, guide RNA; hCas9, human Cas9 endonuclease; hU6, human U6 promoter; pGK, mouse phosphoglycerate kinase 1 promoter; puro, Puro gene; 2A, "self-cleaving" 2A sequenc. (**b**) High-resolution transmission electron microscopy (HR-TEM) and dynamic light scattering (DLS) analysis of the nanoparticles. HR-TEM image of chondroitin/protamine/Cas9-sgRNA plasmid DNA (A) and PLNP/DNA-a (B), DLS analysis of the PLNP/DNA-a and Lipo2000/DNA-a in serum-free Dulbecco's modified Eagle's medium (DMEM) (C), Opti-MEM reduced serum medium (D) and 10% fetal bovine serum (FBS) DMEM (E). (**c**) Confocal laser scanning microscopy (CLSM) and flow cytometry (FCM) analysis of CRISPR/Cas9 system transfection in vitro. A375, PC3, and MCF-7 cells were transfected with Cas9-sgPLK-1a (DNA-a), Lipo2000/DNA-a, and PLNP/DNA-a, respectively. Images were taken 48 h after transfection. (Adapted from [134])

5.3 Porous-Based CRISPR/Cas9 Delivery System

Synthetic bio-organic vectors have been explored to surpass the drawbacks of viral vectors. Similarly, inorganic silica-based NPs have also provided an innovative possibility for gene therapy with DNA/RNA delivery. The mesoporous silica NPs (MSN) have the most attractive properties including stable molecular structure, modifiable particle size, large pore volume, suitable surface reactivity, and natural

biocompatibility [136]. During transmembrane transport, MSNs can resist different biological disintegrations throughout intracellular transport. Post internalization, MSNs can lead toward the cytoplasm avoiding the endosome, thereby protecting the cargo from any destructive damage. Moreover, features involving focusing on and enigmatic covering functionalization to MSNs can adjust dynamic focusing on, layer transport process, forestall the spilling of stacked cargoes, and endosomal departure of MSN [137]. MSN nanocarriers have been designed as an effective cancer cell targeting, stimuli response release, and drug controlled-releasing system [138]. The stacking capability of siRNA in MSN is 10–100 times higher and has significant security in the physiological circumstances in comparison to LNPs [139]. Zhang et al. [140] designed a polyamidoamine-aptamer-coated hollow MSN for the co-delivery of sorafenib and CRISPR/Cas9 to inhibit angiogenesis, demonstrating >60% EGFR-altering productivity without off-target impacts. The co-delivery of CRISPR/Cas9 with sorafenib harmoniously hindered the EGFR expression and the PIK3-Akt downstream pathway with zero askew effects, steering dynamic antiangiogenesis effect. Almost 85% growth restraint in a mouse model was achieved with efficient EGFR gene therapy. In vivo and in vitro accumulation of the nanocomplex at the tumor site was extraordinary. The aptamer adjustment on the outer layer of NPs progressed their take-up by HCC cells lessening the symptoms of sorafenib. The NPs applied cytotoxicity to HCC cells by definitively joining the EpCAM receptor on the cancer cell layer. The nanocomplex exhibited nontoxic nature without injuring major organs. This nanocomplex filled in as a multipurpose conveyance approach for proficient co-stacking of gene-drug blends, endorsing characterized gene altering, and cooperative prevention of cancer development with minimalized secondary effects and annihilation on ordinary tissues [140].

Another strategy includes biomimetic lipid-coated MSN for introducing new possibilities toward secured CRISPR component conveyance and heightened gene editing. One such effective delivery of CRISPR RNP and plasmid into A549 lung and HeLa cervical cancer cells along with prosperous gene editing in the brain neurons of murine models showed remarkable results. However, the attained editing proficiency proved to be subpar, since there were some adverse outcomes in gene editing, with regard to the size of the plasmid. Nonetheless, the gene-editing tool encapsulated MSN accomplished 10% editing in reporter cancer cells in vitro including over 70% release of RNP within 72 h. In vivo injection of NPs showed 10% tdTomato expression in the brain neurons of an Ai9-tdTomato reporter mouse model albeit the restriction in the area of editing [141]. Liu et al. [142] developed MSNs modified to the state of “viruslike nanoparticle (VLN)” for co-conveyance of the CRISPR/Cas9 system and smaller-sized drugs targeting malignant tumors. This nano-drug delivery system is comprised of a hardcore/shell structure, loaded with the drug and CRISPR/Cas9 system encoded with sgRNA-targeting programmed death-ligand (PD-L) encapsulated in the MSN-based core, embodied within a lipid shell. The complexity of this structure promotes the stability of VLN in blood flow. Surface-thiolated (MSN-SH), had pores encapsulated with axitinib. The disulfide bonds were used to conjugate RNP to MSN-SH. After internalizing NPs, the disulfide bond present among RNP and MSN-SH is broken up by the elevated levels of

glutathione in the tumor environment. The released RNP enters the nucleus for further PD-L1-targeted gene editing. Multiple immunosuppressive pathways were inhibited opting for this approach along with concealment of the development of melanoma in vivo. This platform demonstrated new heights of application intravenously and co-delivery of gene-drug combinations with room for improvement in the enhancement of the VLN build for a more explicit focus on the tumor cells [142]. Polymer coating of MSN with poly (dimethyl diallyl ammonium chloride) (PDDA) demonstrated prominent safeguarding against denaturation and forestalled untimely delivery. The pH-responsive disaggregation of the cationic PDDA covered MSNs acquired supported cellular uptake (16 h) and endosomal escape within 4 h. Nuclear localization sequence (NLS) was conjugated on the surface of a polymer-coated MSN functionalized with a fluorescent dye (Cy5.5), unveiling elevated cargo-loading capability toward the paxillin (PXN) targeting GFP-Cas9-paxillin_ gRNA, plasmid, and repair plasmid. The fruitful GFP-tag knock-in of the PXN genomic sequence in human bone osteosarcoma epithelial cells (U2OS) by the CRISPR/Cas9 plasmids was conceived due to the presence of NLS [143]. Regardless of the staggering advances, the accomplishment of clinical preliminaries of silica-based NPs for different disease treatments and imaging is as yet lingering profoundly behind the examination. Subsequently, it would be really useful to mix upgraded appeal for embarking on clinical preliminaries and interpretation into clinical work on utilizing MSNs-based nanosystems.

5.4 Gold Nanoparticles

Gold NPs have been extensively applied in biomedical sciences, from imaging specialists to inert carriers of various constituents. The inert nature of gold NPs restricts the unintended immune response post-delivery, which thereby intensifies their security profile. The large surface area of the NPs is also another attractive feature for the conveyance of the CRISPR/Cas9 gene-editing system. Linking of anionic medications including siRNA and plasmid DNA to gold NPs with cationic polymer surface can be made possible through the method of covalent bonding or electrostatic interaction [144]. Attachment of cell-explicit targeting components to gold NPs can suffice active cancer-explicit delivery of the drug and passive enhanced penetration and retention (EPR) effects. Modification of shape, size, and composition of gold NPs can be made possible through the surface plasmon resonance (SPR) effect. This SPR effect is brought about by the aggregate intelligible swaying of the free electrons in gold NPs. These properties allow AuNPs to be utilized as nanoprobes for biomedical analysis [145, 146]. Engineering of Cas9/sgRNA RNP with gold NPs to create a complex for drastically enhancing the cytoplasmic/nuclear delivery with simultaneously effective gene editing and causing the desired mutation. The Cas9 system was direct to the cytosol with the aid of supramolecular delivery vehicles. The nuclear accumulation was maintained via an attached NLS. Almost ~90% of cells were conveyed with the gene-altering instrument with rapid altering in the

PTEN (30%) and AAVS1 (29%) gene loci. This combination can enhance spatiotemporal control of gene transcription and imaging chromatin dynamics for future contributions to this genomic editing system. Although this study yielded promising in vitro outcomes, the fundamental pertinence of this delivery vector has not yet been illustrated [147]. CRISPR-Gold NPs were also designed by Lee et al. [148] for the direct delivery of Cas9 RNP and donor DNA in vivo via local administration for inducing homology-directed repair (HDR). Conjugation of gold NPs is done through 5′ thiol-modified single-stranded DNA sequences hybridized to single-stranded donor DNA. The fabricated NPs were later coated in a silicate and an endosomal disruptive polymer, poly(N-(N-(2-aminoethyl)-2-aminoethyl) aspartame) PAsp(DET). Due to the presence of cationic PAsp(DET), the internalization of CRISPR-Gold NPs by cells via endocytosis was effortless. Following the successful endocytosis, the PAsp(DET) polymer on CRISPR-Gold triggers endosomal disturbances prompting the discharge of the NPs system into the cytoplasm. Significantly, once in the cytoplasm, DNA is let out of the gold core of CRISPR-Gold due to the excess glutathione level, triggering the immediate release of Cas9 RNP and donor DNA. Achievement of 3–4% HDR productivity in different human cell types, including primary bone marrow-determined dendritic cells and embryonic stem cells, was made possible. Approximately 5.4% of the mutated Duchenne muscular dystrophy (DMD)-causing dystrophin gene was amended and demonstrated recuperated expression of dystrophin with a solitary infusion of the CRISPR-Gold NPs. Proper efficiency was also observed in primary myoblasts from mdx mice exhibiting reduced levels of fibrosis and partial restoration of muscle function. These results support the application of this nonviral delivery using gold NPs as vehicles in a plethora of genetic pathologies [148]. Moreover, the CRISPR-Gold system utilized for intracranial injection to murine models of fragile X syndrome (FXS) demonstrated articulated and unpredictable mannerisms reliable with this illness. The conveyance framework was intended to convey the RNA-guided endonucleases Cas9 and Cpf1 to adult mice cerebrums and lead gene editing in Thy1-YFP and Ai9 mice. The mGluR5 gene was targeted to lower the amplified mGluR5 signaling in the FXS mouse striatum. CRISPR-Gold-intervened mGluR5 restraint was used to free striatum-subordinate overstated monotonous practices, as estimated by the marble-burying assays and hopping behavior. Following CRISPR/Cas9-assisted knockdown of mGluR5, the treated animals displayed stable and normal behaviors along with remarkably diminished disease symptoms after 2 weeks. One major concern of the developed CRISPR-Gold system was to prioritize effective endosomal escape [149].

5.5 Cell Membrane-Derived Nanoparticles

Exosomes are nanoscale membrane vesicles emitted by a wide range of cells and steadily exist in every single natural liquid and deliver bio-information intercellularly. Their diameter ranges from 30 to 100 nm and can be used to communicate an

assortment of molecules, including microRNA, mRNA lipids, and functional protein [150]. Exosomes are also capable of avoiding rapid phagocytosis by mononuclear phagocytes, containing themselves stably in the circulation, and pass through vascular endothelium to target cells [151]. Exosomes are also capable of crossing over the most stringent biological barriers including blood-brain barrier (BBB) and the placental barrier. They have great tissue or cell-focusing on inferable from the presence of surface protein like tetraspanin. The application of hybrid exosomes for the encapsulation of enormous nucleic acids, including the CRISPR/Cas9 expression vectors into, has been studied. Hybrid exosomes loaded with the cargo are endocytosed by mesenchymal stem cells (MSCs) and aid the release of the cargos inside. Endogenous exosomes from SKOV3 human ovarian cancer cells (SKOV3-Exos) loaded with CRISPR/Cas9 were intravenously injected. The expression of the PARP-1 gene was inhibited, and subsequently, apoptosis of ovarian cancer cells was attained by the delivery of a gene-editing tool [152]. The transfection of HPV- or HBV-specific CRISPR/Cas9 expression plasmids into HeLa cells and HuH7 cells, respectively, was studied in endogenous exosomes. This transfection destroyed the HBV genome of neighboring cells [153]. Albeit endogenous exosomes are continually utilized as a protected and successful conveyance vector for the CRISPR/Cas9 gene-editing tool, there are concerns in regard to the off-target impacts and security issues of gene-altering innovation. Recent studies have testified that hybrid NPs can elevate the efficient encapsulation of plasmid DNA [31]. The hybridization of exomes with lipid to form NPs fruitfully stimulated the expression of the encapsulated genes in MSCs, which would not have been transfected by the liposome alone. These hybrid NPs guarantee in vivo gene manipulation through the delivery of CRISPR/Cas9 gene-editing systems [154]. McAndrews et al. [155] successfully demonstrated that the loading of CRISPR/Cas9 in exosome nanoparticles knocked out the mutant KrasG12D oncogenic allele in pancreatic cancer cells in vitro. This editing framework had the option to stifle proliferation and restrain tumor growth development in syngeneic subcutaneous and orthotopic models of pancreatic cancer in vivo.

6 Applications of Genome Editing

The emergence of the CRISPR/Cas9 tool as a pioneering and amazing asset to control the genome for helpful designs is obvious. The CRISPR/Cas9 innovation has been applied for the identification of the gene, gene performance investigation, and disease/infection model foundation. The two main clusters of clinical trials including CRISPR/Cas9 could be arranged into adoptive cell treatment (ACT) and in vivo therapy. In ACT applications, CRISPR/Cas9 system is used for precise genome editing of stem cells or immune cells isolated from patients, followed by the transplanting of the engineered cells back into the patient's body. The altered cells can be examined to ensure altering proficiency and exactness. Two of the mature delivery strategies designed to deliver the CRISPR/Cas9 gene-editing system into cell lines

to procure the engineered cells are electroporation and viral vectors. This method of import can avoid unintended immune responses by the host when encountered with gene-editing reagents [156]. On the contrary, the in vivo genome-editing therapies accompany a difficult grip because of the gene-editing efficiency, tissue and cell sensitivity, and biosafety. However, clinical studies of EDIT-101 have demonstrated tremendous improvement in in vivo gene therapy. Leber congenital amaurosis (LCA) is a debilitating monogenic disease resulting in childhood blindness caused by the *CEP290* gene mutation. So far, no treatment options were available for this condition. However, with EDIT-101 CRISPR/Cas9 can straightforwardly be delivered into the retina of LCA patients explicitly with the intronic IVS26 transformation. In EDIT-101, the expression of Cas9 is controlled by the photoreceptor cell-specific GRK1 promoter, which can improve the specificity of tissue and cell selectivity for gene editing after subretinal injection inducing reduced side effects. Either an erasure or reversal of the IVS26 intronic area is progressed by Cas9, and both guides prevent the aberrant splicing and thereby allow successive translation of the functional protein [157]. Manipulation of postmitotic and highly differentiated cells is possible through in vivo studies; however, isolation and in vitro editing of different cell types are limited. Nonetheless, gene correction of various diseases can become a reality through the mode of various vectors for in vivo CRISPR/Cas9-mediated genome editing [35]. Another application of CRISPR/Cas-9 in clinical trials is targeting sickle cell disease (SCD) therapy and β-thalassemia. The two main advances of CRISPR/Cas-9 treatment for SCD involve either direct repairing the hemoglobin S gene or boosting fetal γ-globin. However, the commonly applied method in a clinical trial is boosting fetal hemoglobin. Initially, the removal of bone marrow cells from the patient is conducted followed by the CRISPR/Cas-9 disabling of the B-cell lymphoma 11A (BCL11A) gene that deactivates fetal hemoglobin production. The gene-engineered cells are later imbued back into the body. The disabling of this gene using CRISPR/Cas-9 can increase the production of fetal hemoglobin comprising γ-globin in the red blood cells, consequently letting the severity and the manifestations of SCD relieved [158]. Presently, technologies involved in gene exploitation and molecular targets are likewise being investigated in other diseases including Alzheimer's [159] and cystic fibrosis [160]. Although it is in the beginning phases of advancement, the utilization of CRISPR/Cas-9 technology for genome editing reflects immense possibilities in the management of various diseases.

The importance of CRISPR/Cas9 has shifted throughout history (Fig. 4a) [161] especially in cancer treatments starting with in vitro and in vivo followed by its introduction into clinical trials (Fig. 4b) [162]. The first clinical phase 1 CRISPR-based therapy was directed to treat patients with refractory lung cancer. T-cells were refracted from three patients' blood, and utilizing the CRISPR/Cas-9 tool *TRAC*, *TRBC*, and *PD-1* genes was deleted meddling in the inhibition of cancer cells. The designed T-cells were subsequently imbued once again into the patients. Targeting of specific antigens by the engineered T-cells effectuates the killing of cancer cells. The modified T-cells were distinguished as long as 9 months post-mixture with no fundamental systemic side effects identified [163]. Taking into consideration the

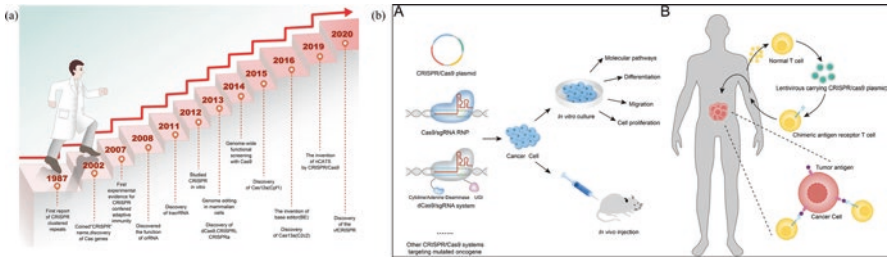

Fig. 4 (**a**) Development history of the CRISPR/Cas9-based gene-editing tools. The "CRISPR" repeat sequence was reported in 1987 and named in 2002. In 2012, in vitro experiments demonstrated that mature crRNA formed a special double-stranded RNA structure with tracrRNA by base complementary pairing, thus directing Cas9 protein to cause double-stranded fracture on the target DNA. In 2013, the type II Cas system was applied to the cutting of DNA in mammalian cells, which paved the way for the application of the CRISPR/Cas9 system for gene editing. Since then, the CRISPR/Cas9 technology developed rapidly, and several CRISPR/Cas9-based tools were generated for gene editing at both DNA and RNA levels by 2020. (Adapted from [161]) (**b**) Applications of CRISPR/Cas9 technology in cancer research: (A) Modifications of cancer cell genomes with different types of CRISPR/Cas9 systems in vitro and in vivo; (B) CAR T-cell therapy combined with CRISPR/Cas9 technology. (Adapted from [162])

internal barriers of the vehicles hindering gene-editing tool's application in cancer treatments is just a small pit stop until its maximum capacity is restored in the near future (Fig. 5) [115]. Since the discovery that CRISPR/Cas9 could be applied to treat irresistible ailments brought about by microorganisms, research studies demonstrated that the application of the CRISPR/Cas9 gene-editing system in animal models could bring closure to HIV-1 replication and kill the infection from contaminated cells through the excision of the HIV-1 gene. Editing of chemokine coreceptor type 5 (*CCR5*) can block HIV entry into host cells. This was upheld by an in vitro preliminary drive in China that exhibited that CRISPR/Cas9 gene-editing of the CCR5 quality could successfully be shielded from HIV disease and showed no proof of harmfulness (contamination) on cells contrasted with the unmodified cells [164]. Gene editing has only been currently sanctioned in a somatic cell, be that as it may, a dubious CRISPR preliminary in human embryos might have effectively penetrated the moral norms set up for such preliminaries. The genetic engineering of the *CCR5* gene in human embryos, conferring HIV resistance, was conducted in this study. The evidence received so far is limited on the effect of CRISPR/Cas9 in targeting this gene. The gene editing simply initiated DSBs toward one side of the erasure, instead of replicating the naturally noticed and useful 32-base deletion. This allowed NHEJ to fix the impaired DNA while initiating irregular, uncharacterized mutations. Thus, the fate of the resultant protein is unclear, whether it will work basically in the same manner as the typically happening CCR5Δ32 protein and give HIV obstruction. The two embryos, named with the pseudonym Nana, had productive alters in the two duplicates of the CCR5 quality; nonetheless, the subsequent embryo, with the pseudonym Lulu, had compelling altering in just one duplicate. Irrespective of the outcome, the two incipient organisms were embedded back inside their mom, realizing that the HIV opposition will be sketchy in Nana and nonexistent in Lulu [165, 166].

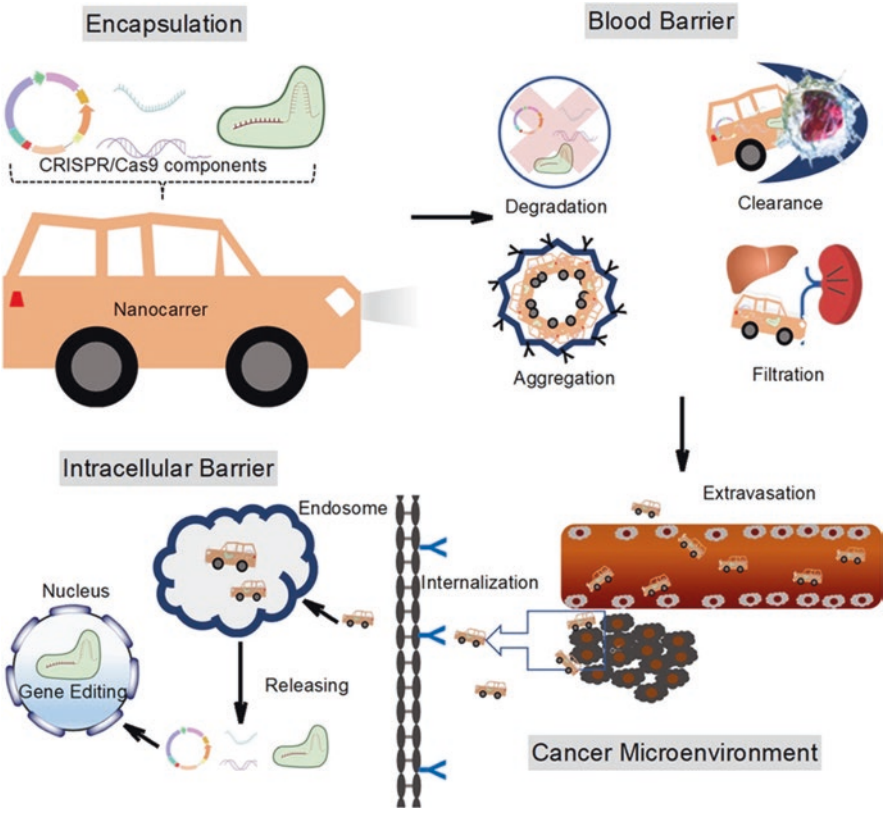

Fig. 5 Schematic illustration of different barriers in the process of CRISPR/Cas9 delivering for cancer therapy. (Adapted from [115])

7 Future Directions and Challenges

CRISPR/Cas9 genome-editing system without no doubt has conquered genome engineering with advanced editing tools in vitro and in vivo. However, the safety and efficacy of gene editing are still a concern. Addressing many challenges can aid in the identification of the full capacity of CRISPR/Cas9 systems. Within the system itself, the concern lies in off-target cutting. The resolution to this problem includes the engineering of Cas9 nickases and mutants that diminishes vague DNA restricting; however, they are an imperfect solution. The advancement of a few prescient software sgRNA design tools is required to understand sgRNA binding and mismatch; nevertheless, research studies of off-target effects remain deficient [57]. Prime editing can address gene mutations without initiating double-stranded breaks joined by undesirable focuses during CRISPR/Cas9 gene editing. Catalytically weakened Cas9 is meddled to reverse transcriptase and channeling is guided by a

prime-editing guide RNA (pegRNA). The latter directs the framework to the designated DNA site and encodes the ideal adjustment. Upcoming studies are necessary to ascertain the capability and off-target edits of this new editing tool [167]. In vitro and ex vivo applications of CRISPR/Cas9 gene-editing systems are rapidly increasing with the advance in research possibilities and technology. Ex vivo modification of hematopoietic stem cells can be conducted prior to the autologous transplantation of the altered/changed cells back into patients but is very expensive. Therefore, induced pluripotent stem cells (iPSCs) produced from effectively available cell types, for example, fibroblasts and peripheral blood cells, are more well-known in gene-editing platforms. After human leukocyte antigen classes, I and II, are knocked out in iPSCs, the differentiated cell items are contemplated "off the shelf" and universally viable, modernizing the application of ex vivo cell therapies [29]. In spite of the exceptional gene-editing capability of CRISPR/Cas9, in vivo genome editing is arguably difficult for application in clinical trials. The incapacity of the delivery systems is one cautionary reason for this delay. The CRISPR/Cas9 cargo is nothing like the customary biomolecular drug, such as therapeutic mRNA, siRNA, or protein. It can either be in the pattern of Cas9/sgRNA RNP, plasmid (DNA), or a mixture of Cas9 mRNA/sgRNA, which is cumbersome for the vehicles to successfully wrap and convey. In addition, when methodically administrating the obligation of performance in the specific target cells, tissues and organs managing reduced off-target effect are very crucial [168]. Genome editing is distinguished as an irreversible iterative process; the protection of CRISPR/Cas9 therapy needs multidisciplinary research studies. Examining the drawn-out impacts of CRISPR treatment and possible off-targets is as yet inadequate. Strong ethical considerations are resilient pillars behind the fundamental purpose of CRISPR/Cas9 editing in humans. One of the advantages of the gene-editing system lies in the fact that it is somatic rather than germline. Therefore, results of the CRISPR/Cas9 gene editing will be manifested just in the treated individual and not passed down to next generations [169].

Every delivery system of CRISPR/Cas9 has both advantageous and disadvantageous properties. All around planned delivery systems ought to moderate different restrictions and accomplish a few victories with respect to (i) encapsulation of a large cargo inter- and intracellular uptake, (ii) improved loading efficacy and elevated gene-editing capability, (iii) escaping the endosomes, (iv) subduing toxicity and immunogenic responses, (v) specificity of targeting cells/tissues and preventing undesirable off-target editing, (vi) precluding the leakage/degradation of cargo intracellular, and (vii) delivering unblemished gRNA and functional Cas9 proteins to the nucleus [116]. An important factor of translatability is remaining during the delivery of the protein; the Cas9 expression can be difficult, and when detached its nuclease movement is lost surprisingly fast [170]. Currently, limited information is existing on the fate of nanoparticle delivery systems after internalization, including the period of their circulation, and if there is any lasting toxicity related to any part of the nanoparticle that relates to the body. This is a crucial aspect for the extended investigation of CRISPR/Cas9 gene-editing systems to fathom its promise.

8 Conclusions

The outlook of genome editing and genome regulation has been modernized by the CRISPR/Cas9 gene-editing system. Although in vivo applications of Cas9 remain apprehensive, a few tweaks in the administration of current delivery approaches clarify the performance of the CRISPR/Cas9 system. Since 2011, the interest in the CRISPR tool is evolving at a mind-blogging pace, with a 1453% surge in the research articles regarding CRISPR/Cas9 [57]. Mitigating the inadequacies connected with plasmid-based, viral, mRNA-based, nonviral, and protein-based delivery methods for the CRISPR/Cas9 system can be addressed with the rise of research technology involving multidisciplinary participation. The elements connected to safety, efficiency, and feasibility are of fundamental significance in assessing the practicality of each methodology. The advances in the customization of the delivery medium with the exceptional ability for delivering CRISPR/Cas9 gene-editing systems can surpass many obstacles that once obstructed its translatability. RNA-guided CRISPR/Cas9 system is the modern face of genomic editing, simplifying the preceding cumbersome genetic manipulation. Lately, CRISPR/Cas9 system is introduced to therapeutic applications through disease modeling. Unquestionably, with improved technology, its application will extensively be more prosperous and beyond expectation shortly.

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Lipoplexes and Polyplexes for Targeted Gene Delivery

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1 Introduction

Targeted gene delivery is a scientific approach with numerous advantages in the fields of bio- and personalized medicine [1–10]. The success of targeted gene therapy relies on gene transfer to cells and subcellular organelles and effective transgene expression. The in vivo effectiveness is strongly dependent on the delivery system, the physicochemical properties of the gene, the route of administration, and the target organelles [1–10]. Firstly, different chemical modifications have been used to alter the pharmacodynamic and pharmacokinetic behavior of nucleic acids and to achieve targeting to cells and issues. This scientific approach presented many limitations. For this reason, the nonviral delivery vectors and the nanosystems were the ideal carriers for the improvement of the stability of nucleic acids. According to the literature, up to 2016, "*Nonviral gene therapy has maintained its position as an*

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approach for treating cancer. This is reflected by the fact that more than 17% of all gene therapy trials employ nonviral approaches. Thus, nonviral vectors have emerged as a clinical alternative to viral vectors for the appropriate expression and delivery of therapeutic genes" [7]. Nowadays, this percentage has increased by more than 30% [10]. Additionally, they can ameliorate their administration, distribution, metabolism, and Excretion (ADME) profile of them because they can achieve cellular and tissue targeting. Furthermore, these nanosystems that have been modified by proteins and/or peptides are also able to facilitate nuclear translocation and enhance the efficacy of gene expression [5]. The overcome of intracellular barriers and the sustained targeted expression can also be designed by the nanosystems [3–8].

Delivery systems based on nanomaterials have been already used as nanomedicines and nanovaccines, as marketed products, and they exhibit higher loading capacity and ease of fabrication [9, 10]. Formulation scientists can manipulate their characteristics (i.e., surface charge, size, etc.) and the method of their preparation to achieve the ideal properties in vitro and in vivo. Namely, a positive surface charge allows carriers to interact electrostatically with anionic nucleic acids and antigens. In fact, cationic lipids and polymers can be utilized as carriers in gene therapy, especially for cancer treatment [1–10]. Except for cancer treatment, gene therapy is also widely utilized in ocular gene delivery and cardiovascular diseases. For gene therapy, lipid and polymer gene vectors condense negatively charged oligonucleotides and form a well-organized complex, also called lipoplex and polyplex, respectively. In general, these complexes because of Coulomb attractions and ion-pair mechanism interact with plasma and endosomal membranes leading to rapid cellular uptake, endosomal escape, and as a result gene silencing efficacy. However, it is well-known that cationic vectors exhibit extensive cytotoxicity and rapid clearance from the bloodstream, also called as "polycation dilemma" due to positive surface charge, which induces reactive oxygen species (ROS) formation and rather significant interactions with blood cell membranes and proteins [5–10]. There has been extensive research to solve these problems and a prominent solution is a PEGylation, although there are limitations to this approach, such as the "PEG dilemma," and efforts are needed to overcome them [9, 10].

This chapter aims to present the technology and the applications of lipoplexes and polyplexes in the field of targeted gene delivery. Special attention will be given to the mechanisms by which lipoplexes and polyplexes are used for the delivery and the release of the complexed nucleic acids. Several examples from the recent literature will be discussed.

2 The Technology of Lipoplexes

In the late 1990s, Radler et al. described the complexation process of cationic liposomes with DNA [11]. The formation of multilamellar lipid/DNA complexes was observed by several techniques and was fully characterized regarding their structure

and their properties. The mechanism of lipoplex formation is well established in the literature [11–15]. The negatively charged nucleic acids are attached to positively charged lipid molecules. This formation mechanism generally produces multilamellar liposomal structures [11–15]. The thickness of the lipid bilayer is around 4 nm and is spaced 2 nm apart from each other lipid bilayer by the negatively RNA/DNA biomacromolecules. These complexes opened a new horizon for gene delivery in several biomedical areas [11–15].

The main lipids that are used for the formation of lipoplexes are the ones with quaternary ammonium function, i.e., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methyl sulfate (DOTMA). DOTAP is also characterized as pH-sensitive lipid and is used for lysosome delivery. Synthetic lipids are also used for the preparation of lipoplexes [11–20]. For example, Sato et al. synthesized a cationic pH-responsive lipid to formulate a multifunctional envelope-type nanodevice for gene delivery and targeting useful in cancer immunotherapy [15]. Cholesterol and other "helper" lipids are also components of the lipoplexes [16]. Their role is important because lipoplexes possessing cholesterol domains exhibit higher transfection efficiency in systematic circulation. Generally, the "rich" cholesterol domains in the lipid-DNA complex do not bind serum proteins such as albumin, and this may enable these moieties to enhance transfection efficiency by ameliorating membrane interactions and the final fusion [16, 17]. Cholesterol and the "helper" lipids influence the physicochemical and biological characteristics of the lipoplexes and for this reason, are extensively used for the development of lipoplexes [15–17].

From the technological point of view, several factors are crucial for the design and delivery of lipoplexes [18–26]. Firstly, the lipid/nucleic acid ratio and the chemical treatment of lipid before the complexation process with the nucleic acids are the main preformulation studies that should be done before choosing the formulation protocol [18]. Several studies in the literature showed that different lipids exhibit different transfection efficiency of DNA [18]. According to the literature findings, the chemical structure and the concentration of salts and biomolecules (i.e., serum components) present in the lipid/nucleic acid complexation medium are also crucial preformulation parameters [12, 18]. Except the lipid composition, the size, the size distribution, the fluidity, and the lamellarity of cationic liposomes affect the transfection efficiency of lipoplexes [19]. For example, Ramenzani et al. developed liposomes using several types of cationic lipids like 1,2-dioleoyl-3-trim ethylammonium-propane (DOTAP) or 3-beta-[N-(N′N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-CHOL) in combination with other (helper) lipids including 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), egg L-alpha-phosphatidylcholine (EPC), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) [19]. The different formulations caused lipoplexes with different sizes, lamellarity, and consequently different transfection efficiency of the attached nucleic acid [19]. According to Koynova [20], "*the phase evolution of lipoplex lipids upon interaction and mixing with membrane lipids appears to be decisive for transfection success: specifically, lamellar lipoplex formulations, which were readily susceptible*

to undergoing lamellar-nonlamellar phase transition upon mixing with cellular lipids and were found rather consistently associated with superior transfection potency, presumably as a result of facilitated DNA release." Furthermore, the formation of cubic phases of lipoplexes composed of two novel cationic lipids (O-alkyl-phosphatidylcholines, 1,2-dioleoyl- sn-glycero-3-hexylphosphocholine (C6-DOPC) and 1,2-dierucoyl- sn-glycero-3-ethylphosphocholine (di22:1-EPC)) showed that the transfection efficiency is also related to lipoplex microstructure and microfluidity [21]. On the other hand, it seems therefore that the lipoplex structures have not any influence on transfection efficiency and cytotoxicity in the experimental procedure followed by Congiu et al. [22]. It should be highlighted that according to other authors, the morphologies of lipoplexes should be evaluated at two levels, size and self-assembled structures of lipoplexes, and understanding these aspects would be very important for formulation scientists to develop innovative systems with high transfection efficiency and low toxicity [23]. Regarding the formulation protocols, thin-film hydration method, ethanol injection, and a modified ethanol injection method have been used for the preparation of lipoplexes with DNA [23–25]. The selection of lipids is also crucial for the selection of the preparation protocol [23–25].

The aforementioned parameters, i.e., structure, size, lamellarity, and method of preparation, are important for the serum protein attraction, the toxicity, and the transfection efficiency [26–28]. Additionally, the coating of the lipoplex surface with hydrophilic polymers like polyethylene glycol (PEG) is a strategy to ameliorate the stability of the prepared complexes and the selectivity of cellular targeting [29–31]. In the case of PEG or PEGylated lipid utilization, the biodistribution of the complexes is also ameliorated because the blood concentration is elaborated and the targeting of specific tissues is achieved [29–31]. Lipoplexes can be administrated by different routes including the i.v. delivery, nasal route for brain delivery, intramuscular, etc. [11–31].

On the other hand, there are some limitations in the use of lipoplexes that the formulation scientists should overcome for their effective utilization in gene delivery and targeting [32–36]. Firstly, the toxicity of cationic lipids should be considered in several studies, i.e., evaluation of toxic effects in physiological cell lines and long-term adverse reactions (mutations, etc.) [32–36]. Additionally, the transfection efficiency of lipoplexes is low in comparison to other engineered nanoparticles and viral vectors. Some authors believe that the deeper study of interactions of the lipoplexes with the cellular membranes is the crucial step to understanding the toxicity and the low transfection efficiency of the lipoplexes [32–36]. Marchini et al. proposed the formation of multicomponent lipoplexes to overcome the low transfection efficiency of lipoplexes [36]. This kind of complex exhibited a distinctive ability of endosomal escape and release DNA into the nucleus [36].

3 The Technology of Polyplexes

Polyplexes are the complexes between polymers and nucleic acids (DNA/RNA). Several parameters ranging from the structure of polymers to the physicochemical characteristics of the resulting complexes are crucial for the design and development of polyplexes. Tang and Szoka [37] studied the ability of four cationic polymers to interact with DNA, forming polyplexes and delivering their cargo to cultured cells. The polymers that they used exhibited different chemical structures including polylysine, intact polyamidoamine dendrimer, fractured polyamidoamine dendrimer, and polyethyleneimine. All these cationic polymers interact via electrostatic attractive interactions with DNA forming a unit structure with nanoscopic size and variable morphology. On the other hand, the morphology of the resulted complexes was found to be strongly dependent on the structure/architecture of the polymer [37]. The charge density of chitosan, a biopolymer, and the number of charges per chain were found to be the crucial factors for the morphology and colloidal stability of its DNA complexes [38]. The presence of surfactant is also important for the control of size and colloidal stability of the resulting polyplexes [39]. The branched and linear polyethyleneimines, poly[N-ethyl-4-vinyl pyridinium bromide], polyamidoamine dendrimer, poly(propyleneimine) dendrimer, and a conjugate of Pluronic P123 and polyethyleneimine (P123-g-PEI(2K)) also block copolymers that have been studied for gene transfection [40]. The highest transfection activity and lowest cytotoxicity were achieved by the linear structures in comparison to branched ones [40]. The particle size, the colloidal stability, the cellular uptake, and the resistance to nuclease degradation were also studied by the same researchers [41]. Zheng et al. demonstrated that poly(ethylene oxide) grafted with short polyethyleneimine gives DNA polyplexes with superior colloidal stability, low cytotoxicity, and potent in vitro gene transfection under serum conditions [42].

Petersen et al. studied the influence of polyethyleneimine-graft-poly(ethylene glycol) copolymer block structure on DNA complexation as a gene delivery system. The blood compatibility, cytotoxicity, and transfection activity were also evaluated [43]. The molecular weight and the degree of PEG grafting were found to be crucial for the biological activities of the resulted polyplexes [43]. Other studies also revealed that the copolymer block structure significantly influenced not only the physicochemical properties of complexes but also their cytotoxicity and transfection efficiency [44–46].

Except for the colloidal stability, the particle size/size distribution and the surface charge of polyplexes are also crucial for their in vitro and in vivo transfection [47, 48]. These parameters also influence the attraction toward serum components [47, 48]. The serum protein binding can lead to the alteration of polyplex structure and properties [47, 48]. For example, poly-L-lysine (PLL) polyplexes are quickly removed from blood circulation because they interact extensively with plasma proteins [49–52]. The steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes complexed with plasmid DNA showed colloidal stability in vitro, extended circulation times, and tumor targeting in mice [50]. The same polymers

were also used for gene transfer into human ovarian carcinoma cells via active targeting [51]. The importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation has also appeared in the literature several years ago [52]. Oupicky et al. studied polyplexes containing DNA. They used PLL or polyethyleneimine (PEI) systems, surface-modified with PEG, or multivalent copolymers of N-(2-hydroxypropyl)methacrylamide (PHPMA) via intravenous administration [52]. According to the findings, the molecular characteristics and the dose of the coating were the most important parameters for the prolonged circulation of the complexes in the plasma [52]. Furthermore, the ideal ratio of cationic and hydrophobic content of PEGylated siRNA polyplexes can ameliorate the colloidal stability, endosome escape, blood circulation half-life, and bioactivity/effectiveness of the polyplexes in vivo [53]. A polyplex library was designed for the investigation of combinatorial optimization of PEG architecture and hydrophobic content [54]. These design parameters can improve ternary siRNA polyplex stability, the pharmacokinetics of the cargo, and effectiveness in vivo [54]. Sarett et al. demonstrated that hydrophobic interactions between polyplex vector and palmitic acid-conjugated siRNA improve PEGylated complexes' colloidal stability and enhance in vivo pharmacokinetics, as well as the tumor gene silencing [55]. In the same line, Jackson et al. declared that charge ratio optimization maximizes the safety and avoids the rapid clearance of polyplexes from circulation [56]. Recently, thermo-responsive polymers were utilized as vectors for the transfection of nucleic acids [57]. The combination of polyoxazoline moieties, possessing great biocompatibility, with DNA-binding PEI into a single copolymer chain was found as a good candidate for improved transfection efficiency [57]. It should be pointed out that in the majority of the published data, PEGylation is a successful strategy to improve gene delivery via polyplexes and enhance the prolonged circulation times [49–58].

4 Lipoplexes for Targeted Gene Therapy

In the following section, we are going to discuss several examples from the recent literature regarding the targeted gene therapy of lipoplexes. An interesting approach to evaluate the efficiency of the non-covalent association of folate to lipoplexes (FA-associated lipoplexes) was demonstrated by Duarte and coworkers. They studied the ability of this novel gene delivery lipoplex system in two different cancer cell lines (SCC-VII and TSA cells). They found that the addition of 40 μg of FA to lipoplexes was positive for transfection and permitted to get over the prohibitive action induced under physiological conditions. Also, they compared the transfection efficacy between the FA-associated lipoplexes and FA-conjugated lipoplexes. The data presented that the electrostatic association of FA to the lipoplex results in significantly higher levels of biological action than that involving the covalent coupling of FA. Furthermore, the FA-associated lipoplexes provide better DNA protection than FA-conjugated lipoplexes. In conclusion, the FA-associated lipoplexes

seem to have better efficacy in gene delivery than FA-conjugated lipoplexes, and this fact made them potential candidates for in vivo gene delivery [59]. Buñuales et al. designed a rapid, simple, and reliable technique based on lipoplexes, which was utilized in gene delivery [60]. For this reason, they prepared liposomes with a common cationic lipid, 1,2 diodeoyl-3-trimethylammonium propane (DOTAP), and cholesterol (Chol) as the neutral helper lipid. Mixing of cationic lipid and DNA at variable ratios leads to the spontaneous formation of lipoplexes by electrostatic interactions. This work aimed to determine the ability of targeted lipoplexes to improve transgene expression in EGF receptors (EGFR, overexpressed in tumor cells) utilizing lipoplexes. The EGF-lipoplexes presented sizes at the nanoscale and were able to transfect different cancer cell lines effectively in comparison with non-targeted systems. Also, these EGF-lipoplexes showed an augmentation transfection action and were noncytotoxic and extremely capable of protecting DNA from DNase I attack. These observations indicate that these vectors could be a sufficient alternative to viral vectors for gene delivery [60].

The impact of the different structural orientations of amide linkers in modulating in vitro gene transport efficacy of cationic amphiphiles is reported by Srujan et al. They found that the reversible structural orientation of amide linkers strongly affected the serum compatibility and lung transfection efficacy of these amphiphiles. Significantly, cationic lipoplexes of the amide linker-based amphiphiles presented a more effective mouse lung eclectic gene transport ability than a commercial lipid (DOTAP) utilized in liposomal lung transfection [61]. Duarte and coworkers developed a novel formulation of lipoplexes via electrostatic non-covalent interactions of folate (FA) to 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC)/cholesterol liposomes composed of various lipid/DNA ratios. They investigated the potential of the biological action in different cell lines, and they found that the FA-lipoplexes had highly improved transfection efficiency in both cell lines and decreased the tumor growth in an animal model of oral cancer [62]. Furthermore, Cardarelli et al. prepared two different lipoplex formulations to explore the efficiency of intracellular delivery of DNA. The first lipoplex formulation was formed by DOTAP and schizophrenic helper lipid dioleoylphosphocholine (DOPC), while the second one was prepared by the cationic lipid 3β-[N-(N,N-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and the schizophrenic lipid dioleoylphosphatidylethanolamine (DOPE). They utilized a combination of pharmacological and imaging approaches, and they concluded that both DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes are taken up in Chinese hamster ovary living cells, mainly via fluid-phase micropinocytosis. The obtained results demonstrate that lipoplex micropinocytosis is a cholesterol-responsive uptake process. Also, the exploration of the intracellular fate of these lipoplexes was accomplished, and it was found that low efficacy DOTAP-DOPC/DNA lipoplexes are highly degraded in the lysosomes, in contrast to DC-Chol-DOPE/DNA lipoplexes that successfully escape the endosome pathway [63]. In another important example, a group of formulation scientists utilized O-(2R-1,2-di-O-(1′Z-octadecenyl)-glycerol)-3-N-(bis-2-aminoethyl)-carbamate (BCAT) to construct targeted cationic lipoplexes with mannose-poly(ethylene glycol, MW3000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Mannose-PEG3000-DSPE) and

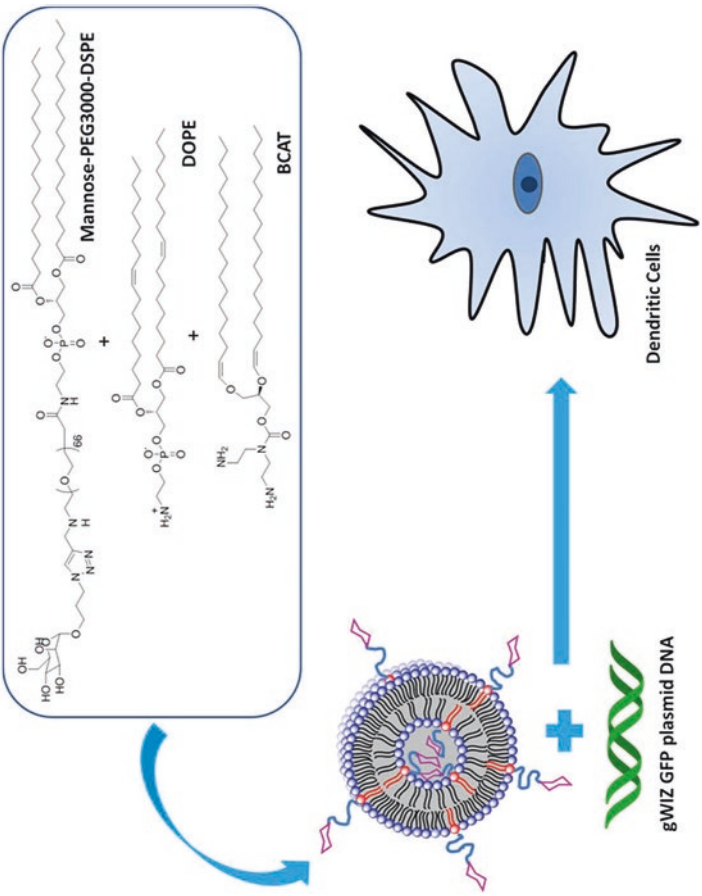

Fig. 1 Conceptual diagram of gWIZ GFP pDNA delivery to DCs via lipoplex composed of Mannose-PEG3000-DSPE, BCAT, and DOPE. (Adapted from Ref. [64])

DOPE for effective delivery of gWIZ GFP plasmid DNA into dendritic cells (DCs) [64]. The structure of the system is visualized in Fig. 1. The transfection performance presented by Mannose-PEG_{3000}-DSPE (10%), BCAT (60%), and DOPE (30%) demonstrates that the most productive delivery into DCs takes place by the synergistic interaction between mannose targeting and acid-labile fusogenic BCAT/DOPE formulations. These outstanding results suggest that these cationic lipoplexes could be potential candidates for gene delivery vectors to DCs [64].

Xu et al. reported the preparation of lipoplexes containing the ideal lipid composition with different conjugates of the folate ligand included in or kept out from the cholesterol component. Using a xenograft tumor model, it was able to evaluate the influence of locating the ligand within the cholesterol segment. The acquired results of the lipoplexes containing the ligand within the cholesterol component revealed a considerably higher luciferase expression and plasmid accumulation in tumors, in comparison to lipoplexes in which the locating ligand was kept out of the cholesterol component. These important data indicate that the environment of the ligand can influence gene delivery to tumors [65]. Wojcicki et al. designed a lipoplex system containing hyaluronic acid (HA) and the lipid DOPE. Subsequently, HA-DOPE has complexed with DNA at different lipid/DNA ratios, where the most efficient formulation of them was the 10%w/w HA-DOPE including lipid/DNA ratio of 2. The transfection efficacy was defined on the CD44-expressing A549 cells via flow cytometry. The desired HA-DOPE rate leads to the creation of the most effective formulation for transfection and increases significantly the GFP expression. Also, a slow transfection of these lipoplexes was presented when a higher level of GFP was achieved after 6 h of incubation. The acquired data indicate the strong ability of DNA targeting via the CD44 receptor utilizing HA as a ligand [66]. Hattori and coworkers reported a promising approach to treating blood lipoplex aggregation in the context of systematic gene delivery work. Specifically, they focused on the coating of liposomes based on DOTAP and Chol, with a series of anionic polymers such as HA, chondroitin sulfate (CS), and poly-L-glutamic acid (PLE), which obstruct accumulation in the lung and enhanced DNA expression in tumor via inhibition of the lipoplex interactions with erythrocytes. The dimensions of HA, CS, and PLE-coated lipoplexes were ca. 200 nm. CS and PLE-coated lipoplexes did not exhibit aggregation after mixing with erythrocytes, while the HA-coated lipoplexes revealed aggregation. Furthermore, CS and PLE-coated lipoplexes did not reveal high gene expression levels in the lung and mainly accumulated in the liver. Their results for CS and PLE-coated lipoplexes determined that these anionic polymers-coated lipoplexes are exceptional tools for effective and secure gene delivery [67].

An interesting work was reported by He and coworkers. They utilized a folate receptor α (FRα)-targeted lipoplex which then was complexed with short hairpin RNA (shRNA) targeting claudin3 (CLDN3) gene forming a folate-modified lipoplex (F-P-LP/CLDN3) to investigate the pharmaceutical properties of the complex. Afterward, the antitumor study of F-P-LP/CLDN3 was carried out in an in vivo model of ovarian cancer. In vivo antitumor experiments presented benign differentiation of tumor and managed to achieve about 90% tumor growth inhibition.

Considering these lipoplexes, it is observed that they are an ideal formulation for ovarian cancer therapy [68].

Additionally, Puras et al. developed a novel niosome system based on the 2,3-di(tetradecyloxy)propan-1-amine cationic lipid in combination with squalene and a nonionic surfactant, namely, polysorbate 80, to investigate the transfection efficiency in rat retinas. These new niosomes, acquired by the solvent emulsification-evaporation procedure, were mixed with pCMSEFGP plasmid to form lipoplexes. In vitro experiments were carried out to determine transfection efficacy and viability in HEK-293 and ARPE-19 cells. The results displayed the successful transfection of HEK-293 and especially of ARPE-19 cells, without affecting the viability. Furthermore, the lipoplexes entered mainly retina cells by clathrin-mediated endocytosis, whereas HEK-293 cells exhibited an important caveolae-dependent entry. This novel niosome formulation leads to a promising process to deliver genetic material into the retina for the treatment of inherited retinal illness [69]. Naicker et al. explored the effect of PEGylation degree in terms of physicochemical properties, cytotoxicity, and transfection efficiency of lipoplexes including cationic liposomes. These lipoplexes contained the cytofectin 3β-[N-(N′-, N′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T), helper lipid (DOPE), the asialoglycoprotein receptor (ASGP-R) targeting cholesteryl-β-D-galactopyranoside (Chol-β-Gal) ligand, and plasmid DNA in ASGP-R-negative (HEK293) and receptor-positive (HepG2) human cell lines. The results presented important advantages of PEGylated lipoplexes in comparison to non-PEGylated lipoplexes such as well-defined and colloidally stable nanoparticles. Moreover, the transgene efficacy raised by 63% and 77% when HepG2 was encountered by the 2 and 5mole% PEGylated lipoplexes, respectively, in comparison to non-PEGylated lipoplexes. In the case of Chol-T Chol-β-Gal 5% PEG, lipoplexes recorded an achievement of 164% augmentation in transfection activity in the ASGP-R-positive cell line (HepG2) in contrast to HEK293(ASGP-R negative) [70]. Huang's team developed a series of cyclen-based cationic lipids with histidine-containing ester groups or amide bonds in the backbone for gene delivery. The preparation of cationic liposomes was done by mixing the lipids and the neutral lipid DOPE in a proper molar ratio. The synthesized liposomes presented excellent stability and formed lipoplex-encapsulated pDNA into nanoparticles. Cell viability studies based on CCK-8 cells revealed low-level cytotoxicity of these lipoplexes in comparison to the commercial Lipofectamine® 2000. Green fluorescent protein and luciferase experiments were performed to explore the in vitro transfection efficacy of these lipoplexes. The experimental data presented that the structures of the hydrophobic chain and the linking bond influenced notably the transfection efficiency. Also, the imidazole group is indicated to have an important role in the transfection by such a type of lipid. In summarizing, these data recommend that the cyclen-imidazole cationic lipids could be a promising nonviral gene delivery vector [71].

Luo et al. reported an FRα-targeted lipoplex loading plasmid interleukin-12 (PIL12) forming a folate-modified lipoplex (F-PLP/PIL12). In vivo studies were performed, such as an in vivo model of CT26 colon cancer to explore the antitumor effect of these lipoplexes. F-PLP/PIL12 could prevent about 56.6% tumor growth,

and also IL12 expression rise in the F-PLP/PIL12 group, while at the same time, FRα expression was downregulated. Furthermore, toxicity studies of F-PLP/PIL12 indicated no toxicity in the mice. Therefore, the F-PLP/PIL12 could be a promising formulation for clinical colon cancer immunogenic therapy [72]. Zheng's team synthesized two amino acid-based cationic lipids, which included an α-tocopherol moiety and a biocompatible amino acid head group (histidine or lysine) linked by a biodegradable disulfide and carbamate bond. These lipoplexes were evaluated in cell culture experiments as nonviral DNA delivery vehicles. DNA and cationic liposomes formed lipoplexes which present low cytotoxicity and comparable transfection efficacy compared with the commercially available Lipofectamine 2000. Under physiological conditions, especially in the presence of 10% serum, the transfection efficacy of the cationic lipid based in histidine was 4.3 times higher than that of branched polyethylamine. The acquired results indicate that the amino acid-based lipids are capable of a safe and efficient gene delivery [73]. Cardarelli et al. constructed a Lipofectamine/DNA lipoplex to evaluate the intracellular trafficking mechanism of them in live cells. They discovered that Lipofectamine (LFN), in comparison with alternative formulations, could effectively avoid intracellular transport along microtubules and the following encapsulation and degradation of the payload within acidic lysosomal components. This observation is accomplished by following the random Brownian motion of LFN-including vesicles into the cytoplasm (Fig. 2). Also, they indicate that Brownian diffusion is an effective process for LFN/DNA lipoplexes to avoid metabolic degradation, thus resulting in optimal transfection. Based on their results, it would be possible to developed new generations of more optimized nonviral, lipid-based, gene delivery vectors [74].

Another important work was reported by Rak et al. They utilized a set of cationic polyprenyl derivatives (trimethylpolyprenylammonium iodides (PTAI)) as part of effective DNA vectors. Optimization experiments were accomplished for PTAI combined with lipid DOPE on DU145 human prostate cancer cell lines. The acquired data present that the lipofection action of PTAI enhances transfection efficiency of pDNA complexes in negatively charged lipoplexes into cells with no important side effects on cell physiology and incidence of eukaryotic cell proliferation. Considering these results, the PTAI-based lipoplexes could be promising candidates for gene delivery to eukaryotic cells [75].

Another interesting study was the construction of the DNA-liposome complex (lipoplex) by Rasoulianboroujeni et al. This work refers to cationic liposomes and their modified preparation process utilizing the dry lipid film method, including lyophilization, for DNA delivery applications. Using a particle size instrument, it was possible to determine the dimensions of liposomes before and after lyophilization and of course the mean particle size of DNA-lipoplexes. Transfection assays were accomplished by using human embryonic kidney 293 (HEK-293) cell lines. Overall, the acquired data indicated that the DNA expression of these lipoplexes is almost equal to the Lipofectamine® 2000. Furthermore, the acquired cellular protein of the developed lipoplexes was at higher levels than in Lipofectamine® 2000 based on studies [76]. Mohammed-Saeid et al. developed a novel cyclic arginyl-glycyl-aspartic acid (cRGD)-modified gemini surfactant-based lipoplexes for use and

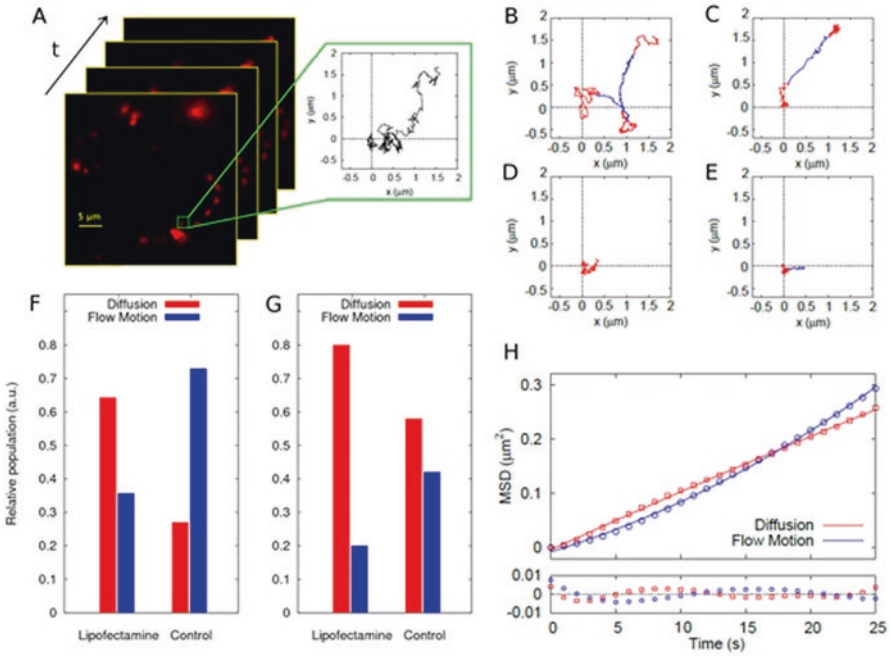

Fig. 2 (**a**) Schematic evaluation of a single particle track from a set of confocal images acquired within 300 s, with a time lapse $\Delta t = 1$ s. Representative trajectories of complexes in not treated Chinese hamster ovarian (CHO) cells: (**b**) Lipofectamine/DNA; (**c**) DOTAP/DOPC/DNA (DD/DNA). Representative trajectories of complexes in nocodazole (NCZ)-treated CHO cells: (**d**) Lipofectamine/DNA and (**e**) DD/DNA. Diffusion (red) and flow motion (blue) segments are shown. Relative populations of the acquired tracks for Lipofectamine and DD in not treated (**f**) and NCZ-treated (**g**) CHO cells. (**h**) Mean square displacement (MSD) analysis of two representative tracks. MSD calculation was used for the measurement of the dynamic parameters, i.e., diffusion coefficients and flow speed. (Adapted from Ref. [74])

evaluation in an in vitro human melanoma model (A375) cell line as a different option to conventional chemotherapy. These peptide-modified lipoplexes exhibited an important enhancement in gene transfection action in A375 human melanoma cell lines in contrast to the standard non-targeted formulations, specifically when RGD was chemically coupled to the gemini surfactant (RGD-G). The IFN-γ expression in A375 cells at 48-h posttreatment with lipoplexes is presented in Fig. 3. These results demonstrate the useful action of RGD-modified gemini surfactant-based lipoplexes in melanoma therapy [77].

A colloidal stable lipoplex formulation of single unilamellar vesicles (SUV) containing a PEGylated stearyl amine (pegSA), which maintains the SUV properties after complexation with DNA, was designed for targeted purposes [78]. The pegSA lipoplexes presented a lower N/P ratio (1.5) for BMP-9 gene complexation, suitable for intravenous infusion for delivery to bone marrow mesenchymal stem cells via sinusoidal vessels in the bone marrow. Furthermore, these lipoplexes exhibited low

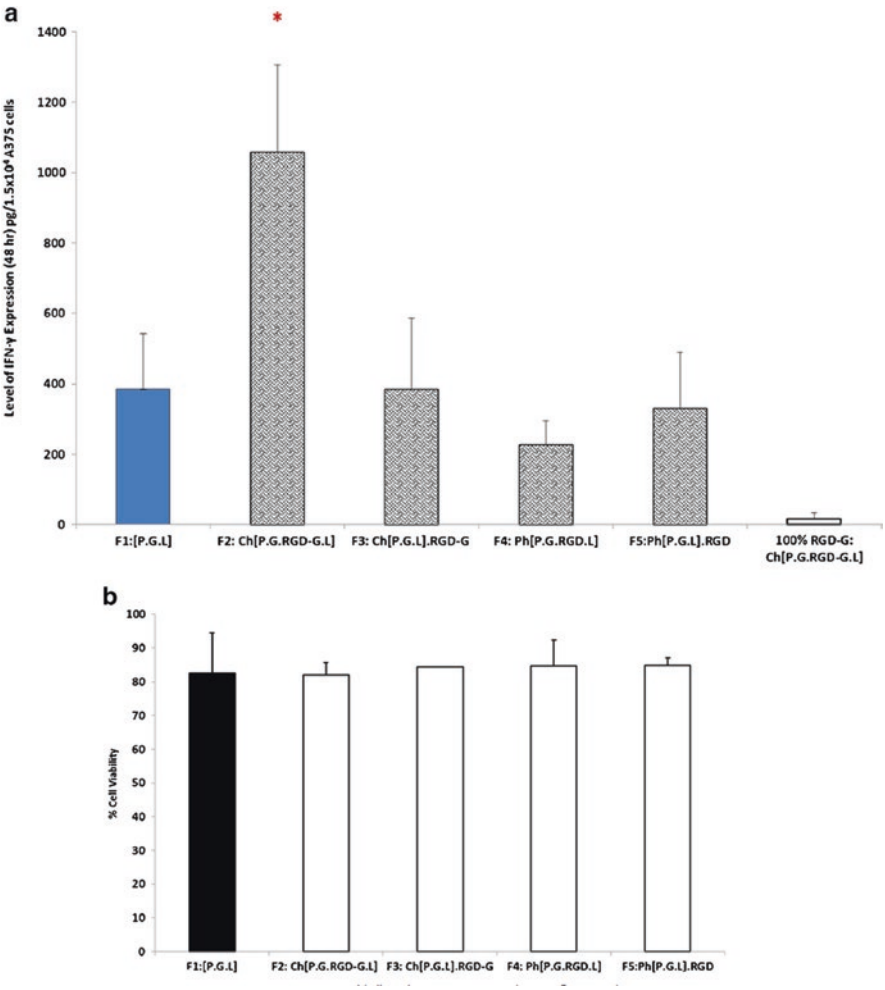

Fig. 3 (**a**) IFN-γ expression in A375 cells at 48-h posttreatment with lipoplexes constructed at 1:10 −/+ charge ratio. P, pDNA; G, 12-7 N(GK)-12; L, DOPE; RGD-G, 12-7 N(RGD)-12; RGD, RGD peptide. (Ch) indicates that the lipoplexes were built using chemically conjugated RGD-gemini, and (Ph) indicates a physical co-formulation of free RGD with the non-targeted lipoplexes. IFN-γ level was determined by ELISA. Significant increase (*$p < 0.01$, one-way ANOVA) in IFN-γ expression was observed when cell treated with RGD chemically conjugated lipoplexes (F2: Ch[P.G.RGD-G.L]) compared to non-modified lipoplexes (F1:[P.G.L]). (**b**) Cell viability in A375 cells after a 48-h treatment with RGD-modified lipoplex formulations as determined by MTT assay. Cell viability was calculated as % relative to non-transfected cells. Four wells of each formulation were loaded in three different experiments. The results are expressed as mean of the three experiments ($n = 3$). Bars represent standard deviation. * Indicates significance at $p < 0.01$ in comparison to standard formulation [P.G.L] (F1). (Adapted from Ref. [77])

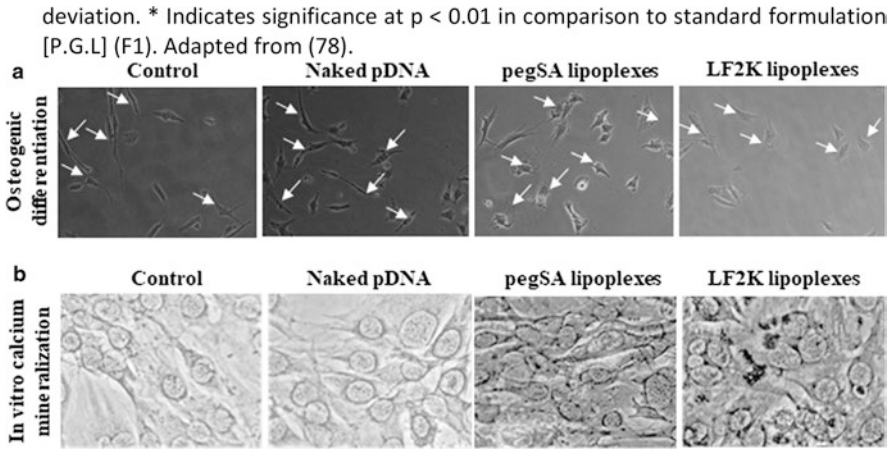

Fig. 4 In vitro BMP-9 transfection in C2C12 cells by pegSA lipoplexes. (**a**) Osteogenic differentiation of C2C12 cells by BMP-9 lipoplexes (n = 3), (**b**) in vitro calcium mineralization after transfection by BMP-9 lipoplexes (n = 3) (All images were captured on a Nikon-2000 microscope, Nikon, Japan). (Adapted from Ref. [78])

toxicity to the C2C12 and NIH3T3 cells and erythrocytes. Also, transfection experiments presented an effective gene delivery to C2C12 cells inducing osteogenic differentiation via BMP-9 expression (Fig. 4). Complementary in vivo studies further proved the safety of the constructed lipoplexes [78].

Lipoplexes based on pH-responsive cationic liposomes were also developed by varying molar mass ratios with respect to pDNA utilized [79]. The authors evaluated the lipoplex abilities for in vivo tumor-targeted gene transfection compared to the conventional reagent Lipofectamine® 2000. In vitro hemocompatibility estimation of pDNA lipoplexes presented <8.5% of hemolysis in contrast to the hemolysis by Lipofectamine® 2000 which was 15.9%. Cell viability studies exhibited >80% values along with 4.42, 5.18, and 5.00 higher transfection efficacy than Lipofectamine® 2000 in MCF-7, HeLa, and HEK-293 cells, respectively. Also, pDNA lipoplexes revealed higher tumor transfection compared with Lipofectamine® 2000, demonstrating outstanding abilities for in vivo gene delivery [79]. Nie et al. by utilizing a flash nanocomplexation controlled procedure mixed the 1,2-dimyristoyl-rac-glycero-3-methoxy poly(ethylene glycol)-2000(DMG-PEG)/DOTAP liposomes with plasmid DNA and formed lipoplex nanostructures with small sizes (60 nm). DMG-PEG acted as a hydrophilic and neutral layer-coated cationic lipid in the DNA-containing nanoparticles to decrease the barrier of penetration via the mucus. Lipoplexes with a PEG surface showed improvement of transportation in the mucus layer of the GI tract. A repetitive transgene expression was verified, and the expressed insulin was found to retain the blood glucose level for 24 h, presenting repetitive therapeutic properties by multiple doses. The results indicate the fast translation effects of DMG-PEG/DOTAP-DNA nanostructures in type I diabetes through oral delivery [80].

Buck et al. developed a combinatorial process for the synthesis of short-chain aminolipids with various headgroups, containing aliphatic and heterocyclic groups [81]. These lipids in combination with the cationic lipid DOTAP can be used to increase the delivery of DNA. This combination leads to the development of novel lipoplex systems able to complex minicircle DNA and explore the transfection efficiency in human liver-derived cell lines (HuH7). Cytotoxicity studies revealed that the combination of these lipoplexes remarkably mitigated the cytotoxicity and increased the transfection ability in HuH7 cells in vitro, in contrast to common DOTAP/chol lipoplexes. These new lipoplex systems would be a potential candidate to promote effective DNA delivery [81].

Recently, Harrys and coworkers reported an interesting work aimed to investigate the utility of LFN as a lipid-based alternative to positively charged polymers and lentiviral transduction for T-cell gene delivery. The cationic lipid LFN facilitates the formation of a lipoplex containing negatively charged DNA and positively charged liposomes capable of transfecting Jurkat cells. Transfection of Jurkat cells was accompanied with high efficiency by transfecting cells with LFN in X-VIVO15 media. On the other hand, a much lower transfection efficacy was observed in T cells. This observation, made by confocal microscopy, revealed that the lipoplexes did not enter the primary T cells. Pyrin and HIN (PYHIN) DNA sensors which could prompt apoptosis after complexation with cytoplasmic DNA were also recorded at high concentrations in primary T cells. Consequently, transfection of primary T cells seems to be restricted in the process of cellular uptake [82].

5 Polyplexes for Targeted Gene Therapy

As mentioned above, polyplexes are complexes formed by cationic polyelectrolytes and nucleic acids via the development of electrostatic interactions. Polyplexes comprise an alternative, safer, and propitious approach, toward addressing or averting acquired and hereditary diseases since the cationic polymer carriers facilitate the nonviral delivery/distribution of DNA or RNA in diseased tissues [83–86]. Since the breakthrough investigation by Tang and Szoka, who introduced cationic polymers as nonviral carriers for nucleic acids directly to target cells [37, 87], fundamental advancements involving design approaches, preparation, and exploration of polyplexes by numerous scientific groups have been added to the constantly increasing field of targeted gene therapy. New-generation vectors based on synthetically and physicochemically evolved cationic polymer classes have overcome several barriers concerning polyplex detection by the host's immune system, while their dismissal from the organism has been averted [88–90]. The most essential qualities that polyplexes should possess are the decreased cytotoxicity to block unwanted adversary actions in patients and to maintain the functionality of the transfected cells to employ therapeutic efficacy, the obstruction of the nuclease-intervened decomposition prior to approaching target cells, and the evasion of host eviction procedures, such as filtration in the spleen and absorption by collector cells [91].

The fact that DNA undergoes extensive folding and is finally condensed when connected with cationic polymers has been confirmed by numerous investigations [92]. Hitherto, polyplexes of globular, rodlike, and toroid structures have been reported as the outcome of cationic polyelectrolyte complexation with DNA molecules [93–95], poly(amidoamine) (PAA) [96], poly(dimethylamino ethyl methacrylate) (PDMAEMA) [97], and poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] [98] being some typical examples of positively charged polyelectrolytes. Nevertheless, polyplexes tend to aggregate when the complexation process occurs at charge stoichiometric conditions [88]. Incorporation of a neutral hydrophilic block such as poly(ethylene glycol) (PEG) to a cationic block toward the preparation of block copolymers can inhibit further aggregation and permits the compaction of a DNA molecule inside a polyplex nanostructure protected by a PEG corona (Fig. 5) [94].

A detailed study regarding the effect of PEG functionalization of polymeric carriers on the efficient distribution of gene material to target cells was presented by Clima and her collaborators [99]. They created a collection of vectors consisting of the hydrophobic component PEGylated squalene SQ-PEG-NH_2, namely, poly-(ethyleneglycol)-bis(3-aminopropyl)) (NH_2-PEG-NH_2) of different molecular weight (Mn 1500, 2000, and 3000 Da), and branched polyethyleneimine (bPEI) of low molecular weight (Mn 800 Da). The concept behind the creation of the nonviral vector collection was to alter the composition of the vectors by slowly increasing both the H_2N-PEG-NH_2 ratio and the molecular weight of H_2N-PEG-NH_2 (from 1500 Da to 3000 Da). TEM and DLS techniques showed that an increase in molecular weight of H_2N-PEG-NH_2 induced the formation of structures of smaller dimensions due to steric interactions among PEG clocks and the scaffolding components (Fig. 6). Additionally, it was determined that increasing the molecular weight of H_2N-PEG-NH_2 to 3000 Da in the carrier composition pDNA-binding capability was becoming more fragile, resulting in the formation of larger polyplexes, ascribed to the shaping of a protective veil over the bPEI800 units triggered by PEG units. The group also performed biological evaluation studies on HeLa cell culture to determine transfection efficacy and cytotoxicity parameters of the aforementioned nonviral vectors. All examined samples exhibited transfection efficacy higher at an N/P ratio equal to 100 than at an N/P ratio equal to 50. The chain length of H_2N-PEG-NH_2 exhibited an important effect on both the cytotoxicity and transfection efficacy. Finally, the group concluded that the presence of H_2N-PEG-NH_2 component with comparatively high molecular weight as part of the polymeric vector contributed at the enhanced biocompatibility of the latter, yet reduced transfection efficiency of the cells.

Haladjova et al. studied the physicochemical properties of DNA carriers based on novel poly(vinyl benzyl trimethylammonium chloride) (PVBTMAC) homopolymers and block copolymers [100]. The investigations involved the utilization of two types of linear DNA in terms of chain length. The outcome of the complexation process between the homopolymers and block copolymers with the DNAs was determined by factors such as the length of a cationic block, total polymer composition and architecture, N/P ratio, and salt concentration, which were expected to

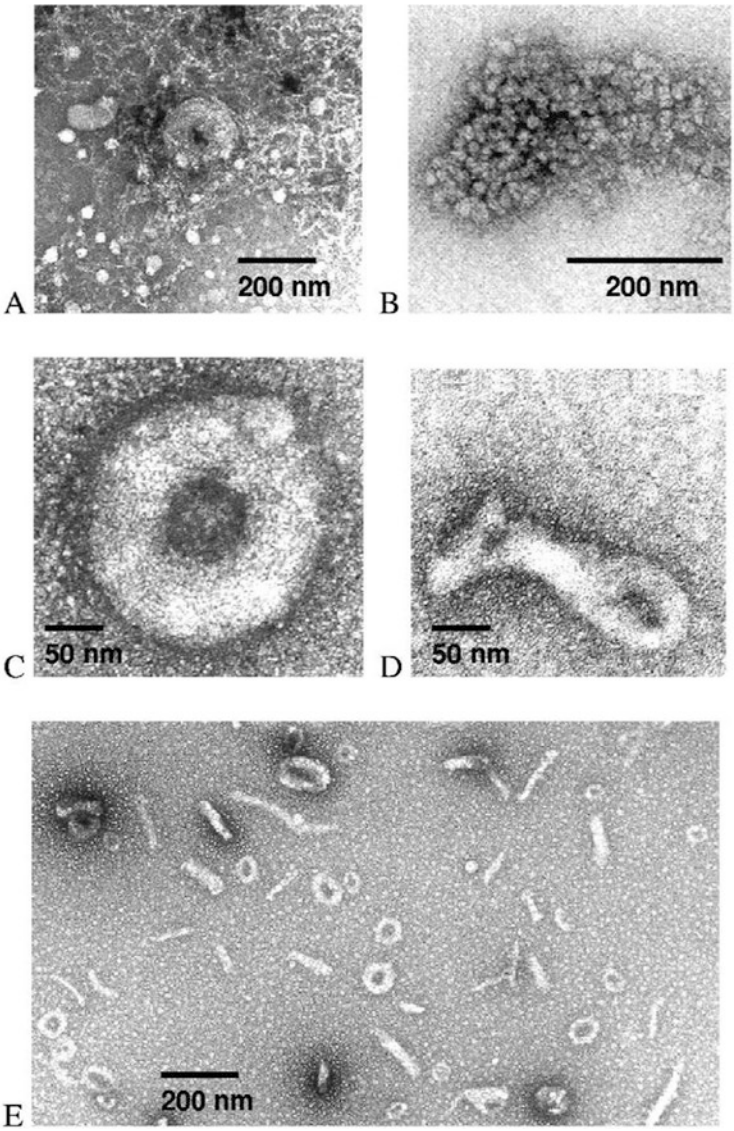

Fig. 5 Transmission electron microscopy pictures of poly-L-lysine/pDNA complexes at 1/2 charge ratio 4:1. Toroid "in a net," size of 200 nm formed by unpegylated third-generation dendrimer (**a**). Aggregate of unpegylated linear PLL 20 kDa, about 500 nm in size (**b**). A perfect complex of unpegylated third-generation dendrimer and plasmid DNA (**c**). The size is about 200 nm. Twisted complex formed by pegylated fifth-generation dendrimer (**d**) ⌊250 nm size. General picture of usual forms of rods and toroids with grafted PLL (**d**); linear PLL showed similar forms. The size of rods is about 150–250 nm and toroids about 100 nm. (Adapted from Ref. [94])

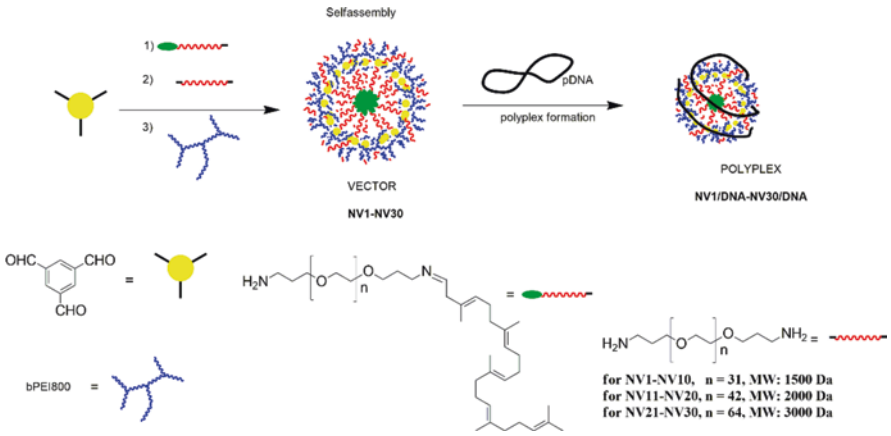

Fig. 6 Schematic representation for the formation of vectors NV1–NV30 and polyplexes. (Adapted from Ref. [99])

affect the structure, stability, and efficacy of the formed polyplexes. The group ascertained that the presence of POEGMA groups provided stabilizing and shielding functionality that eventually prevented aggregation and precipitation and assured colloidal stability to the polyplexes. In fact, precipitation phenomena were monitored only in the case of complexation of the shorter DNA with the shorter PVBTMA homopolymer (20 K), as the absence of POEGMA block allowed the polyplexes to aggregate by forming structures of high dimensions at a specific solution ionic strength. Moreover, they established PVBTMAC as a very promising gene vector as they discovered that the high content of charged segments and the average hydrophobicity contributed to compressing DNA dimensions. Specifically, they declared that as long as the molecular weight of the polyelectrolyte block increased, it acted more efficiently in shrinking DNA regardless of the molar mass and secondary structure of the nucleic acid.

Another important contribution to the field was reported by Tan and coworkers [101]. The group compared the efficacy of the poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) homopolymer and poly(ethylene glycol)-block-poly(2-(dimethylamino) ethyl methacrylate) (PEG-b-PDMAEMA) double hydrophilic diblock copolymer pair along with poly(2-(dimethylamino)ethyl methacrylate)-block-poly(n-butyl methacrylate) (PDMAEMA-b-PnBMA) amphiphilic diblock copolymer and poly(ethylene glycol)-block-poly(2-(dimethylamino)ethyl methacrylate)-block-poly(n-butyl methacrylate) amphiphilic triblock terpolymer pair, as plasmid DNA (pDNA) carriers. Upon complexation with pDNA, PDMAEMA and PEG-b-PDMAEMA formed polyplexes, while PDMAEMA-b-PnBMA and PEG-b-PDMAEMA-b-PnBMA micelleplexes. The polyplexes were aggregates of high mass and size in the case of PDMAEMA and of globular morphology in the case of PEG-b-PDMAEMA. In both cases, pDNA was compressed in the core of the nanostructure. On the contrary, micelleplexes formed beads-on-a-string morphologies with pDNA chains surrounding and connecting with

multitudinous micelles. The results of the study demonstrated that even though both kinds of complexes exhibited similar cytotoxicity, micelleplexes outperformed polyplexes regarding transfection efficacy. Indeed, the micelleplexes presented four times greater transfection efficiency than the polyplexes. The fact is correlated to multiple aspects of the nanosystems. First, the micelleplexes seem to be incorporated more efficiently. Moreover, the introduction of a greater number of amine segments to each micelleplex might help endosomal getaway. More significantly, the "beads on a string" morphology of the micelleplexes imitate the way the cells enclose DNA all over histones in chromatin and preserve the endogenous geometry of helix B of DNA chains. The preservation of DNA geometry is essential since it prompts more beneficial protein expression relative to that where DNA is located within polyplexes, in complexed and compacted conformation, and with its B-form considerably modified.

Equally interesting is the case of the double DNA delivery ensemble, nanoparticle-in-microsphere (NIM) toward DNA vaccine formulations, proposed by Lu and his group [102]. They designed and developed a hybrid system (NIM) that would combine the features of nano-dimensional polyplexes along with the continuous release microsphere concept for DNA vaccine preparations. Specifically, they synthesized polyethylene glycol-graft-polyethyleneimine (PEG-g-PEI) copolymers that acted as DNA vectors. In the next step, lyophilization of DNA along with PEG-g-PEI occurred to condense DNA into a nanostructure for DNA micronization. The resulted polyplexes were formulated into NIMs by following the solid-in-oil-in-water (S/O/W) emulsion protocol to encapsulate DNA polyplexes. In this case, PEG contributed to the colloidal stability of the hybrid system and to maintain the solubility of the polyplexes in a nonaqueous environment. The authors stated that by implementing this strategy, the DNA vaccine could be shielded by increased homogenization and by avoiding the water-oil interface that would trigger DNA denaturation. NIMs comprise a novel, beneficial technology for gene distribution to phagocytic cells that would speed up the intracellular release of DNA vaccine. Further investigations proved that this particular DNA vaccine ensemble presents the ability to provoke immune responses even at low DNA doses in big animals. Therefore, this type of vaccine technology offers great potential for application in humans to treat life-threatening diseases.

Similarly, Soares et al. proposed a novel approach to developing vaccine formulations [103]. They introduced nonviral vectors consisting of poly(β-amino ester) (PβAE) and poly[2-(dimethylamino)ethylmethacrylate] (PDMAEMA) polymers to address low transfection efficiency and immune endurance, parameters that create obstacles to the effectiveness of DNA vaccine technology. The group via a series of methods, such as size measurements, gel retardation studies, cell viability assays, transfection experiments, blood compatibility investigations, etc., established that pDMA/PDMAEMA/PβAE polyplexes can be adjusted to acquire a small size by increasing the pDNA ratios and are eligible for utilization in mice vaccination investigations. Subsequently, they accomplished to include two types of β-glucan in vaccine formulations to finally collect polyplexes of ameliorated transfection efficiency in RAW 267.4 macrophages. The conducted experiments revealed that the

cytotoxicity and the hemocompatibility are strongly influenced by the dose rate. Thus, a secure operating set was arranged. Mice vaccination investigations through the subcutaneous route were not successful as originally anticipated producing a 40% HBsAg seroconversion, unaffected by the β-glucan presence.

Other works involving the formation of polyplexes for nonviral gene delivery include that of Faria and coworkers [104]. Hitherto, conventional delivery vehicles that present serious limitations such as restricted lifetime in the patient body and undesirable side effects are the ones mostly used in combating most cancer cases. The group conducted studies that combine drug and gene delivery strategies. Specifically, they incorporated the anticancer drug methotrexate (MTX) into the polyplexes that resulted from the complexation of polyethyleneimine (PEI) with p35 encoding pDNA. The polymeric vehicles, apart from being capable of encapsulating MTX, acquire a plethora of advantages such as size, structure, surface charge, and cargo complexation for intracellular transportation. The MTX cancer cell-targeting quality tested over folate receptors has been established and produces proof for receptor-intervened endocytosis. Confocal microscopy studies established the integration of vectors into cells and pDNA access in the nucleus, along with the regulation of the cell transfection efficacy with the aim of ameliorating protein expression rates.

Another group that has dealt among others with the formation of DNA polyplexes is that of Valente. In one particular case, they studied the complexation ability of chitosan (CH) or polyethyleneimine (PEI) with different lengths of pDNA [105]. ζ-Potential and encapsulation efficacy experiments confirmed the hypothesis of synthesizing cationic polymeric vehicles, able to load DNA in dependence on the ratio of both components. Cell viability assays were conducted in cases of CH/pDNA at N/P = 0.75 and PEI/pDNA at N/P = 100, where the encapsulation efficiency, zeta potential, and size values were evaluated as the most suitable. Cytotoxicity evaluation demonstrated that CH/pDNA polyplexes were biocompatible, but PEI/p53-pDNA polyplexes presented exiguous cytotoxicity in healthy cells which could prevent their future employment as therapeutic agents. The results obtained by transfection assays showed that all the studied polyplexes can transfect the cell lines utilized (Fig. 7). However, higher transfection rates were monitored in the case of complexation with the smaller DNA. In the next step, the group investigated the possibility of P53 protein expression via employing the Hela cancer cell line. P53 rates increased up to 54.2% and 32% when chitosan and PEI polymers, respectively, acted as vectors. A direct comparison between the two types of polymers based on experimental conditions and the obtained results defined chitosan/pDNA polyplexes at N/P = 7.5 as less cytotoxic, more productive regarding cell transfection, and more effective triggering agents toward protein expression. To sum up, chitosan/pDNA polyplexes are more eligible to pDNA delivery approaches relative to PEI/pDNA polyplexes.

Sentoukas et al. described the complexation process of (2-[dimethylamino]ethyl methacrylate)-b-poly(hydroxypropyl methacrylate) (PDMAEMA-b-PHPMA) dual-responsive block copolymers, in terms of pH and temperature, and their quaternized derivatives QPDMAEMA-b-PHPMA with short, 113-base DNA [106].

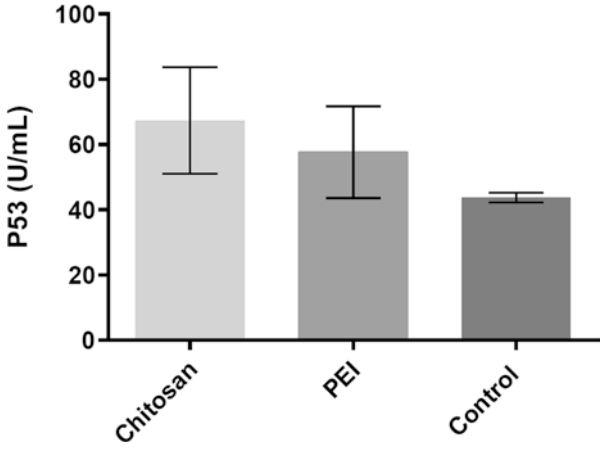

Fig. 7 Evaluation of p53 protein expression following administration of p53-pDNA polyplexes of CH and PEI in HeLa cervix carcinoma. Data are represented as mean ± S.D., $n = 2$. (Adapted from [105])

Studies have shown that complexation with a block copolymer of smaller length resulted in well-defined polyplexes only at N/P = 1, while the one with the longer chain length formed well-structured polyplexes at all N/P ratios. However, salt addition induced the formation of aggregates of large dimensions due to the occurrence of charge screening effects that eventually triggered a decrease in the electrostatic interactions between the positive charges of the block copolymer and DNA. The occurrence of stronger interactions between DNA molecules and the PDMAEMA segment compared with the quaternized one was determined by optical absorption studies. Fluorescence quenching studies demonstrated that the block copolymer interacts efficiently with DNA, suggesting a distinctive inner association of the preorganized copolymer nanoparticles, regardless of the low ionization degree of the PDMAEMA block. Surface charge measurements indicated that the most suitable N/P ratio is equal to 1, producing polyplexes under the highest complexation with DNA. Finally, the authors suggested that for all cases of polymers used, the most possible scenario about the location of the DNA is on the surface of the resulted complexes, because the cationic segments, interacting with DNA, should also be sited on the surface of the preorganized polymer aggregates.

Along the same lines, Chroni et al. proposed an innovative multirole nanosystem that entailed the cationic poly[oligo(ethylene glycol) methacrylate]-b-poly[(vinyl benzyl trimethylammonium chloride)] (POEGMA-b-PVBTMAC) diblock copolymer, combined with hydrophilic negatively charged magnetic nanoparticles (MNPs), and its subsequent complexation with linear DNA, comprised of 113 base pairs [107]. The preassembled nanostructures were spherical. The design and investigation of the novel triple-functional DNA delivery system were based on the development and subsequent monitoring of the electrostatic interactions between the positively charged PVBTMAC segments and the negatively charged magnetic nanoparticles and DNA phosphate groups. Parameters such as solution

concentration, solvent, and ionic strength strongly affected both the self-assembly behavior of the diblock copolymer and the co-assembly of DNA and cationic block copolymer with the magnetic nanoparticles. DLS measurements showed the formation of magnetopolyplexes resulting from the complexation of the hybrid MNPs/copolymer aggregates along with the short DNA molecule. The obtained magnetopolyplexes presented a hydrodynamic radius equal to 283 nm at N/P = 4. Physicochemical investigations revealed that surface charge, size, mass, and complexation ability between the hybrid MNPs/copolymer aggregates and DNA were regulated by the N/P ratio. The group observed the formation of magnetopolyplexes of lower mass as the salt concentration increased, while the size was relatively constant. Analysis of the images obtained by cryo-TEM disclosed an important inclination toward aggregation phenomena of the NMPs/copolymer structures upon interaction with the DNA molecules. The observed behavior is assigned to the accretion of the DNA molecules on the surface of the hybrid NMPs/copolymer complexes. The magnetic properties of the MNPs were preserved after the complexation process with DNA, prompting the multifunctional MNP/polyelectrolyte copolymer/DNA system eligible for therapeutics and bioimaging applications.

Another noteworthy study was reported by Rumschöttel et al. [108]. The group explored polyplexes formed between DNA extracted from salmon tastes along with either hyperbranched poly(ethyleneimines) (PEIs) of molecular weights equal to 5000 g/mol and 25,000 g/mol or modified PEI (5000 g/mol) with maltose segments (PEI-Mal), according to molar N/P ratio adaptation by implementing DLS, surface charge, DSC, STEM, and cryo-SEM techniques. Particularly, polyplexes derived from hyperbranched PEI and DNA exhibit dissimilar tendencies, depending on the N/P ratio. Polyplexes of small dimensions (ca. 80 nm) were observed when DNA content was higher relative to that of the polymer. Those polyplexes were formed by relaxed stacked DNA network regions, linked with DNA strands. However, when cationic PEI content was in excess, every DNA molecule was located inside the cationic polyplex. At the specific N/P ratio equal to 8, the majority of DNA molecules are placed in the core of spherical configurated polyplexes, encircled by PEI chains. At an extremely high excess of PEI (ca. N/P = 40), polyplexes of onion-like configuration and 200 nm dimension are detected. Therefore, the morphology of the resulted polyplexes is tremendously affected by the N/P ratio. Moreover, the authors discovered a deviation at the melting point of DNA from 88 °C to 86 °C attributed to the strong electrostatic interactions developed between DNA and PEI. In the case of interaction of the less toxic PEI-MaI with DNA, polyplexes of positive charges were detected at lower N/P ratios. Moreover, the participation of PEI-Mal instead of unmodified PEI forges larger aggregates a tendency ascribed to the occurrence of more hydrogen bonds. According to DSC studies, solely on the occasion of a high quantity of PEI-Mal at N/P ratio above 40, dense polyplexes characterized by two melting points are detected. Relatively to polyplexes obtained as the result of the interaction between the unmodified PEI and DNA, the ones derived by PEI-Mal and DNA are of spherical morphology and dimensions of 100–250 nm. The latter exhibited enhanced stability, attributable to the combination of electrostatic and H-bonding interactions.

6 Conclusions

In this chapter, we presented the current technology and the applications of lipoplexes and polyplexes in the field of targeted gene delivery. These nanosystems are very useful for the delivery of nucleic acids for the treatment of several diseases like cancer. We also gave special attention to the mechanisms by which lipoplexes and polyplexes are used for the delivery and the release of the complexed nucleic acids. The physicochemical characteristics of nucleic acid complexes were discussed, in some detail. Several examples from the recent literature were analyzed. Current research which has already utilized several types of lipo- or polyplexes to target a plethora of molecular and cellular mechanisms has been presented. The research outcomes show that both of these cationic carriers open new horizons for the fast clinical translation of nanomedicines with added value for the treatment of several diseases.

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Aptamer-Based Targeted Drug Delivery Systems

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1 Introduction

Aptamers are short, chemically synthesized generally single strands of RNA or DNA oligonucleotide having the ability to bind to a specific target [1]. Most aptamers are made up of sequence of nucleic acid, where various nucleotide chains are linked with sugar at 50 and the 30 extremities through phosphodiester bonds [2]. Further, the flexible nature of these nucleotides provides possible interactions with others and the associated surrounding environment [3]. As a result, these molecules can form a variety of structures through combination with or without proteins (Fig. 1). Frequently, these complex structures are composed of several secondary

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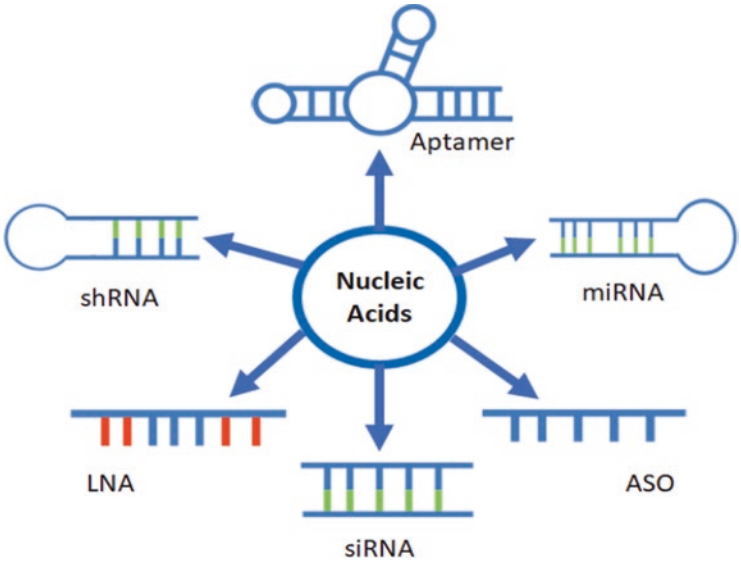

Fig. 1 Aptamer architectures for therapy

structure motifs and formed through Watson-Crick base pair methods [4]. Moreover, sequences of these stems may transform into bulges or loops to get three-dimensional complex structures [5]. Currently, several structural prediction software have been developed that can be utilized to predict exactly the transformation of stems from the primary nucleic acid sequence [6]. However, many times these predictions are not that much of perfect to consider secondary structures as rigid elements. The major reasons are due to the flexible nature of both single-stranded and secondary structure loops, formation and breaking of base pairs, and development of flex at helical regions [7].

The size and ease of synthesis of aptamers make them as a ligand of choice when compared with counterpart antibodies during medical research for targeted delivery [8]. Moreover, high specificity which is critical for functioning, especially to biomarkers of cancer, makes them a highly applicable choice of ligand for drug targeting and delivery system development. Additionally, the high affinity of aptamers can be achieved as similar to antibodies. Moreover, ease of modification and modeling, lower toxicity, higher stability, shorter screening cycles, and immunogenicity are some of the additional features of aptamers over antibodies [9].

Geysen and collaborators in 1984 reported for the first time the use of a novel combinatorial synthesis approach to develop viral protein aptamers of 213 amino acids, which was further tested for their affinity toward antibodies and resulted to have a binding and recognition of each other. At the later stage, similar approaches have been widely explored with peptides and some molecules of chemical origin by others [10].

Bereft of oligonucleotides, peptide-based aptamers, or affirmers are other types of aptamers also included in this group [11]. All aptamers are unique in features with constant at 5′ and 3′ end regions and having random nucleotide bases in the middle region [12]. The random region of the aptamer is mainly responsible for heterogeneity and is mainly utilized for library formation of the aptamer. SELEX (systematic evolution of ligand by exponential enrichment) is an iterative method, to incubate a library of aptamer followed by a further selection of targets [13].

Aptamers are mainly utilized in nanomedicine for the diagnosis of cancer cells [14]. The unique features of aptamers are their small molecular weight and high affinity to the target molecules enabling them for targeted therapy [15]. The aptamers exhibit to reach the cancer cells' core via the internalization process through the endosomal pathway. This is mainly due to higher flexible configuration, which causes target specific and high binding recognition [16].

Interestingly, aptamers can be further suitable to be utilized for the production of nanoimaging agents via linking with radionuclide probes or imaging nanomaterials [17]. The present chapter covers the various roles and applications of targeted delivery through aptamer delivery systems after reviewing several published studies carried out in the recent past. Further, the application of aptamer is included and discussed at the end of this chapter and also has been summarized recently with reference to a recently published study [18].

2 DNA Aptamers as Targeted Drug Delivery

DNA aptamers are known for the folding of short single-stranded nucleotide sequences (versatile molecular species), which exhibit significant advantages for multiple therapeutic applications. Generally, two important therapeutic functions can be achieved through DNA-based aptamer delivery system.

DNA aptamers specifically bind to the target protein and function as agonist or antagonist by promoting the activity of the target protein or inhibiting the protein-protein interactions, respectively [19]. Although the non-immunogenicity of DNA-based aptamers provides them an edge as future therapeutics, which in terms make them similar to antibodies [20]. This is due to easier uptake considering their small size [21] and through recognition of a broad range of target species such as proteins, smaller molecules, and cells. Moreover, DNA-based aptamers are more economical in production cost than correspondence antibodies due to the easiest design and selected based on simpler in vitro methods. These characteristics of DNA-based aptamers mentioned above suggest the enormous clinical value of DNA aptamers [16]. Some of the important innovative development in this connection is discussed herewith:

2.1 AS1411

Antisoma is an aptamer rich in guanine base, having a guanine quadruplex structure and nucleolin (a eukaryotic with nucleolar phosphoprotein targeting for anticancer therapies) protein [22, 23]. The guanine quadruplex structure of AS1411 improves the resistance toward nuclease degradation and the cell uptake process. Further, AS1411 also exhibits antitumor activity in vitro in breast cancer cells [24] and acute myeloid leukemia [25]. In 2009, clinical trial (NCT00740441) phase 2 of AS1411 aptamers to treat carcinoma of renal cells was completed with significant activity (NCT01034410). In another clinical trial (NCT01034410) phase 2, accomplishing treatment of acute myeloid leukemia disease was completed in 2011.

2.2 ARC1779

ARC1779 is a PEGylated DNA aptamer by Achemix, which selectively identified platelet ligand-receptor, i.e., von Willebrand factor, to mediate recruitment of platelet [26]. It induces an antithrombotic effect by blocking the binding between the blood platelet and the von Willebrand factor. Further, platelet inhibition activity has been demonstrated by ARC1779 during clinical trial phase 2 for treating platelet dysfunction associated with von Willebrand factor (NCT00632242). A recent study demonstrated that ARC1779 is also effective in preventing thromboembolism [27]. Further, in phase 2 clinical study in patients with congenital thrombotic thrombocytopenic purpura, it was observed that ARC1779 demonstrated favorable pharmacokinetics and pharmacodynamics parameters [28].

2.3 NU172

It is an unmodified DNA aptamer targeting the thrombin protein (ARCA biopharma), to prolong the blood clotting process [29]. The antithrombin activity of NU172 was further validated in the clinical trial (NCT00808964) phase 2 in patients undergoing off-pump coronary artery bypass graft (CABG) surgery [30].

2.4 E10030

It is a PEGylated DNA aptamer (from Ophthotech) and acts as a platelet-derived growth factor antagonist. Evidence suggested that E10030 can effectively prevent angiogenesis when given in combination with anti-VEGF (vascular endothelial growth factor). In this context, a phase 3 clinical trial study (NCT01944839) is

ongoing against the treatment of wet-associated age-related macular degeneration in combination with ranibizumab (Lucentis®) of E10030 as a platelet-derived growth factor antagonist [31].

2.5 Miscellaneous

Further, many other DNA aptamer candidates are currently in preclinical studies against various diseases including viral infections, somatic tumors, and diseases of the central nervous systems. A recent study is based on DNA aptamer, where recognition of the receptor for binding influenza A hemagglutinin region was effective against viral infection in an animal model. The antiviral effect of these aptamers on different influenza strains showed a 90–99% reduction of the lung-treated mice virus burdens [32]. An earlier study (included both in vitro and in vivo) demonstrated that DNA-based aptamer NAS-24 induces the mouse ascites apoptosis of adenocarcinoma cells which was binding with vimentin [33]. Another study also depicted that DNA aptamers NAS-24 were able to retard the gastric cancer tumorigenic growth in mice by targeting human epidermal growth factor receptor 2 (ErbB-2/HER2) with superior activity in comparison with anti-ErbB-2/HER2 monoclonal antibody [34].

3 RNA Aptamers as Targeted Drug Delivery

Therapeutic potentials of several RNA-based therapeutics have been extensively studied in the past, including short hairpin RNA (shRNA), ribozymes, small interfering RNA (siRNA), and antisense oligonucleotides (AS OGNs), microRNA (miRNA), and RNA aptamers [35]. RNA aptamers have unique advantages that they can directly target the extracellular molecule and activate the target specific functions.

The RNA-based therapeutics are generally uptaken by the cells to carry out their functions [36]. Due to the binding ability of RNA aptamers with cell surface proteins, RNA aptamers were able to deliver several therapeutic agents including peptides, small molecules, and specific cell type-based RNA therapeutics.

RNA aptamers which have developed against a variety of cell surface markers associated with several human diseases have shown many potential applications including RNA interferences (RNAi): siRNA, shRNA, or miRNA [37]. Thus, RNA-based aptamers have provided a unique opportunity for specificity and delivery to the targeted site [38]. Herein, a few potential examples are illustrated on recent advancements in using RNA-based aptamers as therapeutic agents including delivery tools for the cell-specific site [39].

3.1 Prostate-Specific Membrane Antigen

Prostate-specific membrane antigen (PSMA) is a prostate cancer marker which was highly expressed at the prostate cancer cells' surface and vascular endothelium tumor without affecting normal prostate epithelia [40].

In past, RNA-based aptamers (both A9 and A10) have been isolated against PSMA and used for delivery of therapeutics intracellularly, i.e., use of siRNAs as elaborated earlier [41]. The application of a non-covalently coupled A9 biotinylated aptamer with siRNA against lamin A/C or GAPDH via streptavidin was studied. As a result, knockdown of target genes was demonstrated successfully for specific targeting of these particles without affecting PSMA non-expressing prostate cancer cells [42].

Additionally, RNA conjugated aptamer, i.e., A10 to siRNAs against survival genes of cancer [polio-like kinase 1 (*PLK1*) and *BCL2], was* tested in prostate cancer cell growth in both in vitro and in vivo settings [43]. These A10-siRNA chimeras downregulate the expression of survival genes and subsequent cell death through successful internalization and processing by Dicer, as an evidence from in vitro study [44]. In an in vivo study using a mouse prostate cancer xenograft model, A10-Plk1 siRNA and control mutant A10-PlK1 siRNA chimeras were injected into prostate tumors. It was found that mutant A10-Plk1 siRNAs were able to prevent the tumor growth of the prostate to mediate regression of the tumor.

Moreover, PSMA-based aptamer has also been reported to use in multifactorial approaches, e.g., it can deliver siRNAs to induce tumor immunity followed by ionizing radiation sensitivity for prostate cancer therapy [16]. A10-based aptamer was utilized for the delivery of siRNA against both Smg1 and Upf2 [regarded as a key target of the RNA surveillance or non-sense mRNA decay (NMD) pathway], which was found to be responsible for preventing mRNA expression from premature termination [43]. It was demonstrated that in both in vivo and cell culture xenograft studies, A10 conjugated Upf2 and Smg1 siRNA can specifically target and inhibit the tumor cells' growth. Another aptamer A10-Smg1 was found to be superior in tumor growth inhibition activity over-vaccination with irradiated tumor cells expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) [45]. Similarly, A10 aptamer-shRNA chimera targeted to DNA-activated protein kinase (DNA-PK) demonstrates the improvement in radiosensitivity of prostate cancer cells expressing PSMA [46]. High-throughput screening of siRNA library is carried out to identify the important gene members responsible for the cell's repair mechanisms in PSMA-expressing prostate cancer cells. During the both in vitro and in vivo (in a human prostate tissue specimen and xenograft models) studies, DNA-PK was found to be one of the potential targets of prostate cancer cells which could be specifically downregulated using the A10-3 aptamer-DNA-PK shRNA. Further, A10-3-DNAPK shRNA chimeras administered intravenously improve sensitization of PSMA-positive prostate tumor toward ionized radiation. Thus, low levels of radiation are required to treat cancer cells to avoid surrounding healthy tissue deprivation such as the gallbladder and rectum [46].

3.2 CD4 Aptamer and pRNA Nanodelivery System

The major challenge and limitation associated with RNA-based therapy is systemic and intracellular delivery of RNA therapeutics (e.g., siRNA, ribozyme, etc.) to the desired and specific target cells. RNA aptamers also could open a new avenue to provide cellular targeting specificity. However, they suffer from accelerated body clearance [47]. Thus, pRNA nanoparticles are the first choice for the delivery of RNA therapeutics as they are composed of several types of RNA. Further, retention of added advantages of RNAs as therapeutic agents is always due to maximum utilization of all types of RNA delivery systems [48]. Additionally, pRNA monomer is an RNA molecule of about 117-nt long and 11 nm in size constituting the DNA subunit packaging motor of bacteriophage phi29. The pRNA is composed of two functional domains, i.e., pro-head binding and DNA translocation domain, that can fold independently. Structurally, the DNA translocation domain has a 3′/5′ double helix loop in which left- and right-hand loops are of the pro-head binding domain. Term survivin is used for replacing pRNA sequence (at 3′/5′ double helix loop) with siRNA against a pro-survival gene. A study (included both in vivo and in vitro) demonstrated for the first time that this replacement improves proper folding to knock down the target gene expression followed by tumor growth inhibition. One major significant feature of pRNA-based delivery system is to form pRNA dimers or multimers by interactions of right- (A) and left-hand loop. Subsequently, pRNA dimers were generated with one pRNA(a′-B) having RNA aptamer against CD4 receptor, while the other pRNA(A-b′) was conjugated with siRNA against survivin to inhibit the cancer growth. In conclusion, evidence from the studies suggested that the developed dimer can target specific CD4-positive lymphocytes, reduce cell viability, and silence the target gene expression [49].

3.3 gp120

Research on human immunodeficiency virus (HIV) infections utilized RNA aptamer against gp120 target for targeted delivery of siRNA [50]. It was demonstrated that people infected with HIV-1 are susceptible to target cells expressing the CD4 receptor. Cell surface protein known as glycoprotein gp120 (express during infection) is primarily used in the HIV-1 virus to recognize the CD4 cell receptor followed by membrane fusion initiation and following the delivery of both viral RNA and enzymes intracellularly [51]. Using this mechanism of HIV, siRNA and gp120 aptamer chimeras targeting the HIV-1 tat/rev region were developed. It was observed that these chimeras can channelized into the cells expressing gp120 to abolish the target gene expression. Evidence from a study suggested that these gp120 aptamer-siRNA-based chimeras are potent and sustainable in action toward inhibition of HIV growth by T cells without any interferon response [52].

4 Aptamer Selection Methods

Oligonucleotide-based aptamers have been popularized for target specificity and affinity. Generally, the selection of aptamers is based on a large set of nucleic acid libraries as a random candidate through relative interaction with the target. As per the stochastic hybrid model, which illustrates the combined impact for success of many important evolutionary factors including randomness, competition, and changes in the environment. For this, the culture environment may be modified with parameters such as target concentration, competition variance (aptamer-target binding affinities differences during initial distribution), and random events (sometimes may eliminate even the ligands with the highest affinity) followed by SELEX approaches. This process dictates an unknown population distribution of fitness parameters, shown by the binding affinities, toward SELEX targets. These uncertainties are created to isolate high-affinity ligand aptamers. Bereft this, the stochastic hybrid model is also defined in terms of the evolutionary selection method of aptamers to explore the impact of these unknown parameters. A single copy of a high-affinity ligand in a pool of billions can strongly influence population dynamics; however, their survival is highly unpredictable. Another simulation technique, Monte Carlo simulation, which is suitable to analyze the impact of environmental parameters including target concentration and selection efficiency in SELEX and identify methods to control these uncertainties was better which leads to ultimate improvement in the outcome and rate of this time- and resource-intensive process accelerated.

4.1 Positive Selection

This process includes aptamers' library incubation with potential targets immobilized on support molecules or vice versa [53]. Such incubation is generally performed at room temperature with an incubating solution that is composed of monovalent salts and can convert into a secondary structure from a linear single strand for improved aptamer affinity toward a target [53]. Subsequently, during incubation high-affinity aptamers make a non-covalent bond with target molecules, while low-affinity aptamers remain in the suspension. The bounded aptamers are segregated from unbound ones, and the bond between the aptamer-target complex is broken down using EDTA, urea, and high temperature [54]. These aptamers are amplified by the PCR method for the next round of positive selection with the target. The whole process is iterative in nature and carried out to remove low target affinity aptamers. The process of positive selection is carried out around in 10–15 rounds to get high-affinity aptamers for the designed target molecule [55].

4.2 Counterselection

Counterselection through SELEX only chooses aptamers that have a higher affinity for the target without using the information on the selective nature of these synthetic molecules [56]. Counterselection is generally carried out to evaluate selectivity via incubating aptamers with counterselector molecules immobilized on support molecules. Generally, these molecules are very similar to the target but not identical in nature [57].

4.3 Negative Selection

The abovementioned selection process of aptamers is followed by the third selection step which is also known as negative selection [58]. However, it was believed that the binding of counter/target molecules is never reached with 100% efficiency and several times resulted in a naked form. Further, the selection process begins with incubating aptamers with naked support molecules to find out the unbound aptamers [29].

5 Aptamer Internalization Mechanism

It is observed that the potential value of any aptamers results due to greater target sensitivity. Apart from this, aptamers have better structure recognition on the intracellular epitopes and can also support intracellular targeting and intracellular imaging. Some of the following internalization mechanisms have been recorded earlier:

5.1 Aptamer Internalization Mechanism Using Temperature

Mostly, aptamers are used at body temperature (37 °C) via in vivo bloodstream transport. Subsequently, binding with membrane receptors and internalization mechanisms of aptamers need to be determined at 37 °C [59]. The secondary structure of aptamers is also temperature-dependent and selection is often based on incubation with a library at 37 °C [60]. Thus, the cells are incubated along with aptamers at different temperatures (4 and 37 °C) conditions to assess the internalization mechanism. For example, when DU145 cells were incubated with fluorescently labeled Cy5-labeled DML-7 aptamers at 4 °C or 37 °C for 2 h, fluorescence was observed within cells incubated at 37 °C [61].

5.2 Aptamer Internalization Mechanism by Chemical Inhibitor

It was established that proteinases (e.g., trypsin and proteinase K) can degrade the extracellular protein [62]. To explore the membrane receptors' aptamer internalization mechanism, the cells are often allowed briefly to proteinase digestion followed by incubation with aptamers [63]. The aptamer internalization is compared against the control group to assess the role of membrane proteins [64]. To evaluate the intracellular uptake of fluorescently labeled aptamers, the digestion of extracellular cell surface-bound aptamers is performed after the internalization has been completed [63].

6 Nanocarrier Functionalization by Aptamers for Targeted Drug Delivery

Some potent therapeutic agents exhibit significant toxicity on healthy cells due to the lack of specificity, and this interaction may also lead to severe side effects, intolerance, and effectiveness. Clinical data from several studies have demonstrated that healthy blood-forming cells, hair follicles, and the nervous system are most sensitive to being attacked and affected by the chemotherapeutic agents. It was well established that targeted delivery systems are the key approach to overcoming unwanted cytotoxicity. Thus, aptamers combined with nanocarrier (Fig. 2) for target drug delivery are currently gaining high impetus and are capable of preventing damage to healthy cells, and increasing drug efficacy on tumors without side effects. Some of the possible functionalizations are herewith further discussed:

6.1 Aptamer-Functionalized Liposomes

Liposomes are generally considered to be versatile and self-assembling vesicular carriers containing one or more lipid bilayers of phospholipids and/or cholesterol and are generally used to encapsulate hydrophobic drugs [65]. Liposomes are comparatively easy to produce, nontoxic, and biodegradable in nature, with smaller (approx. 50–150 nm) in size [66] to get access up to the molecular level. Liposomes are effective for loading drugs having suboptimal therapeutic index due to unfavorable pharmacokinetic properties [67]. Many value-added procedures currently added new features to liposomes including coating with polyethylene glycol (PEG), which is mainly responsible for opposing interaction by the mononuclear phagocyte system (MPS) for improvement of circulation time, if injected intravenously [68]. Further, it supports improving the liposome extravasation into the tumor microenvironment compared to healthier counterparts [69]. Currently, virus-derived and non-virus-derived vectors for delivering DNA to the cells are utilized for a clinical trial

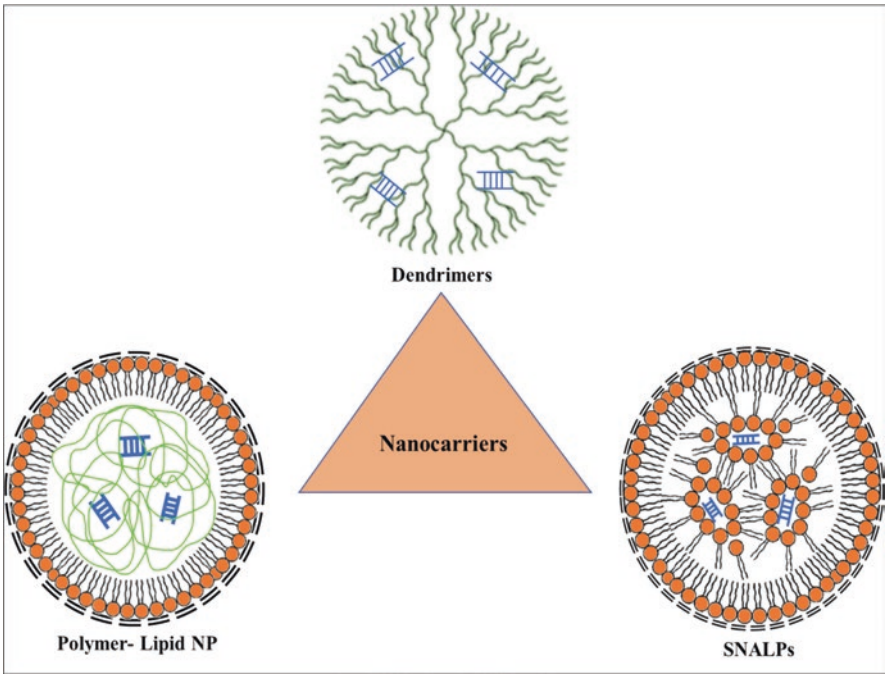

Fig. 2 Schematic illustration of aptamer functionalized nanocarriers

conducted through gene therapy [70]. Additionally, viral vectors are considered to cause higher transfection efficiencies over a variety of cell targets, which many times limits the process of immune response induction and insertional mutagenesis during clinical trials [71]. Therefore, nonviral gene transfer methods are preferred over viral gene transfer. Additionally, nonviral vectors, including liposomes, are a promising alternative to viral vectors, as they are safer, versatile, and easy to develop and scale up.

6.2 Aptamer-Functionalized Micelles

Micelles are generally regarded as self-assembly of amphiphilic copolymers block in certain solvents above critical micelle concentration (CMC) level. Further, many recently developed amphiphilic diblock polymers used in nanotherapeutics were studied and designed to develop delivery systems and also act as an inhibitor for multidrug resistance [72]. Micelles have been developed in a variety of shapes and sizes including rods, spheres, vesicles, lamellae, and tubules, based on the relative length of hydrophobic/hydrophilic blocks and solvent nature [73].

Additionally, micelle-based nanocarriers offer selective recognition of targets which ultimately increases the cellular uptake and reduces the systematic toxicity.

It was shown that micelle nanocarriers composing AS1411 aptamers, copolymers β-CD-PELA, and amphiphilic polymer Pluronic F127 could be used for targeting DOX delivery to human breast tumors [74, 75]. The study revealed that aptamers AS1411 improved the cellular uptake of DOX via nucleolin-mediated endocytosis through selective recognition of nucleolin-positive MCF-7 cells.

DOX loaded in micelles exhibited stronger anticancer efficacy and lower cardiotoxicity in mice bearing MCF-7 tumors over free drugs. Thus, conjugation of AS1411 aptamers significantly improves the delivery of micelle-based drug nanocarriers [75]. Surprisingly, aptamer-micelle nano-conjugates could also achieve versatile applications by adding multiple functional elements such as responsive stimuli or imaging agents. For an instant, in a recent study, a multifunctional aptamer-micelle nanocarrier was developed via integrating a pH-activated fluorescent probe (BDP-668) as well as a near-infrared photosensitizer (R16FP) to detect target cancer cells, MDA-MB-231. The payload of R16FP was capable of lysosomal degradation of target cells via generating reactive oxygen species (ROS) upon NIR irradiation. The BDP-668 introduction enabled the change in lysosomal pH, to monitor the real-time-based therapeutic progress. Therefore, such a multifunctional approach is a great addition to cancer therapy with effective drug delivery combined with an imaging-guided approach [76].

6.3 Aptamer-Functionalized Nanoparticles

Drug delivery systems utilizing nanoparticles (NPs) have several unaided advantages including treatment of cancer, precise tumor targeting, desired pharmacokinetics, avoiding drug resistance, and comparative least side effects [77]. Besides this NPs offer designing as per the size and required characteristics to act on tumor pathophysiology [69, 78]. Additionally, these NPs have targeting efficiency and positioning effect over tumor cells after absorption [79]. Further, choice of size, surface properties, improvement of half-life, drug localization at the tumor site, specificity to tumor cells/environment (without affecting normal cells), enhancement of retention, and permeability associated with NPs also make them suitable delivery weapons [80]. Formulation of the key novel nanocarriers and the mechanism of pH-induced drug delivery are discussed in Fig. 3 [81].

In this connection, a study proposed a robust aptamer-based Au-NPs co-drug delivery system to co-deliver photosensitizing agent (TMPyP4) and potential anticancer drug doxorubicin. It was observed that an anti-nucleolin aptamer, i.e. AS1411, was assembled on the surface of the nanoparticle by a dsDNA annealing. Then, DOX and TMPyP4 were loaded by physical intercalation with the aptamer sequences. Further, the light induction allows TMPyP4-dependent photodamage, while doxorubicin is released at the target site parallel, thus enhancing the final therapeutic value of developed nanocarriers [81, 82]. Subsequently, novel AuNP-aptamer complexes were used for the anthracycline drug daunorubicin (Dau) for pH-dependent release of the drug adjacent to the leukemia cells [83]. In this study,

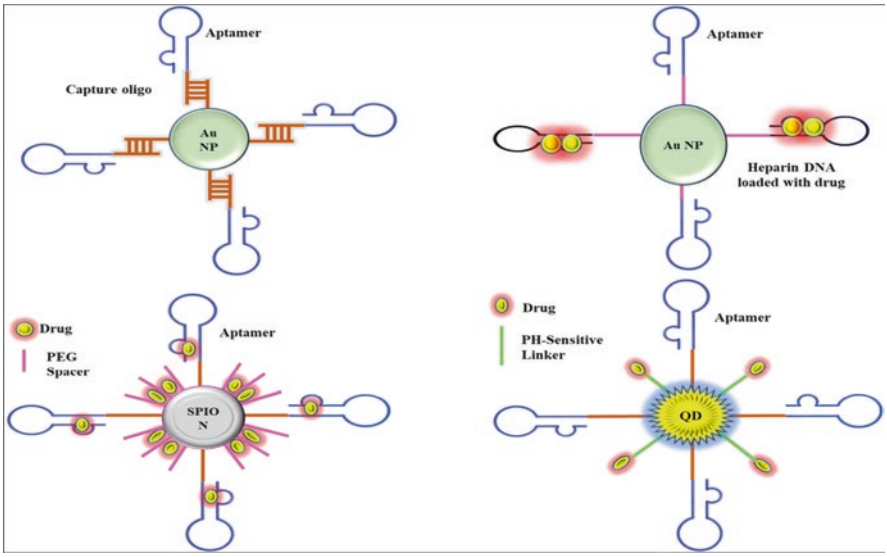

Fig. 3 Formulation of the potential PEG-based nanocarrier drug delivery platform and pH-dependent drugs release the mechanism [81]

anti-PTK-7 sgc8c aptamers were complexed to AuNPs via electrostatic interaction, and Dau was loaded using the electrostatic adsorption at the nanoparticle periphery and also via intercalation into polyvalent aptamers. The developed complex has shown selective binding to the cells and potential cytotoxicity in an in vitro study. Later on, a similar approach was then further developed to obtain a dual-targeted drug delivery vehicle by using blocks of the polyvalent aptamer composed of the anti-PTK-7 sgc8c and anti-nucleolin AS1411 aptamers [83]. In addition to the selectivity, there is a strong possibility to reverse the functional effect of the complex by using antisense of the polyvalent aptamers. The AuNP-based dual therapeutic approach was also demonstrated by using a novel nano-platform having cyclic Arg-Gly-Asp (cRGD) effective against αvβ3integrins, and the AS1411 aptamer [84]. This delivery complex was functionalized either with near-infrared (NIR) fluorescence dye or DOX, to produce a dual-targeting NIR fluorescent probe or tumor microenvironment-sensitive prodrug. In another study, a pH-sensitive anti-CD30 aptamer-hollow gold nanosphere (HAuNS) was developed to deliver the therapeutic molecules after observing change in pH at targeted site [85].

6.4 Aptamer-Functionalized Dendrimers

Dendrimers are generally considered to be outstanding example of molecules of chemical origin having several biological potentials. Apart from this, dendrimers can be functionalized with higher surface groups than a protein of similar size [86].

Recently, aptamer-based dendrimer bioconjugates with potential target specificity have been studied. For an instant, the outermost layer of oligodeoxynucleotides was complexed with poly(amidoamine) (PAMAM) succinamic acid dendrimers to get single-stranded oligodeoxynucleotide-dendrimer (sONT-DENs) conjugates. This complex (i.e., sONT-DENs) was further hybridized with anti-PSMA aptamers to make double-stranded aptamer-dONT-DEN conjugates as well as DOX intercalation. Surprisingly, resulting bioconjugates exhibited antitumor activity and target specificity in animal prostate tumor models [87]. Similarly, PAMAMPEG-based dendrimers conjugated with anti-nucleolin aptamer and encapsulated with 5-fluorouracil (5-FU) which achieve 5-FU accumulation at target sites were demonstrated [88]. Three aptamers, i.e., AS1411, MUC-1, and ATP, which functionalize dendrimer for targeted epirubicin delivery also demonstrated the anticancer activity [89]. It was observed that this complex is specifically uptaken by target cells and has shown effective cytotoxicity through in vitro as well as in vivo settings.

6.5 Other Aptamer-Functionalized Nanocarriers

There are several other nanocarrier-based delivery systems developed for aptamer-guided drug delivery as well as imaging purpose, e.g., metal- or silica-based nanoparticles. The toxicity profile and biodegradation of metal-based nanocarriers may raise concerns. However, they allow a high degree of control over physicochemical properties that is many times not flexible with the aforementioned biomimetic or organic nanocarriers [41]. Particularly, metal-based nanoparticles display higher monodispersity, where several chemical groups can be conjugated with a variety of ligands to achieve drug targeting. Interestingly, metal-based nanoparticles exhibit wide ranges of applications which include magnetic separation, species concentration, and analyte detection for diagnostics besides imaging and therapeutics. The major limitation of these metallic nanoparticles is that they can produce long-term toxicity to normal tissue due to difficulty in elimination from blood circulation. However, these nanoparticles when functionalized with aptamers were found to have promised for the delivery of both imaging and drug molecules as a piece of evidence from many supportive studies [90]. AS1411 aptamer functionalized gold nanoparticles with the improved 17% cell death when compared with free AS1411 aptamer tested against four different types of cancer using 12 cancer cell lines. Interestingly, the visualizing of the active trafficking of AS1411-Au nanoparticles shows the active movement into the nucleus. Further, S1411-Au nanoparticles induced the phenotypic changes to the nuclear envelope which ultimately lead to cell apoptosis [91]. Moreover, the combination of hyperthermia and movement of metal nanocarriers in the cancer cells has been verified by targeting the MCF7 cell line by using multimodal nanoparticles, i.e., Gd2O3 and Ag nanoparticles, bearing the AS1411-based aptamer [9]. Subsequently, the conjugation of the AS141-based aptamer-functionalized doxorubicin with 21 base pairs of (CGATCGA)3 repeats

and to Au nanoparticles allows the effective co-drug delivery of doxorubicin and TMPyP4 photosensitizer. It was observed that the release of doxorubicin in cancer cells was induced by photodynamic stimulation of the constructs [82].

7 Application of Aptamer as a Therapeutic Molecule

Aptamers have shown as one of the promising approaches as evidence that many developing therapeutic agents with several aptamers are either in the preclinical development stage or a few of them have also reached in clinical trial phases (Table 1).

Further, the binding of the aptamers (Fig. 4) to a clinical target to modulate downstream signaling is one of the key features of this delivery system [36].

Other aptamer-based approaches for cancer treatment include target adherence factors (TAFs), receptor tyrosine kinases (RTKs), cell growth modulators, and the immune system [104]. While retrieving information about therapeutic aptamers used for cancer, it was surprising that aptamers have been formulated and investigated for their usefulness in several neurological diseases. Aptamers are flexible to conjugate with many therapeutic nucleic acids as well as small molecules using both covalent and non-covalent approaches (Fig. 5).

This characteristic feature of aptamers has been primarily utilized to develop different chimeric molecules where the aptamer is conjugated with a short hairpin RNA (shRNA) or small interfering RNA (siRNA) to target RNA interference (RNAi). Several nanocarriers may sometimes incorporate the complexes to enhance the delivery of aptamer-siRNA chimeras. The delivery and therapeutic utility (including both cancer and neurological diseases) and application of several aptamers were discussed in the current chapter by citing evidence collected from in vitro as well as in vivo experiments [105]. Several patents as discussed in Table 2 are filled based on novel and innovative approaches toward aptamer-based drug delivery technology and processes. In summary, potential applications of aptamers in several diseases are mentioned below.

7.1 Cancer

The therapeutic aptamers that target the specific molecule on the cell surface primarily modulate the downstream signaling pathways. This activity of the aptamers is due to structural modification in the target molecule, downstream phosphorylation, or inhibiting dimerization to associated molecules. The anticancer activity of aptamers is due to the combination of these effects and can be regarded as one of the leading applications of aptamers [106]. Few aptamers that can distinguish the healthy and tumorigenic cells are especially more helpful for the detection and/or

Table 1 Aptamer-based drug delivery system

Application of aptamers	Outcome application	References
Aptamer-based delivery system of the drug to synovium	Osteoarthritis application	[92]
HER2 aptamer-conjugated, pH-sensitive mesoporous silica nanocarrier	Synergistic cytotoxic effects in HER2-overexpressing cells to improve the efficacy of chemotherapy	[93]
MSN-PEM-aptamer conjugate-based drug delivery system	Promising target-specific intracellular drug transport which may offer better efficacy in cancer therapy	[94]
Cascaded aptamer-governed multifunctional drug delivery system based on biodegradable envelope-type nanocarrier for the treatment of HER2-overexpressing breast cancer	Exhibits improved inhibitory actions on tumor cells overexpressing the human epithelial growth factor receptor. Reduced tumor growth and minimum side effects to the healthier organs. Higher transport precision and biological safety	[95]
Aptamer-modified DNA bodies for targeted cancer therapy	Exhibit an effective paradigm of DNA-based nanostructure for targeted therapy	[96]
Aptamer-modified liposomes for reversible intracellular drug delivery	Cisplatin encapsulating in nucleolin-aptamer-conjugated liposome delivery system successfully demonstrated the enhanced activity. Further, the amount of drug delivery can be controlled using a complementary antidote aptamer DNA	[97]
Aptamer integrated with diverse nanomaterials including gold nanorods, carbon nanotubes, DNA micelles, or hydrogels	Such integration using these nanomaterials exhibits wide applications in nanomedicine, including the treatment of cancer	[98]
Liposomal nanostructure for aptamer delivery	Delivery to target cells with higher specificity results in excellent efficiency	[99]
Silver nanoclusters embedded in zirconium metal-organic framework tagged with aptamer-templated	A novel drug delivery system for targeted cancer therapy was demonstrated	[100]
A novel nanoscale-based dynamic drug delivery system	Application in various diseased microenvironments, e.g., pH, redox potential, etc.	[101]
Aptamer complex	5-FU-CS-CQD-Apt exhibits the potential activity in breast cancer treatment with improved half-life and bioavailability of the drug	[102]
ICG-tagged aptamer targeted against malignant melanoma	AS1411 aptamer demonstrated antitumor activity against melanoma by acting as the delivery system of ligands	[103]

treatment of cancer [107]. Interestingly, aptamers can be designed for targeting cancer cells for selective treatment and abolition of especially malignant cells [108]. This quality of aptamers is most important for cancer treatment, as several other currently used chemotherapeutics are associated with potential side effects

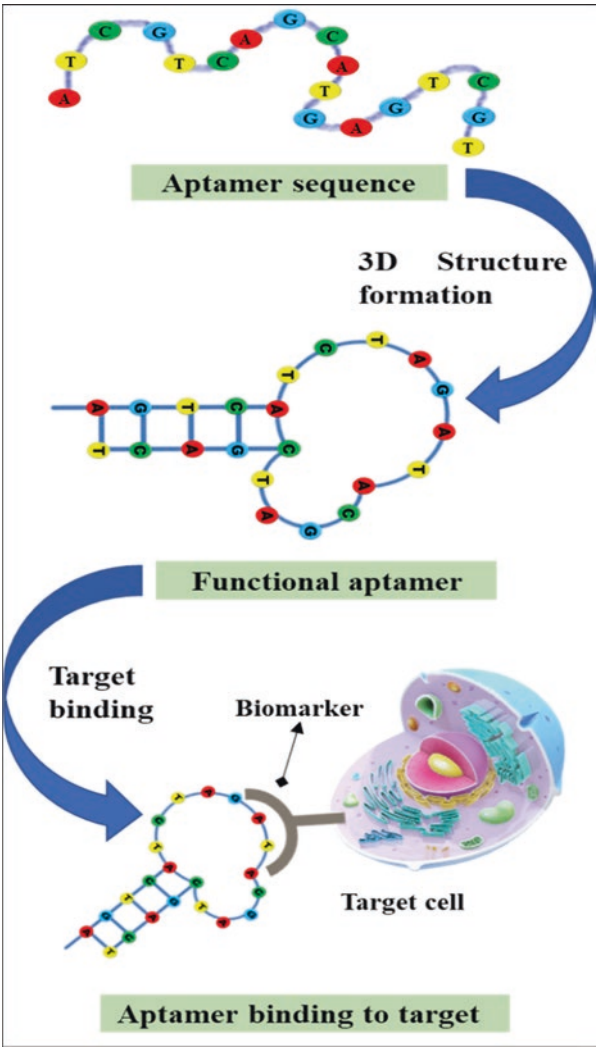

Fig. 4 Schematic diagram showing the aptamer binding to the disease sites

including massive amounts of healthy cell death and also depletion of immune cells which further reduce the activity of the patient's body to fight infectious disease. The properties of specificity and effectivity of aptamers to treat various aspects of tumorigenesis enable them as one of the current choices for developing the cancer drug delivery system [1].

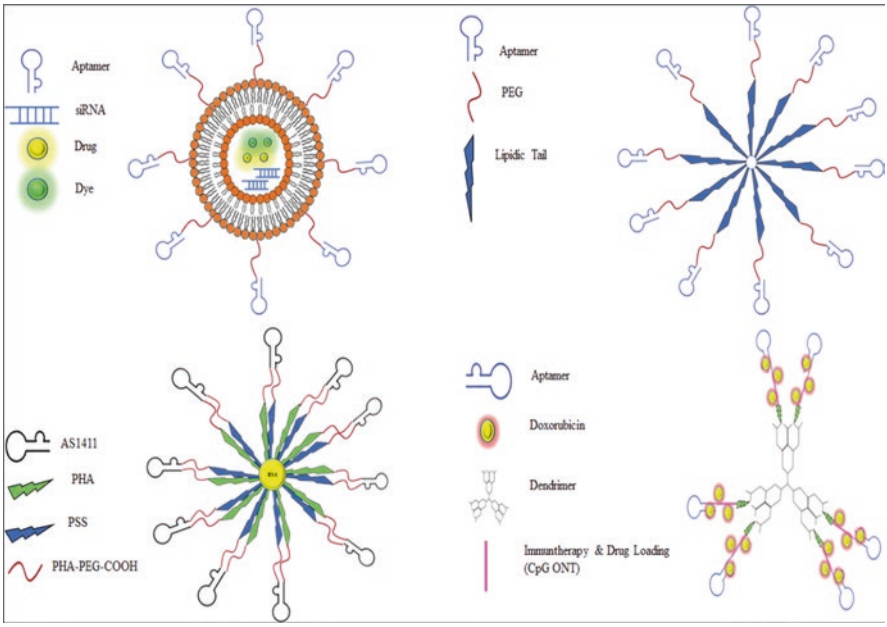

Fig. 5 Schematic diagram showing that the aptamers can be conjugated to the drug using the covalent as well as non-covalent methods

Table 2 Patents filled on aptamer-based innovation and technology

Patent number	Patent title
International patent	
PCT/US2004/039329	Nucleic acid aptamer-based compositions and methods
PCT/US2013/049212	Aptamer-targeted antigen delivery
PCT/JP2014/081954	Nucleic acid aptamer for microvesicle
PCT/US2013/038006	Transferrin receptor aptamers and aptamer-targeted delivery
PCT/IB2015/051964	Aptamers for topical delivery
PCT/US2007/069144	Aptamer-directed drug delivery
PCT/SG2015/050240	Aptamers
PCT/KR2008/002282	Preparation of drug delivery systems using pH-sensitive block copolymer and their application
PCT/US1999/006466	Aptamer-based bacterial inhibition systems (ABBIS)
PCT/KR2016/007410	Aptamer-coated microneedle-based diagnostic skin patch
PCT/KR2013/011851	Protein transduction domain based on gold nanoparticle-aptamer conjugate and method for producing same
PCT/US2005/016201	Aptamer-nanoparticle conjugates and method of use for target analyte detection
PCT/AU2013/000850	Cd133 aptamers for detection of cancer stem cells
PCT/EP2013/071003	DNA aptamers to diagnose mycobacterium tuberculosis bacteria and treat tuberculosis disease, specific for *M. tuberculosis* bacteria

(continued)

Table 2 (continued)

Patent number	Patent title
PCT/JP2014/060458	Conjugate composed of EpCAM-binding peptide aptamer and phosphorylcholine polymer copolymer
PCT/NL2011/050509	Tunable, biodegradable linker molecules for transient conjugation of components in drug delivery systems, and drug delivery systems prepared therewith
PCT/CA2015/050212	DNA aptamers specific to CD200R1 and their therapeutic uses
PCT/IL2016/051232	Functional transfer t-RNA aptamer molecules
PCT/AU2013/000850	CD133 aptamers for detection of cancer stem cells
EP04720328A	Oligonucleotide mimetics
EP15767725.3A	Aptamer for FGF2 and use thereof
EP09807096A	Nucleic acid aptamers
EP13886916.9A	Targeting aptamer for atherosclerosis and preparation method and application thereof
CN201510087938.6A	Complement-binding aptamers and anti-C5 agents useful in the treatment of ocular disorders
CN201510486116.5A	Aptamer specifically bound with glyphosate and application
EP11814899.8A	Interior functionalized hyperbranched dendron-conjugated nanoparticles and uses thereof
EP14842475.7A	PDGF and VEGF aptamers having improved stability and their use in treating PDGF- and VEGF-mediated diseases and disorders
US13/310,287	Aptamer bioconjugate drug delivery device
US13/008,568	Aptamer-based colorimetric sensor systems
US11/880,013	Aptamer-based methods for identifying cellular biomarkers
US11/318,227	Aptamer therapeutics useful in the treatment of complement-related disorders
US13/643,408	Methods of preparing targeted aptamer prodrugs
US14/198,546	Drug delivery systems and use thereof
US13/991,650	Aptamer labeled with F-19 nucleus for targeted molecular imaging by MRI
US10/762,915	Aptamer therapeutics useful in ocular pharmacotherapy
US12/299,596	Aptamers directed to MUCI
US12/422,971	Novel aptamers that bind to listeria surface proteins
US14/498,444	Pharmaceutical compositions for high-capacity targeted delivery
US13/026,244	Lariat aptamer: aptamer candidate exclusion by nuclease digestion
US11/121,165	Aptamer-nanoparticle conjugates and method of use for target analyte detection
US14/801,710	Cell-specific internalizing RNA aptamers against human ccr5 and uses therefore
US14/128,152	Aptamer which selectively binds to ERBB2 receptor and uses thereof
US13/286,847	System and method for delivery of DNA-binding chemotherapy drugs using nanoparticles
US13/092,209	Aptamer sandwich assays
US10/831,632	Gene knockdown by intracellular expression of aptamers

(continued)

Table 2 (continued)

Patent number	Patent title
US12/576,209	Microfluidic platform and related methods and systems
US14/815,193	Selection of RNA-aptamers as anti-malaria agents
US14/114,392	DNA aptamers for promoting remyelination
US10/718,833	Multivalent aptamer therapeutics with improved pharmacodynamics properties and methods of making and using the same
US13/651,265	RNA aptamers against BAFF-R as cell-type-specific delivery agents and methods for their use
US12/221,429	Aptamer based point-of-care test for glycated albumin
Indian patent	
202127031220	Aptamer against irinotecan
202117029646	DNA aptamer specifically binding to yellow fever virus Ediii and use thereof
202117029426	DNA aptamer binding specifically to dengue virus Ediii and use thereof
202117028810	DNA aptamer binding specifically to Chikungunya virus E2 and use thereof
202117025882	A novel aptamer and an electrochemical biosensor for the rapid detection and diagnosis of tuberculous meningitis
202147020075	Method for preventing oxidation of polyphenol by means of aptamer, material thereof, and use thereof
202137006312	Aptamer-based Car T-cell switch
202037056506	Aptamer preparation
202047027164	Anti-chymase aptamer and use for same
202047022945	Aptamer for adamts5, and use for aptamer for adamts5
202017017259	Aptamer against M. Tb Mpt51 and uses thereof
201937038968	Regulation of gene expression by aptamer-modulated RNAse P cleavage
201917035873	Regulation of gene expression by aptamer-mediated accessibility of polyadenylation signals
201937029521	Aptamer-drug conjugate and use thereof
201947020322	TNF-A-binding aptamer and therapeutic use for same
201917013080	Method for preventing oxidation of antioxidant material using aptamer material and use thereof
201817049360	Aptamer-based analyte assays
201817031855	Regulation of gene expression via aptamer-mediated control of self-cleaving ribozymes
201817031856	Regulation of gene expression through aptamer-modulated polyadenylation
201811023360	Aptamer against M. Tb Mpt51 and use thereof
201837019590	DNA aptamer binding to cancer cell
201837019347	DNA aptamer capable of bonding to VWF
201717031044	Regulation of gene expression by aptamer-mediated modulation of alternative splicing
201711001246	Aptamer against M. Tb HUPB and use thereof
201637039785	Aptamer for bonding to autotaxin and inhibiting biological activity of autotaxin and use for same

(continued)

Table 2 (continued)

Patent number	Patent title
201637036215	Aptamer-inhibiting biological activity of autotaxin by binding with autotaxin and use thereof
201644035860	Neural network for processing aptamer data
201637035389	Aptamer for FGF2 and use thereof
201611021901	Aptamer against mycobacterial malate synthase and uses thereof
6499/CHENP/2015	Aptamer to Il-17 and use thereof
3147/DEL/2015	Aptamer, method of detection of SEB and KIT thereof
636/DELNP/2015	EPCAM aptamer for detection of cancer stem cells
8861/CHENP/2014	Compositions comprising an anti-PDGF aptamer and a VEGF antagonist
2647/KOLNP/2014	Aptamer-based multiplexed assays
2720/DEL/2014	A combinatorial selex method for selection of aptamer(S) for target protein
6796/DELNP/2014	Aptamer method
8436/DELNP/2013	Aptamer-coated measurement and reference electrodes and methods using same for biomarker detection
3036/KOLNP/2012	Aptamer for NGF and use thereof
6773/CHENP/2012	Method and apparatus for forming of an automated sampling device for the detection of *Salmonella enterica* utilizing an electrochemical aptamer biosensor
5029/KOLNP/2011	Aptamer for chymase and use thereof
1551/KOLNP/2011	Aptamer for NGF and use thereof
891/CHENP/2011	Aptamer against Il-17 and use thereof
1863/MUMNP/2009	Methods for detecting a target nucleotide sequence in a sample utilizing a nuclease-aptamer complex
2166/KOLNP/2009	Aptamer against midkine and use thereof
3357/DELNP/2009	Nuclease-resistant RNA aptamer-inhibiting replication of hepatitis C virus replicon
3208/KOLNP/2007	Aptamer therapeutics useful in the treatment of complement-related disorders
3103/DELNP/2007	Multidomain RNA molecules comprising at least one aptamer for delivering double-stranded RNA to pest organisms
774/KOLNP/2007	Aptamer medicinal chemistry
1400/DELNP/2005	A biopharmaceutical composition comprising an siRNA or antisense oligonucleotide or ribozyme or antibody or aptamer or spiegelmer and a pharmaceutically acceptable vehicle

7.2 Immune System Manipulation

It is well established that tumorigenesis is frequently associated with the modulation of the immune system. Tumor cells are generally responsible for the inactivation of the immune system to diminish the tumor clearance activity of immune cells [109]. Aptamer-based cytotoxic T-cell antigen 4 (CTLA-4) inhibition is one of the possible therapeutic strategies to prevent immune system inactivation, which

consequently will promote tumor elimination. Moreover, CTLA-4 also acts as an "off" switch for T-cell differentiation and therefore diminishes the T-cell-mediated immune responses. Additionally, anti-CTLA-4 aptamer induces T-cell proliferation and thus produces antitumor immune activity in mice. It also increases the susceptibility of weakly immunogenic tumors in chemotherapeutic treatment. These aptamers can also be utilized to act as an adjuvant to tumor vaccine through simultaneous delivery of tumor antigen to the dendritic cells. Besides this, along with antibodies, the aptamer can be used to improve the antitumor property in a tetrameric form [110].

The inhibition of the activity of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) is another potential approach to cancer treatment. These cells can increase angiogenesis, inhibit antitumor immunotherapy, and promote tumor cell invasion on activation of the IL-4Rα signaling pathway [111]. Therefore, anti-IL-4Rα aptamer binding leads to TAM and MDSC depletion, as it was evident in tumor progression inhibition and tumor size in the mice model [112].

7.3 Neurological Disease

The complexity of the nervous system is always interfering to identify the right neurological diseases. Although brain biopsy and some methods support the investigation, scarce options exist for clinical treatments [113, 114]. In this connection, aptamers provide viable options for treatment due to higher affinity and smaller size, which further facilitate deep tissue penetration and blood-brain barrier passing, and reduce adverse effects, in a more controlled manner [105]. Many of the neurological therapies discussed in the entire chapter are similar strategies which can be utilized for cancer treatment. Further, immune system regulation also plays a key role in treating neurological diseases [105].

7.4 Multiple Sclerosis

Multiple sclerosis (MS) is an incurable condition due to CNS inflammation and results in leukocyte infiltration in the CNS. Generally, treatment available merely opposes the disease progression and also the treatment of the phenotypic symptoms is low [115]. Experimental autoimmune encephalitis (EAE) is a similar disease in animals that closely resembles MS and is used as a pharmacological model of MS (using a mouse) to study the disease progression. Since the exact pathology of MS is doubtful, that's why the utilization of aptamer is more significant for the determination of both EAE and MS biology to provide new strategies for their treatment [116, 117]. A study including the IL-17 and midkine aptamer shows that IL-17 aptamers can hinder the IL-17-mediated pro-inflammatory activity, which is

followed by reduction of neurological symptoms as observed in the EAE mouse model [118].

7.5 Stroke

Ischemic strokes generally occur due to blockage in blood flow to the brain or due to the presence of blood clots [119]. Besides this, inflammation and restricted blood supply in ischemic stroke may lead to brain damage. Further, tissue plasminogen activator (TPA) is the only approved treatment available that acts by dissolving blood clots to resume normal flow. However, TPA is generally recommended within 4.5 h of onset of stroke [120], which restricts the number of patients who can receive the TPA during the prescribed time, and one of the associated demerits is to cause hemorrhaging. Taking all together, it is worth searching for better therapeutic option strategies [121]. Surprisingly, aptamer has a similar activity as TPA (Factor IXa, RB006 from Regado Biosciences) and is currently under clinical trials in phase 2 against acute coronary syndrome (NCT00932100 and NCT00113997) [122]. Further, hemorrhaging can be prevented through aptamer inactivation via the use of aptamer antidote RB007. In summary aptamer-based treatment of stroke is to be a safe and possible alternative to TPA [122].

7.6 Alzheimer's Disease

The reason for Alzheimer's pathology is still unknown; however, the deposition of β-amyloid (Aβ) proteins is one of the leading factors which interrupts the connectivity of neurons in the brain's healthy areas. Further, Aβ is also associated with the accumulation of synuclein in the brain of AD and PD. Certainly, diagnosis occurs through brain biopsy, and still limited or null treatment options are available for both AD and PD due to a lack of precise evidence about Alzheimer's disease progression. Several biomarker assays based on SOMAmers have been developed for both AD and PD [123]. SOMAmers are the class of aptamers having different functional groups on uracil residues that was able to enhance both the stability and functionality of the aptamer. This assay can identify the age-related differences in proteins present in the cerebrospinal fluid. Subsequently, over 200 biomarkers associated with aging which may be used to support in diagnosis and eventually treatment of AD or PD have been identified to date. Progressively, an aptamer-based delivery targeted to the β-secretase (BACE1) protein was developed to inhibit the formation of Aβ for the treatment of AD [112, 124]. As Aβ is created due to the cleavage of β-amyloid precursor protein by BACE1, the anti-BACE1 aptamer may support elucidation of the primary reason for AD and provide new possibilities in treatment options for Aβ accumulation. However, it needs to undergo further testing in culture or animal models to validate the activity.

8 Conclusions and Future Perspectives

As per the description provided throughout the chapter, it is well established that aptamer-based research provides an opportunity for precise drug delivery options focusing on targeting therapeutic molecules. Moreover, the possibility to deliver drug substances in response to specific stimuli molecules enables the aptamer-based drug delivery systems as a unique therapeutic strategy for providing spatially and temporally controlled drug release. Current research on aptamer-based gating is still in the infancy phase without many reports on biological model systems. However, the promising results offered by the aptamer-based drug delivery approach are potentially attractive, and future interest in this area should be much expected. Interestingly, encouraging results obtained by aptamer-based gating suggest huge potential for developing the many future therapeutic applications in the drug delivery area. Thus, the drug delivery research fraternity should benefit from further investigating the aptamer-based nanoparticular drug delivery systems, which can offer the clinical translation of such a therapeutic approach for unmet clinical needs.

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Artificial Exosomes as Targeted Drug Delivery Systems

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1 Introduction

In the last few decades, researchers of Pharmaceutical and Medical Sciences have realized that the development of new active pharmaceutical ingredients (APIs) is less than enough to address current medicinal needs. It is not a rare phenomenon that potentially efficacious APIs do not have a favorable pharmacodynamic and pharmacokinetic profile. This is the main reason why the science of drug delivery systems has flourished. In this area, Pharmaceutical Nanotechnology and Nanomedicine are pioneers due to their unique properties that are inextricably connected with the size of the delivery systems (1 nm = 10^{-9} m). Drug delivery nanosystems function as a Trojan horse that protects the API from biodegradation, drives it to the tissue or organ of desire, and eliminates its toxicity to healthy tissues. The first medicine based on pharmaceutical nanotechnology was approved in 1995 and since then many others have followed [1]. Lipid-based and polymeric nanoparticles

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are the main platforms that are used, though other platforms such as inorganic nanoplatforms and carbon nanotubes are also studied [2]. All of these nanosystems have undoubtedly optimized drug delivery. But what happens when the API is not just a classic small molecule? The recent example of coronavirus disease 2019 (COVID-19) has proved there is a need for innovative platforms that can encapsulate and deliver bioactive molecules, such as proteins or RNAs. Indeed, the majority of the authorized COVID-19 vaccines utilize nanoplatforms to induce human immunity and achieve their protective role [3].

In this chapter, we will discuss exosomes as API delivery nanovesicles, their limitations in clinical practice, and the development of artificial exosomes. The artificial exosomes are fully- or semisynthetic nanoplatforms that can encapsulate a variety of different biomolecules while being free of the main disadvantages of cell-derived exosomes.

2 Exosomes

Extracellular vehicles (EVs) are cell-derived lipid bilayer structures that were first observed by P. Wolf in human plasma [4]. They are produced by both prokaryotic and eukaryotic cells and are classified into three main categories, based on their biogenesis mechanism, cargo, and size: (i) apoptotic bodies (1000–10,000 nm), (ii) microvesicles (100–1000 nm), and (iii) exosomes (50–150 nm). All types of EVs carry bioactive molecules, such as proteins, lipids, and nucleic acids, that are connected to their parent cell. Thus, depending on the endogenous messages they carry, each of these EV types leads to the activation of different biological signaling pathways [5]. The aim of this unit is the presentation of the biological behavior of exosomes, the smallest group of EVs, and their potential role as well as their main difficulties as effective therapeutic delivery platforms.

2.1 Biogenesis

In contrast with the other types of EVs, which are formed by the outward bending of the plasma membrane, exosomes are released after the fusion of the plasma membrane with the endogenous multivesicular bodies. The term “exosome” comes from the Greek words “έξω” (exo), meaning outside and “σώμα” (soma), meaning body. It was first proposed by Johnstone and her team, due to their formation mechanism: these bodies are formed mainly into the cytoplasm and afterward, they are released in the extracellular plasma [6].

2.1.1 ESCRT-Dependent Pathway

Exosome biogenesis is connected with the endosomal formation mechanism. Extracellular molecules, such as proteins, ions, and lipids, are inserted into the cell through endocytosis. In this way, the early endosomes are formed. Interactions between the early endosomes and intercellular organelles, mitochondria, and trans-Golgi network lead to the insertion of intercellular constituents into the endosomes and their transformation into late endosomes [7]. Some additional modifications and content exchange between these blebs and the cytoplasm give rise to the multivesicular bodies (MVBs). During the formation of the late endosomes and the MVBs, the inward invagination of the bleb membrane results in the development of the intraluminal vesicles (ILVs), which are the future exosomes (Fig. 1). Two pathways are responsible for the production of the ILVs: (a) the endosomal sorting complex required for transport (ESCRT)-dependent and (b) the ESCRT-independent, as analyzed below. MVBs can either undergo a degradation process, direct interaction with lysosomes, or degradation after fusion of MVBs with the autophagosomes first, or anchor into the plasma membrane and release their constituents into the extracellular environment [8].

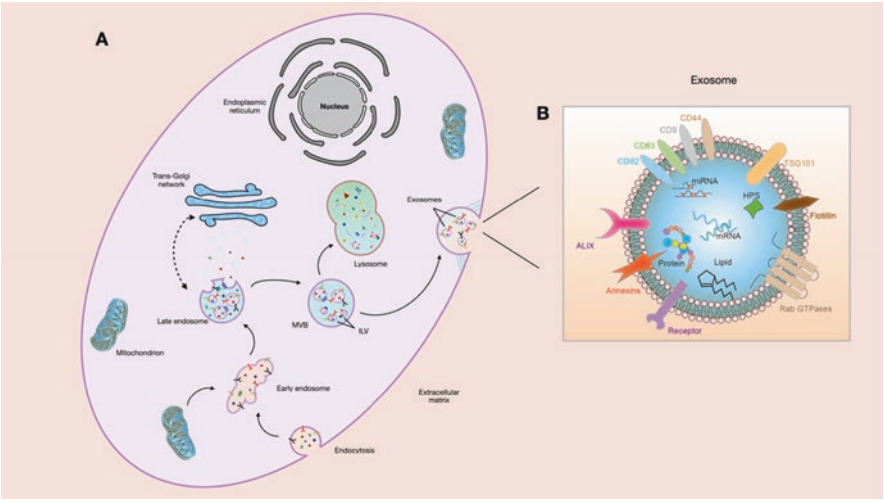

Fig. 1 (**a**) Exosome biogenesis. Extracellular molecules, such as proteins, ions, and lipids, are inserted into the cell through endocytosis and early endosomes are formed. Early endosomes interact with organelles (i.e., mitochondrion) and are converted into late endosomes. Further modifications and interactions with the trans-Golgi network lead to the formation of multivesicular bodies (MVBs) that encapsulate small vesicles that are called intralumilar bodies (ILVs). MVBs can be either degraded or attach to the cell membrane and release their cargo, which contains the modified ILVs known as exosomes, in the extracellular matrix. (**b**) Structure of exosomes. Exosomes carry a variety of biomolecules such as proteins, lipids, and nucleic acids that are produced in the parent cells. (Figure (**b**) adapted from [27])

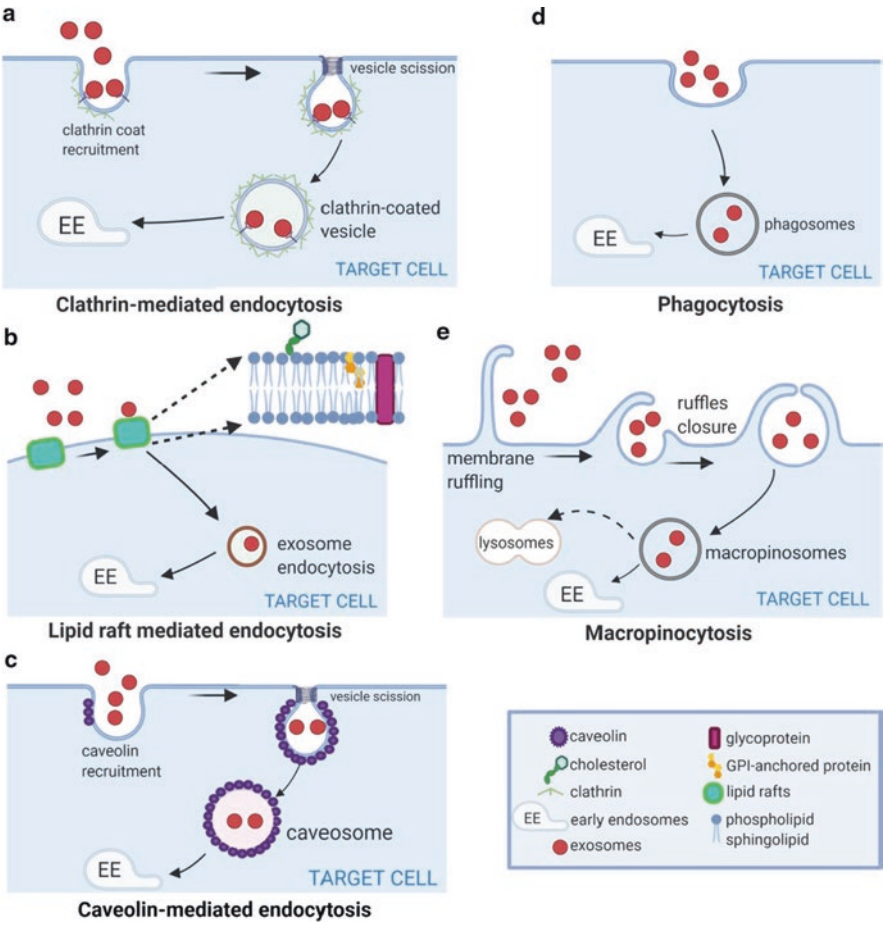

Fig. 2 Possible mechanisms of exosomes internalization by the target cells: (**a**) clathrin-mediated endocytosis, (**b**) lipid raft-mediated endocytosis, (**c**) caveolin-mediated endocytosis, (**d**) phagocytosis, (**e**) micropinocytosis. (Adapted from [8])

Four protein complexes (ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III), as well as the AAA ATPase Vps4 complex (VPS4, ALIX, TSG_{101}), play a key role in the biogenesis of the exosomes via the ESCRT-dependent pathway. ESCRT-0 complex recognizes and binds to the mono-ubiquitinated proteins of the endosomal surface [9]. ESCRT-0 and phosphatidylinositol 3-phosphate (an endosome-enriched lipid) recruit ESCRT-I and ESCRT-II into this area. Subsequently, ESCRT-I and ESCRT-II induce membrane deformation and the development of small blebs into the surface. These blebs enclose soluble molecules and cytoplasm into their center [10, 11]. ESCRT-III and VPS4 (vacuolar protein sorting-associated protein 4) are necessary for the inward endosomal membrane abscission and the release of the ILVs into the late endosomes or MVBs [12]. Next, de-ubiquitylating enzymes de-ubiquitylate the

ubiquitylated proteins so that the vesicles will not be degraded [13]. During all of the mentioned processes, the role of ESCRT-accessory proteins ALIX (apoptosis-linked gene 2-interacting protein X), TSG101 (tumor susceptibility gene 101), and SNARE [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor] complex proteins is of extreme importance as they control the production, the homogeneity, and the value of the ILVS content [14]. For instance, ALIX binds to the de-ubiquitylated proteins and transfer them in the ILVs, with the help of syndecans and tetraspanin CD63 [15]. SNARE complex proteins are responsible for the fusion of MVBs with the plasma membrane and subsequently in the release of exosomes [11].

2.1.2 ESCRT-Independent Pathway

The ESCRT-independent pathway is mainly driven by some areas in the surface of lipidic bilayer membranes that are called lipid rafts or microdomains. Lipid rafts have a very sticked composition and conformation. They are enriched in cholesterol, sphingolipids, glycosylphosphatidylinositol (GPI), ceramides, and saturated phospholipids. The architecture of lipid rafts is stabilized by the high quantity of tetraspanins or other GPI-anchored proteins [16]. These areas seem to be rich in information content, encrypted into the so-called thermodynamic epitopes [17]. The information is translated into an enthalpic content that is capable of interacting with the neighbor molecules [18]. Indeed, lipid rafts are responsible for the encapsulation of certain RNA molecules into exosomes due to enhanced affinity for specific RNA motifs. This process seems to be lipid- and not protein-mediated, while entropic phenomena of the lipidic surface are translated into a “bind-here” message for the RNA motifs [19]. Although this type of information content may be unusual in biological sciences, it is mathematically explained by the Shannon equation of information [20]:

$$H(x) = -\sum_{i=1}^{n} p(x_i) \log_b p(x_i)$$

where H represents the entropy for x_i possible outcomes. $p(x_i)$ represents the probability of the possible outcomes and b is the base of the logarithm used. The corresponding entropy units are bits when $b = 2$.

The above equation can explain the thermodynamic behavior of biological epitopes taking into account the quality and quantity of information (bits) they carry. Thus, the information content of the lipid rafts activates an early-exosome production mechanism that is thermodynamically favored.

Although the complete mechanism of exosome production and release is currently not fully understood, both ESCRT-dependent and ESCRT-independent pathways have an important role on exosomes’ biogenesis. Recent studies support that these two processes act complementary to each other, providing a complex biogenesis algorithm [21–24].

2.2 Biological Role

In the early ages of their discovery, exosomes were thought to be cellular waste carriers so that the unwanted cell cargo is released outside of the cell compartment. However, recent research has proved that their role in cell-to-cell communication is much more complex [25]. Figure X presents possible mechanisms of exosomes' internalization into the receiver cells. As they are carriers of several molecules (i.e., proteins, lipids, or nucleic acids) of the parent cells and have the ability to transfer these contents intercellularly, they affect both physiological or pathological biochemical routes [26]. For instance, their role in the progress of different types of cancer or metastasis [13, 27], in the activation of immune responses [28], or even in progress of the neurodegenerative diseases has been detected [14].

2.2.1 The Role of Exosomes in Carcinogenesis

The function of tumor-derived exosomes (TDEs), also known as oncosomes, is one of the most investigated topics concerning their pathogenesis-associated function. Different cancer types have been studied for this purpose. Breast, gastric, and melanoma cancers are some examples [29–31].

Tumor-derived exosomes that are produced and released by the malignant cells affect the tumor microenvironment and thus the progression of the pathogenicity. Specifically, stress tumor conditions such as hypoxia and oxidative stress increase the rate of exosomal biogenesis and the release by the tumor cells. These exosomes interact with the neighboring area, which subsequently results in the remodeling of the microenvironment [13]. The main cells of the tumor microenvironment (fibroblasts, immune and stromal cells) receive the tumorigenic content of the TDE.

Furthermore, TDE seem to have a crucial role in the metastasis process. TDE can transit their content in more or less distant areas (in transit or satellite metastasis) or even organs [32]. As the development of the pre-metastatic niche is equally important with the cells' mutations in the metastasis formation, the TDE should be taken into account when the secondary tumor tissues are studied [33]. Indeed, according to Emmanouilidi et al., pancreatic TDE were found to carry more than 300 tumorigenic proteins [34], while Hoshino et al. claim that the unique integrins of the TDE drive the organotropic selection of the metastasis site [35]. Moreover, exosomal miR-25-3p induces the modeling of the pre-metastatic niche in foreign sites [36].

Finally, TDE are of importance due to their active role in drug resistance of tumors. On the one hand, TDE can actively remove the chemotherapeutic drugs outside the malignant cells. Notably, HER-2 overexpressing exosomes bind with trastuzumab and inhibit its action [37]. Nevertheless, the basic role of exosomes in drug resistance is attributed to biochemical pathways. For instance, TDE transmit the information for upregulation of certain active molecules such as P-glycoprotein [38, 39] and $GSTP_1$ [40]. Most of the times, nucleic acids are responsible for this upregulation, mainly miRNAs [13, 41, 42].

2.2.2 The Role of Exosomes in the Immune System

As exosomes regulate intercellular communication, they may trigger or suppress the immune response. Interestingly, exosomes that are produced by infected, malignant, or immune cells exert influence on both innate and adaptive immunity.

The innate system, which is the nonselective way of the human organism to fight exogenous, and harmful, factors is composed of macrophages, dendritic and natural killer cells, granulocytes, as well as complement and chemoattractants. Exosomes derived from infected macrophages are considered to enhance pro-inflammatory conditions and activation of the innate system [43, 44]. Zhou et al. suggest that exosomes derived from pancreatic cancer cells are uploaded with miR-203 that downregulate the expression of the membrane toll-like receptor 4 (TLR4) of dendritic cells [35] and thus decrease the capability of dendritic cells to identify bacterial components or host heat shock proteins [45]. Recent studies show that activation of natural killer cells, which are the responsible cells to recognize and cytolyze infected, cancer, or allogenic cells, by TDE might have a beneficial role in the elimination of tumors [46, 47].

On the other hand, the function of adaptive immunity is affected by exosomal messages too. Exosomes produced by activated dendritic cells were found to be more efficient in the immunomodulation than the ones from immature dendritic cells [48]. These vesicles carry in their surface MHC-I and MHC-II complexes that are bound with pathogenic molecules [49]. Notably, humoral immunity ($CD4^+$ T-lymphocytes and B-lymphocytes) induced by the vaccination with dendritic cell-derived exosomes that were infected with *Eimeria tenella* proved to be more efficient than the antigenic-subunit vaccine when intramuscularly administrated in chicken [50]. Moreover, cellular immunity, mainly caused via the T_h1 route and the production of $CD8^+$ T-lymphocytes, is also synergistically activated by exosomes from immune cells [51, 52].

Except for the physiological role of exosomes in the immune system, they seem to trigger or control autoimmune diseases under certain conditions. For instance, miRNAs that are released under rheumatoid arthritis can either up- or downregulate the progress of the disease [53–55]. Miao and colleagues review their role in a variety of autoimmune diseases, such as systemic lupus and sclerosis [56].

2.2.3 The Role of Exosomes in the Central Nervous System and Neurodegeneration

The mechanism of action, communication, and interaction between the central nervous system (CNS) cells is highly complicated and not yet fully understood. However, exosomes have proved to be important information transporters for these mechanisms. Indeed, great studies show that CNS-derived exosomes that were isolated from human fluids such as plasma carry miRNAs and protein aggregates that could help in the very early diagnosis of CNS neurodegenerative diseases like Alzheimer's or Parkinson's disease [57–60].

Microglia, neurons, astrocytes, and oligodendrocytes all produce exosomes that are important factors for neuronal health and normal neural functionality [61–66]. Interestingly, Venturini et al. have recently shown that astrocyte-derived exosomes can be selectively up-taken by neuron cells although the mechanism of action for the above activity is not yet clear. These exosomes transferred neuroglobin, a molecule that is assumed to have a neuroprotective role [67]. Likewise, exosomes from cortical neurons were found to interact only with neurons and not glial cells [68]. This selectivity empowers the perspective that exosomes are not just "junk carriers" but they do support neural homeostasis by selective cell-to-cell communication.

A controversy exists of whether exosomes provide a protective or aggressive profile in the case of neurodegeneration. Recent studies prove that brain-derived exosomes of AD patients have increased rates of pathological proteins and protein complexes such as Aβ oligomers and hyperphosphorylated tau (p-tau) [69, 70]. Some research groups support that these exosomes that are produced by pathological AD parent cells promote the spread of the above toxic proteins and consequently, the evolution of neurodegeneration [69, 71, 72]. On the contrary, some other groups found that exosomes might also have a neuroprotective functionality under certain circumstances: recently, Wei and colleagues proved that in in vitro experiments mesenchymal stem cell-derived exosomes evoke neuronal cell apoptosis in AD models [73]. Moreover, neuronal exosomes were found to aid in the Aβ clearance in nonhuman primates with AD pathologies [74]. Contrariwise, glia-derived exosomes, enriched in α-synuclein, a main pathological protein found in patients with Parkinson's disease, are accused of causing α-synuclein aggregation and low protein clearance of the neurons [75, 76].

2.3 Applications in Preclinical and Clinical Stages

The most common way to develop exosomes as innovative medicinal platforms is through genetic modification of the parent cell. In this way, exosomes are modified to carry increased or decreased quantities of certain cargoes such as proteins or nucleic acids. Notably, Duarte-Sanmiguel and colleagues developed a protocol for the isolation of exosomes derived from modified dendritic cells, mentioning the importance of these exosomes as up- or downregulators of protein biogenesis in the receiver cells [77]. A pioneering study by Alvarez-Erviti et al. achieved more than 60% knockdown of the enzyme beta-secretase 1 (BACE-1) in mice by administration of exosomes derived from genetically modified dendritic cells [78]. Mesenchymal stem cell-derived exosomes that are overexpressing Anti-miRNA-221 or miR-34a proved to be beneficial in the treatment of different cancer types [79–81].

Another way to develop effective therapeutic exosomes is by loading them with drug molecules. The loading procedure takes place after the exosome production via passive or active cargo loading [82]. For instance, a passive process is the incubation of the parent cell with the drug molecule [83], while active ones are sonication [84] or electroporation [78, 85].

Last but not least, certain categories of exosomes, such as mesenchymal stem cell-derived exosomes, show a therapeutic effect due to their inherent regenerative properties without the need of any biochemical or drug loading modifications. For instance, bone marrow mesenchymal stem cell-derived exosomes have recently proved through in vitro and ex vivo models to promote tendon regeneration [86]. Similar regenerative properties have been found in preclinical and clinical trials against musculoskeletal pathologies such as osteoarthritis [87, 88]. Furthermore, mesenchymal stem cell-derived exosomes could have a therapeutic effect in wound healing [89]. Interestingly, Yang et al. showed promising results in the treatment of diabetic wounds after the administration of polymeric hydrogel loaded with umbilical cord-derived mesenchymal stem cell-derived exosomes [90].

Till today, no medicine based on exosomes has been approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Nevertheless, as of February 2022, by using the keyword "exosome" in the US National Institutes of Health (NIH) clinicaltrials.gov, 251 trials appear. Most of these trials concern the diagnosis of diseases, while a few targets in the evaluation of exosomal therapeutic products. Table 1 presents the clinical trials that evaluate the exosomes as advanced therapeutic medicines. Only two trials appear to be in phase III, from whom only one is active (NCT05216562). The active one evaluates the safety and efficacy of mesenchymal stem cell-derived exosomes to reduce hyper-inflammation in COVID-19 patients. The effect of the intravenous administration of the exosomes will be evaluated additionally with the administration of standard COVID-19 treatments. As it is shown, the majority of the clinical trials presented in Table 1 involve mesenchymal stem cell-derived exosomes. Mesenchymal stem cell-derived exosomes are ideal delivery platforms as they are easily modified, nontoxic, and biodegradable [91]. However, other parent cells have also been utilized such as dendritic cells (NCT01159288) or plant cells (NCT01294072, NCT01668849).

2.4 Difficulties and Disadvantages

Although exosomes show tremendous advantages as innovative medicinal platforms, some serious limitations also exist. Firstly, because the production of exosomes requires a parent cell culture, specific quality and safety procedures should be followed. Different cell banks, e.g., mesenchymal stem cells, dendritic cells, or plant cells, require different culture conditions and have unique proliferation rates. Unique microenvironment conditions such as pH, oxygen, or specialized growth factors shall be applied in each case [92]. As it is necessary for every type of medicinal biological factor and in appliance with the guidelines of the worldwide health administrations [93], the culture of cell lines should be done under good manufacturing practice (GMP) conditions, which are stricter for biological products than conventional drug formulations. Indeed, according to the EMA, exosomes belong to the category of advanced therapy medicinal products (ATMPs), and their evaluation

Table 1 Clinical trials of exosomes as advanced therapeutic medicines

Name	Route of administration	Phase	NTC number	Location
COVID-19				
EXOMSC-COV19	IV	2/3	NCT05216562	Indonesia
Exosomes overexpressing CD24	Intranasal	2	NCT04969172	Israel
CovenD24	Intranasal	2	NCT04902183	Greece
COVID-19EXO	Intranasal	2	NCT04602442	Russia
ARDOXSO	IV	1/2	NCT04798716	USA
COVID-19EXO	Intranasal	1/2	NCT04491240	Russia
EXO-CD24	Intranasal	1	NCT04747574	Israel
CSTC-Exo	Intranasal	1	NCT04389385	Turkey
MSCs-derived exosomes	Intranasal	1	NCT04276987	China
Diabetes mellitus type 1				
MSC exosomes	IV	2/3	NCT02138331	
Non-small cell lung cancer				
Dex2	N/A	2	NCT01159288	France
Pulmonary infection				
MPCs-derived exosomes	Intranasal	1/2	NCT04544215	China
Dry eye				
Umbilical mesenchymal stem cell-derived exosomes	Intra-ocularly	1/2	NCT04213248	China
Alzheimer's disease				
MSCs-Exos	Intranasal	1/2	NCT04388982	China
Acute respiratory distress syndrome				
hMSC-Exos	Intranasal	1/2	NCT04602104	China
Degenerative disc disease				
Platelet-rich plasma (PRP) with exosomes	Intra-discal	1	NCT04849429	India
Osteoarthritis				
ExoOA-1	Intra-articular	1	NCT05060107	N/A
Colon cancer				
Curcumin conjugated with plant exosomes	N/A	1	NCT01294072	USA
Familial hypercholesterolemia				
ENDFH	N/A	1	NCT05043181	China
Metastatic pancreas cancer with KrasG12D mutation				
iExosomes	IV	1	NCT03608631	USA
Oral mucositis associated with chemoradiation treatment of head and neck cancer				
Grape exosomes	Per-os	1	NCT01668849	USA
Cerebrovascular disorders				
Allogenic mesenchymal stem cell-derived exosome enriched by miR-124	Stereotaxis/ intraparenchymal	1/2	NCT03384433	Iran

(continued)

Table 1 (continued)

Name	Route of administration	Phase	NTC number	Location
Periodontitis				
Adipose-derived stem cell exosomes	Oral topical injection	Early 1	NCT04270006	Egypt
Ulcer				
Plasma-derived exosomes	Transdermal	Early 1	NCT02565264	Japan
Macular holes				
MSC-Exo	Intra-ocularly	Early 1	NCT03437759	China

follows EMA/CAT/852602/2018 guidelines [94]. As this category of therapeutics is new, there are not yet certain advice or recommendations from the medicine agencies [95].

Moreover, the isolation of exosomes necessitates the development of elegant techniques that are able to distinguish them from other extracellular vesicles, like microvesicles or apoptotic bodies. Fortunately, in 2015, the International Society for Extracellular Vesicles (ISEV) published a position paper regarding the isolation methods that should be applied for EVs that are studied in clinical trials [96], and this paper brought a new era in the exosome clinical trials. However, till today, the distinguished line between the different types of EVs is not yet fully understood and more research is necessary. Recognizing the need for more certain guidelines, ISEV recently published another position paper to promote the need for a legislative framework concerning EV medicinal products. In this paper, ISEV report that according to current EMA guidelines, EV products are categorized in ATMPs, though it supports that certain details differentiate them from the other ATMPs and is correct to be taken into account [97].

Last but not least, exosomes are biological vesicles that contain sensitive cargo. Thus, the storage conditions might be a limitation too. For instance, especially when exosomes are used as RNA carriers that can be affected and inactivated very easily, special storage is required. Indeed, Yamashita and colleagues support that the optimized storage temperature is −80 °C [98]. Such a temperature would dramatically increase the price of these therapeutical products that could eliminate their use in low-income countries.

3 Artificial Exosomes

All of the above create obvious difficulties in the manufacturing production of exosomal medicines. As a result, scientists are currently trying to go a step further and develop new platforms that could acquire the advantages of exosomes and eliminate their disadvantages. The category of artificial exosomes as innovative therapeutic medicinal products is a new area in nano-biotherapeutics with promising results in

the development of safe and effective platforms that could fight currently incurable diseases. In this section, we will discuss the developmental processes of artificial exosomes and their loading efficiency.

3.1 Development Approaches

Three main approaches for producing artificial exosomes can be found in the literature: (a) the bottom-up, (b) the top-down, and (c) the hybrid method (Fig. 3). Each procedure has unique characteristics and limitations that shall be taken into account in each case.

3.1.1 Bottom-Up Method

The bottom-up approach is a method to develop fully artificial exosomes. Building block molecules, e.g., phospholipids, cholesterol, proteins, or peptides, are mixed in the appropriate ratios to self-assemble into biomimetic platforms. The main advantage of the present methodology is that it utilizes the same manufacturing processes with the development of liposomes and other lipid nanoparticle formulations that are already authorized [99–101]. For example, thin-film hydration, microfluidics, or extrusion can be applied for the production of artificial exosomes. This approach, though, requires deep knowledge of the physicochemical properties of exosomes. As it is not possible to utilize all the components of biologically derived exosomes, the choice of certain key molecules leads to a "clean" result when we have targeted certain exosomal properties of interest [102]. Proof-of-concept experiments on the role of each molecule in the surface of exosomes or extracellular vesicles as well as lipidomics analysis are necessary tools for the design of experiment (DoE) methodology to develop such innovative biomimetic nanoplatforms [103–106]. The work of Peña et al. in 2009 is one of the first in the area of artificial exosome development. Although the lipids and the ratios that were utilized do not approach the ones of exosomes, the researchers achieved the complexation of their liposomes with major histocompatibility complex-1 (MHC-1) peptide complexes, enhancing the interaction with T-lymphocytes in a similar way with exosomes [107]. In another study, artificial exosomes carrying DEC205 antibody were developed by a microemulsion process. The conjugation of the antibody, which is negatively charged, is favored due to electrochemical interactions with the positively charged lipids. Interestingly, these platforms do not only have high encapsulation efficiency of the API, but they also show decreased cytotoxicity because of the charge neutralization [108]. Similarly, Staufer et al. developed fully synthetic extracellular vesicles (fsEVs) decorated with tetraspanins, which are naturally present in EVs' membranes and loaded with miRNA molecules. The wound-healing abilities of fsEVs were evaluated by in vitro and ex vivo experiments showing promising results as therapeutic platforms. Although this study is the most accurate concerning the membrane

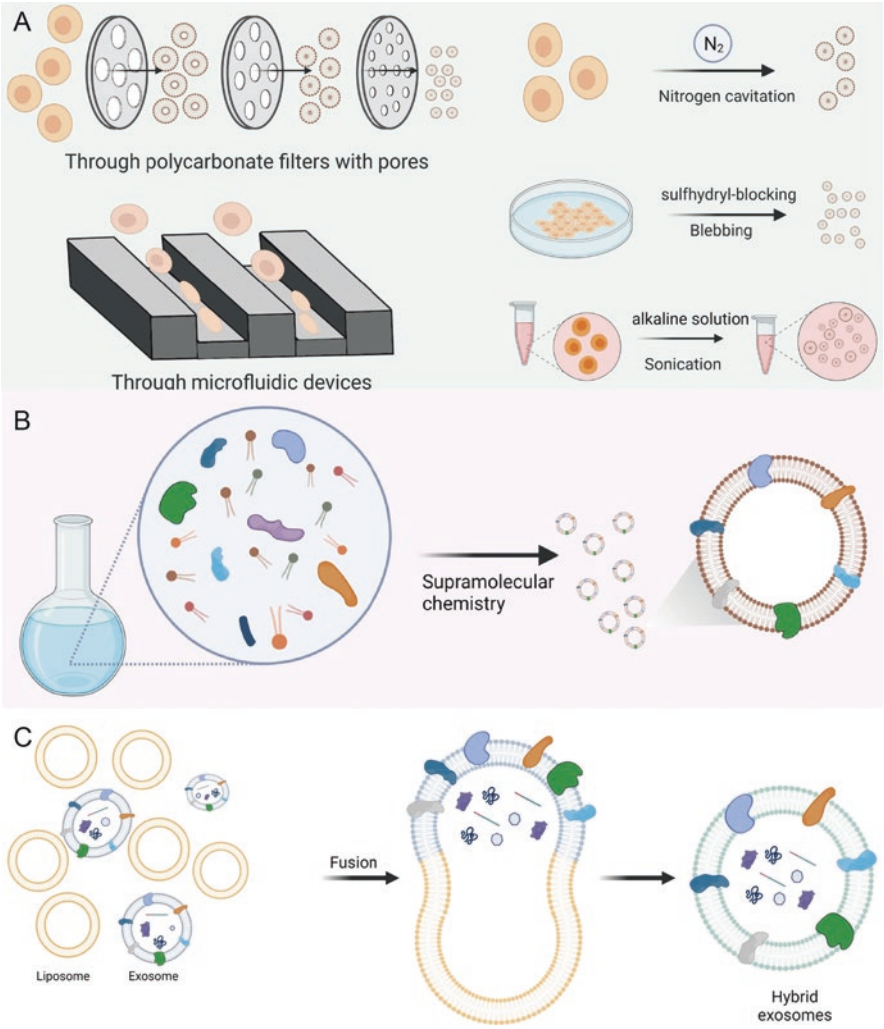

Fig. 3 Development approaches of artificial exosomes. (**a**) Top-down method: cells pass through polycarbonate filters or microfluidics devices and cell membranes are isolated and form pseudo-spherical vesicles. (**b**) Bottom-up method: block molecules, e.g., phospholipids, cholesterol, proteins, or peptides, are mixed in the appropriate ratios to self-assemble into exosome-like particles. (**c**) Hybrid method: exosome-like particles or liposomes and biologically derived exosomes are mixed through fusion techniques and hybrid liposomal-exosomal particles are formed. (Adapted from [102])

composition, the physicochemical characteristics of the fsEVs need further optimization as the polydispersity index (PDI) of the empty platforms is approximately 0.8 [109]. Finally, it is worth mentioning that there is a gray area in the literature of the definition of artificial exosomes which should be based on their functionality and their morphological characteristics. For instance, Aday et al. mention the

development of artificial exosomes loaded with RNA molecules [110]. However, the developed platforms are basically lipid nanoparticles (LNPs) that carry an miRNA that is found in small EVs. In our point of view, the functionality of such nanomorphologies based on their surface characteristics, i.e., the lipid rafts, could be the road map for characterizing such nanoplatforms as artificial exosomal membranes and not as artificial exosomes. However, the platform cargo, such as a single nucleic acid, is not sufficient to characterize the nanovesicles as artificial exosomes without proving their functionality.

3.1.2 Top-Down Method

The top-down method to produce exosome-like particles, also known as nanovesicles, is based on cell membrane fractions that assemble into pseudospherical blebs. Two main processes, extrusion over polycarbonate filters and microfluidics, are utilized for the production. Both methodologies require a cell culture. These cells will then be driven in membrane pressure that conclude in the formation of small nanovesicles. In this way, cell membranes as it is, including the transmembrane proteins and other signal molecules, are part of the final platforms. Thus, the produced artificial exosomes have a very similar proteomic and lipidomic consistency as biologically derived exosomes. Goh et al. presented those data and compared cell-derived nanovesicles (CDNs) with exosomes resulting that CDNs might play an important role as ATMPs [111]. Although a similarity between them is well-established, someone should take into account that differences do exist. For example, in this study, an important differentiation in the percentage of sphingomyelin is observed. Such an observation might seem insignificant, but we believe that it is possibly connected with the formation of lipid rafts, areas of high importance for exosomes, as mentioned above. Various producer cells, such as U937 cells [112], NIH3T3 fibroblasts [113], hMSF10A [113], or ASC cells [114], are cultured to produce NVs. Depending on the desired pharmacological effect, the origin of the NVs is decided as the final vesicles appear with high affinity to their parent cells. Such an approach has tremendous advantages in artificial exosome development. However, many of the limitations that appear in the classic exosome development are also present here.

3.1.3 Hybrid Method

Some recent studies focus on a more complex design by mixing synthetic liposomal formulations with biologically derived exosomal membranes. That semisynthetic approach is a result of the technological combination of both top-down and bottom-up methods and targets the design and development of ATMPs that will take advantage of both synthetic and biological platforms while eliminating their problems in clinical use [110]. A pioneering study that took place in 2016 by Sato et al. presents the successful development of hybrid exosomes by freeze-thaw and their in vitro

evaluation. Notably, the researchers pointed out that the lipids used for the liposomal platforms affected the final behavior of the hybrid systems. Thus, the variety of synthetic lipids could lead to unique biological behavior of the final platform [115]. For instance, the incorporation of PEGylated lipids could eliminate the recognition and neutralization by the mononuclear phagocyte system (MPS), correspondingly to liposome's behavior [116]. At the same time, another research group studied the endogenous production of hybrid EVs after infection of the producer cells with membrane fusogenic liposomes [117]. Results concerning the loading of both hydrophobic and hydrophilic molecules into the produced EVs showed that the efficiency was higher when membrane fusogenic liposomes are utilized in comparison with non-fusogenic highly cationic liposomes.

Two years after, two different research groups applied simple incubation methods to mix synthetic liposomes with mesenchymal-derived EVs [118] or HEK293FT exosomes [119]. According to Piffoux et al., the hybrid systems maintain the intrinsic properties of the EVs while adopting the high loading efficiency and prolonged circulation time from the synthetic cells [118]. Lin et al. benefit from this technology to deliver the CRISPR/CAS9 system into cells. The isolated exosomes were incubated with liposomes that carried the above gene modification tool and created hybrid exosomes that could transfer CRISPR/CAS9 intracellular without the need of a viral vector, as it is often presented [119].

Another method to produce hybrid exosomes is by extrusion through polycarbonate filters. Ryamajhi et al. developed hybrid anticancer exosomes from macrophage-derived EVs and doxorubicin-loaded liposomes. Their results showed enhanced in vitro internalization of the hybrid exosomes into the tumor cells in comparison to the normal cell lines. That observation attributed to the ability of macrophage EVs to actively interact with the cancer cells over the physiological cultures [120]. Moreover, Jhan et al. succeeded to encapsulate siRNA molecules into hybrid EVs. They follow a protocol that the hybrid EVs were first developed by simultaneous extrusion of lung carcinoma (A549) or mouse fibroblast (3T3) cell lines with the liposomal formulation. Afterward, the hybrid systems were loaded with the siRNA via an electroporation process. The scientists achieved an eightfold increase of the hybrid EVs' quantity over the natural production, but believe that further system optimization could lead to less toxic platforms (a parameter that is correlated with the electroporation method) and more effective and "clean" delivery systems [121]. On the other hand, Evers et al. achieved a siRNA encapsulation efficiency of 50–60% into hybrid EV-liposomal systems by adding the siRNA during the liposomes' thin film hydration stage [122].

3.2 Cargo Loading

As it is mentioned above, artificial exosomes have been used for a plethora of different drug molecules or biomolecules. First of all, artificial exosomes have proved beneficial for the loading of small molecules. For instance, Wu et al. developed

advanced platforms that carry doxorubicin. In vitro and in vivo models showed that these novel platforms might potentially aid in the treatment of glioblastoma [123]. Moreover, another study by Go et al. achieved the production of EV-mimetic platforms loaded with anti-inflammatory drug molecules (dexamethasone). The researchers support that their ghost nanovesicles preserve the properties of the EVs, produced by U937 cell cultures. At the same time, the quantity of vesicles is significantly increased and the loading capacity is enhanced [124].

Furthermore, surface molecules of various molecular weights are able to attach in the surface of artificial exosomes. Notably, Yenerni et al. designed a click chemistry methodology that enables the binding of biomolecules in the exosomal surface. A cholesterol-DNA tether is responsible for the interaction with the bioactive molecules. In that study, the researchers attached an immunomodulatory protein, FasL. Nevertheless, due to the nature of click chemistry methodology, the experimental protein is just an example and many other molecules could replace it [125].

Lastly, different nucleic acids have also been delivered in in vivo preclinical experiments by artificial biomimetic vesicles. For example, EV-mimetic nanovesicles loaded with long noncoding RNA H19 that are produced through a top-down extrusion procedure provide promising results in the treatment of diabetic wounds [126]. Similarly, in vivo and in vitro uptake studies showed that artificial exosomes can effectively deliver siRNA [127].

4 Future Perspectives and Conclusion

The development of artificial exosomes provides new possibilities and opens new roads in the area of ATMPs and the technology of delivery systems. Due to their smart design, they combine the benefits of both biological systems and liposomal vesicles. First of all, their small size allows the easy drug or biomolecule delivery when they are in vivo administrated. A lower degree of interaction with the MPS has also been observed, compared to classical liposomal formulations. Moreover, membrane EV proteins that are incorporated in the surface of artificial exosomes might lead to enhanced active targeting to the desired tissue. Finally, the well-established liposome technology allows the production of platforms that can carry a variety of different types of molecules. Small drugs, proteins, nucleic acids, or even complexes, as in the case of the CRISPR/Cas9 complex, could be effectively loaded into artificial exosomes. Although the encapsulating efficiency of the drugs or biomolecules might be lower than in the case of classic liposomes (especially in the case of hydrophilic molecules), the artificial platforms eliminate the toxicity and adverse effects as they provide higher targeting efficiency. Thus, these bio-mimicking platforms might be morphologically complicated, due to the variety of their components, but they present a very clear, noncomplex functionality that we believe is strongly connected with the biophysical routes of biosystems.

However, a lot of points are yet to be cleared or optimized. For instance, although the top-down approaches are the most common ones, as mentioned above, they

often need advanced manufacturing equipment and supplies that increase the cost of the final product. In addition, stricter instructions concerning GMPs are necessary for comparison with fully synthetic nanoparticles. Thus, the acceptance process is more demanding and currently under discussion. On the other hand, bottom-up approaches are still on an early stage, and thus, highly functional nanoplatforms are yet to be developed.

The hybrid systems present interesting behavior as advanced nano-therapeutic medicinal products. They combine the benefits of both synthetic and biological nanoplatforms resulting in the development of innovative procedures in the area of therapeutics. However, thorough design and targeted experiments are necessary to achieve the development of platforms that present the advantages and lack the limitations of these two technologies.

Nevertheless, exosomes and especially artificial exosomes breathe new life into the area of therapeutics, as they bring together the classic synthetic techniques of Pharmaceutical Sciences and the biological processes that are used in bio-medicinal products.

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Nanoarchaeosomes in Drug Delivery

Eder Lilia Romero and Maria Jose Morilla

1 Introduction: The Archaea Domain – Ubiquitous and Relevant but Poorly Known Life Forms

Archaea lipids have been the subject of academic research for nearly 20 years before archaea were classified as a new form of life, separated from bacteria and eukaryotes. Today, a meaningful amount of information is available on a notable product, the archaeal enzymes or extremozymes. Extremozymes catalyze reactions in harsh media, where extreme pH and T, osmolarity, and absence of water make the activity of ordinary enzymes impossible. In particular, because of its high fidelity and strong thermostability, the family B of archaeal DNA polymerases has found extensive application in high-fidelity PCR, DNA sequencing, and site-directed mutagenesis. Currently, the DNA polymerases from the genera *Pyrococcus* (Pfu, Pwo, Deep Vent™, Platinum® Pfx) and *Thermococcus* (KOD1, Tli, 9°N-7) are the only archaeal extremozymes commercially available [1–3].

This chapter, therefore, will not be focused on archaeal extremozymes, but on archaeal lipids or archaeolipids: the building blocks of a promising new type of nano drug delivery system, known as nanoarchaeosomes. The leading structural features of archaeolipids will be described, followed by an updated survey on the most relevant preclinical therapeutic uses of nanoarchaeosomes. To meet that aim, the environmental conditions where archaea groups thrive will be first overviewed. Next, the more significant structural features of polar and nonpolar archaeolipids will be highlighted as a function of their original environment. Finally, the main

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preclinical applications of nanoarchaeosomes according to their administration route will be described and compared with their liposomal counterparts.

In 1977, Woese and Fox published a seminal paper reporting a new unicellular life form, whose phylogeny was determined as a function of a molecular chronometer, a different type of 16S ribosomal RNA (and not on phenotypic criteria, ordinarily used to separate bacteria from eukaryotes such as the absence of nuclear envelope and histones, or low DNA content) [4]. Such microorganisms, initially known as archaebacteria and later renamed archaea, were comprised into a new major evolutionary line or domain Archaea that was added to the Eukarya and Bacteria domains [5]. Under the microscope, archaea may look like bacteria, displaying comparable sizes [between 0.2 and 10 μm], volumes (from one of the smallest organisms, close to a theoretical size limit for the life of 0.008 μm^3 [6] to 2.5 mm^3, which is ideal for maximizing the surface area-to-volume ratio, to optimize the exchange of nutrients and reaction products via diffusion), and shape (looking like cocci, straight or curved cylinders, filaments, discs, or squares). Archaea may be Gram-positive or Gram-negative, live in anaerobic or aerobic conditions, or be facultative. Archaea are broadly distributed in Earth's ecosystems, their lifestyles being highly heterogeneous. Despite not all archaea being extremophiles, many of them live in hostile environments and are involved in processes of global significance, such as the methanogens and Thaumarchaeota members (see below) [7].

Archaea are classified into Euryarchaeota, Crenarchaeota, Korarchaeota, Nanoarchaeota, and Thaumarchaeota phyla, but the rigorous taxonomic location of each member is complex, controversial, in constant revision, and out of the scope of this chapter. To our aims, it will be sufficient to distinguish two big phenotypic groups as (a) sulfur-dependent and (b) the reminder archaea.

1.1 Sulfur-Dependent Archaea

These are considered the oldest archaea; they use inorganic forms of sulfur in diverse ways and are mostly thermoacidophilic; and their molecules display structural features radically different from those of the reminder archaea. Sulfur-dependent archaea dwell in extreme environments such as hot acid springs (pH < 2, 80–90 °C), submarine volcanic areas, and smoldering piles of coal tailing. Their respiration can be either anaerobic or aerobic; some of them display a chemolithoautotrophic anaerobic metabolism involving H_2, CO_2, and elemental S. Some can be used to remove S from coal aiming to minimize the pollutant effects of sulfur-containing gases emitted during combustion. The *Sulfolobales*, hyperthermophilic and slightly halophilic organisms first cultivated from volcanic hot springs at pH values of 2–3 and 80 °C, and the anaerobic, hyperthermophilic *Thermococcales* cultivated at >90 °C are examples of ecologically and biotechnologically important sulfur-dependent archaea.

1.2 The Reminder Archaea, Divided According to Their Life Conditions (Niche) or Metabolism

1.2.1 Halophilic Archaea (Haloarchaea)

These archaea grow optimally in 2.5–5.2 M NaCl and lyse at concentrations below 1.7 M. All are aerobic chemoheterotrophic microorganisms that oxidize simple organic compounds employing molecular oxygen as the electron acceptor for respiration. Halophilic archaea can be found from marine solar salterns, alkaline salt lakes, and natural brines, with salt concentrations as high as halite saturation [8] to the Atacama Desert, the Argentinean Patagonia, or the Dead Sea. Halophilic archaea can survive extreme desiccation, starvation, and radiation, sometimes apparently for millions of years, found worldwide in rock salt deposits, from the Pliocene (5.3–1.8 million years) up to the Silurian (419 million years) [9].

1.2.2 Methanogens

These are obligate anaerobic archaea that reduce CO_2 to the greenhouse gas methane, from a few simple molecules such as H_2 and 1C. Methanogens depend on other organisms for a supply of substrate such as volatile fatty acids, alcohols, or gases in the terminal step of the anaerobic food chain [10]. They are widely distributed around the globe, and their estimated contribution to the global carbon cycle is foremost, nearly 1 Gt carbon yr-1 [11]. Methanogens are responsible for the formation of marsh gas and can be found in anaerobic sediments, sewage treatment plants, cattle rumen, insect guts, and human intestine, as well as in hot vents in the deep oceans and ice-cold permafrost soils.

1.2.3 Psychrophile Archaea

This group of archaea is capable to proliferate in cold environments at 0–10 °C, metabolizes in snow and ice at −20 °C, is predicted to metabolize at −40 °C, and can survive at −45 °C. Most (~75%) of the Earth's biosphere is cold (alpine and polar habitats, terrestrial and ocean subsurface, upper atmosphere), and psychrophiles can be found in permanently cold environments (≤5 °C) [12]. Remarkably, vast amounts of psychrophile archaea (estimated in ~10^{28} cells) thrive in the deep ocean covering 70% of the surface of the planet [13]. The members of the big archaeal phylum Thaumarchaeota [7] are a metabolically diverse group of microbes found in almost every environment ranging from marine waters and arctic soils to the human skin biome. Many cold-adapted archaea are Thaumarchaeota members capable to perform ammonium oxidation, the only biological process converting reduced to oxidized inorganic nitrogen species on Earth and the first and

rate-limiting step in nitrification [14]. Methanogenic Euryarchaeota, together with Thaumarchaeota members resident in the open ocean, significantly contribute to the global N-cycle and C-cycle [13]. The largest proportion and greatest diversity of archaea exist in cold environments, including methanogens, Thaumarchaeota members, and some halophilic archaea [15]. Curiously, despite their relevance, cold-adapted archaea remain understudied [16].

2 The Archaea Domain as a Source for Extremolytes and Novel Biomaterials for Drug Delivery

The resemblance between archaea and bacteria abruptly diverges when their genome sequence, information processing machinery, and, particularly, membrane lipid structures [17] are compared.

Such divergence captured first the interest of the academic community and later became attractive for members of the biotech and nanobiotech field. Unique properties were identified on nonenzymatic proteins such as those conforming the outer wall and, on the lipids, forming the membranes of archaea. The glycoproteins conforming the outer wall or S (surface) layer [excepting the members of *Thermoplasmatales,* which lack outer wall] constitute a self-assembled monomolecular armor conforming a repetitive arrangement of low surface energy [18]. The S layer displays antifouling properties; provides surface hydration, resilience to osmotic stress and proteases; is involved in surface recognition and cell shape maintenance; and acts as a molecular sieve and as a trap for ions and molecules [19]. S layers from archaea are soft and predominantly of hexagonal symmetry (p6); importantly, they lack lipopolysaccharide and murein [20], which are replaced by n-acetylated glucosamine, galactosamine, L-lysine, L-glutamic acid, L-alanine or threonine, and n-acetyl talose derived acid (talose is a sugar only found in archaea). Since prokaryote biomass overcomes that of eukaryotes, S layer proteins are among the most abundant biopolymers on the planet [21]. Specifically, S layer proteins from archaea are considered of high technological value because their ability to retain structure and function either in highly saline environments or extreme pH or temperature, according to its archaeal source [22].

Both wild and engineered archaea can be used as cell factories for other valuable raw materials different from proteins, for instance, biofuels (such as biomethane produced by methanogens, biohydrogen produced by membrane-bound hydrogenases from hyperthermophilic non-methanogenic archaea that use ferredoxin as reducing equivalent), bioethanol or biobutanol, and bioplastics such as polyhydroxyalkanoates (PHA) (used as intracellular carbon and energy storage compounds and produced by halophilic archaea). Archaea are also a source for chemical precursors (e.g., acetate, 2,3-butanediol) needed for the industrial synthesis of expensive chemicals.

A striking advantage of using halophilic archaea as cell factories is that because of their extremophile nature, it is feasible to culture them under non-sterile conditions, employing cheap feedstocks that may be toxic to other microorganisms and significantly reducing cultivation costs [22]. In such sense, halophilic archaea are of major biotechnological relevance compared to other extremophile archaea that require heterologous systems as production platform because of their low yields of products [23, 24]. The other side of halophilic cultures is the need for massive amounts of salt, linked to a subsequent need for desalinization and culture media recycling [25, 26].

Extremolytes are small organic molecules such as sugars, polyols, heterosides, amino acids, and derivatives produced by extremophiles, which protect macromolecules or cell structures in extreme environments currently drawing great industrial interest [27]. Examples of that are archaeal osmolytes (also known as compatible solutes), the negatively charged versions of carbohydrates, and polyols used as osmolytes by bacteria and eukaryote. The negative charge is provided by phosphate, carboxylate, and sulfate groups; such osmolytes act as counterions for K^+ in halophilic archaea [28]. α-D-mannopyranosyl-(1 → 2)-D glycerate (mannosylglycerate (MG) also known as digeneaside, or firoin) and di-glycerol-phosphate levels, for example, are increased in response to osmotic stress by halotolerant and halophilic microorganisms adapted to hot environments [29]. These osmolytes stabilize and protect proteins under salt, heat, and freeze-drying stresses as well as against aggregation. The effect of MG has been preclinically explored to reduce the aggregation of beta-amyloid peptides [30]. Examples of other extremolytes are the di-myo-inositol-1,1′-phosphate (DIP), which tends to increase in concentration according to the growth temperature [31], glucosylglycerate, and sulfotrehalose.

A portfolio plenty of new biomaterials is in theory available from different genders and species of archaea. The industrial manufacture of these biomaterials, however, is limited by the extreme life requirements of archaea, their slow growth rate, and their low production yield. To objectively analyze their biotechnological potentialities, a recent report has positioned each archaeal biomaterial within a scale of nine biotechnological readiness levels (B-TRL) (Pfeifer et al. [22]), linking each level to a determined work achievement. The status of the first four levels corresponds to basic and applied research: level 1 (*fundamental research/ideation:* identifying fundamental concepts, identification of suitable archaea or components thereof); level 2 (*proof of principle:* development of experimental designs; basic principles observed on plate/flask scale experiments; systematically screening suitable archaea or components thereof); level 3 (*concept demonstration:* identification and optimization of scale-up parameters and cultivation systems; identification of possible downstream processes <10 L); and level 4 (*proof of concept*: transferring optimized parameters to a bioreactor level; optimization of up−/downstream processes 10 <100 L). Level 9 is the highest level, corresponding to commercialization, a technology, or system that has shown to function continuously and economically and/or financing and construction of commercial production. Currently, the only commercially available products of archaeal cell factories are bacterioruberin,

squalene, bacteriorhodopsin, and diether/tetraether lipids all of them employing halophilic archaea [27]. Their B-TRL, however, do not exceed the level 3. This signifies that none of them are produced in industrial amounts and that despite of being commercialized, their demand is satisfied selling small amounts at very high prices.

3 Lipids from Archaea

3.1 Nonpolar Archaeolipids

About 7–20% total lipids from halophiles and thermoacidophiles and up to 30% in methanogens are nonpolar lipids, mainly isoprenoid and hydroisoprenoid hydrocarbons. The most abundant are the acyclic isoprenoids, such as C30 hexaisoprenoids (squalenes, available in different hydrogenation degrees, from dihydrosqualene to squalane), C25 pentaisoprenoids, and C20 tetraisoprenoids (phytanes). The major hydrocarbon in the cell is the squalene, a natural antioxidant and a key intermediate in the formation of eukaryotic sterols and bacterial hopanoids [32, 33]. Because of its nonpolar structure, the incorporation of squalene into biological membranes is limited, being mostly accumulated in lipid storage compartments. In membranes the squalene may, however, adopt a sterol-like conformation and span the bilayer from side to side. It is hypothesized that in membranes, squalene plays a role equivalent to cholesterol, inducing local order by moving away the tails of the isoprenoid chains from the surface and prompting a tighter packing. As a consequence, the membrane fluidity is reduced, the average area per lipid molecule is condensed, and the formation of domain with increased average height at the edges is favored [34, 35]. Halophilic archaea produce also C40 and C50 carotenoids. Bacterioruberin is a bright red C50 carotenoid that acts as cell membrane stabilizer increasing its rigidity, confers protection against the natural exposition to high UV and gamma radiation, and displays potent antioxidant activity [36, 37].

The plasma membrane of archaea also contains isoprenoid quinones that function mainly as electron and proton carriers in photosynthetic and respiratory electron transport chains, some of them acting as antioxidant. Menaquinones (MK) are a type of naphthoquinones that act in respiratory and photosynthetic electron transport chains of bacteria. Archaea possess thermoplasmaquinone (TPQ) and methionaquinone (MTQ) differing in length and saturation of the C-3 polyprenyl side chain. The isoprenoid sulfoquinones from thermophilic and aerobic archaea Sulfolobales participate in electron transfer during chemosynthetic oxidation of sulfur compounds, and aerobic respiratory chain contains an additional sulfur heterocyclic ring [38]; and these are known as caldariellaquinone, sulfolobusquinone, and benzodithiophenoquinone (CQ, SQ, SSQ type, respectively) [39, 40]. These benzothiophene quinones possess higher midpoint redox potential than the corresponding naphthoquinones and are supposed to appear later than MKs during evolution while adapting to aerobic conditions (Fig. 1).

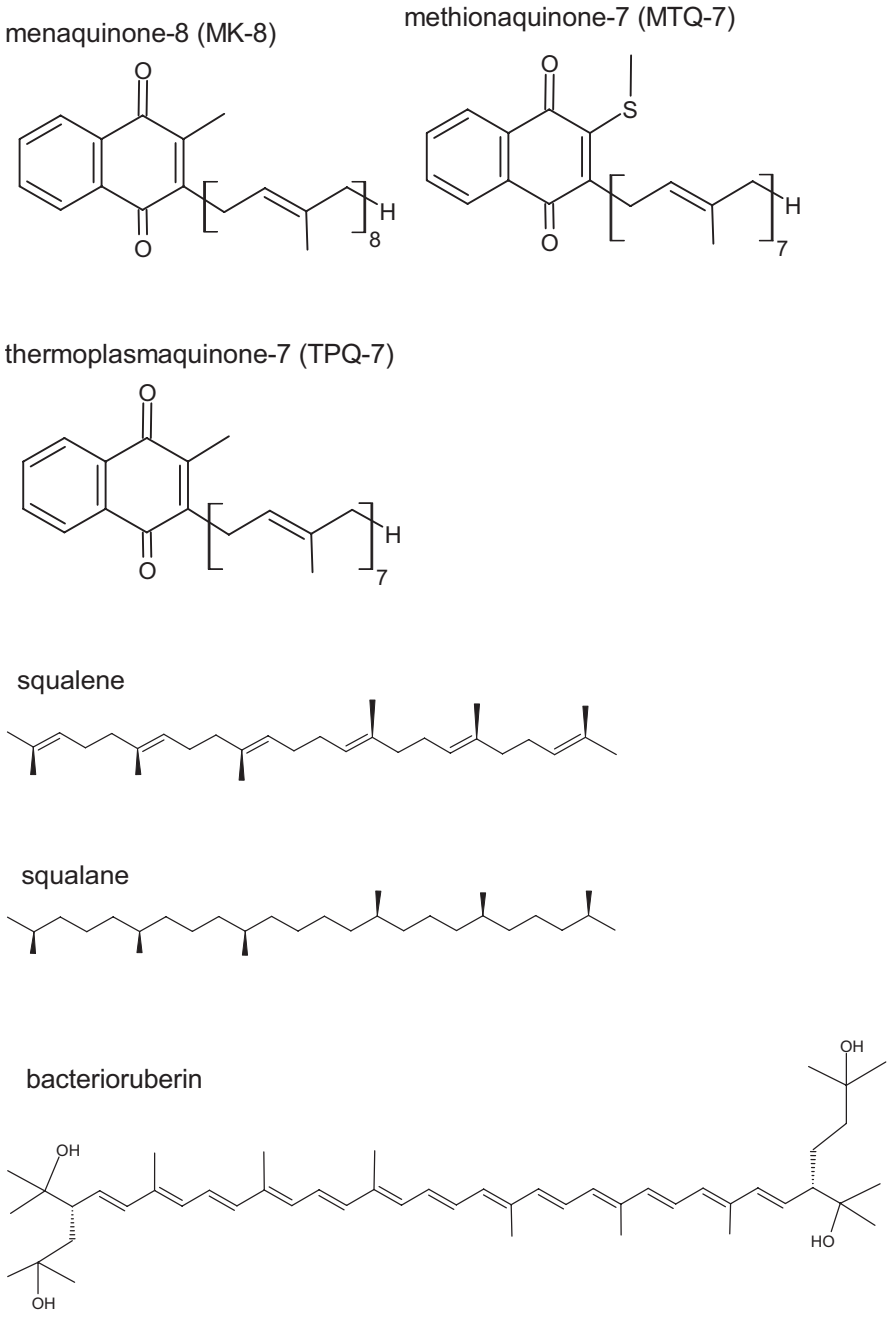

Fig. 1 Representative nonpolar archaeolipids

3.2 Polar Archaeolipids

In this section, an overview on structures and properties of polar archaeolipids, unique molecules of unusual chemical structure considered as taxonomic markers of archaea [41], and the building blocks of nanoarchaeosome bilayers, is provided.

The plasma membrane from bacteria and eukaryotes is mainly constituted by glycerolipids, amphiphile esters of glycerol with fatty acids, named acylglycerols (glycerides). Because of the glycerol C2 asymmetry, the acylglycerols are levorotary having the D- or sn-1,2-glycerol stereoconfiguration, being their polar heads attached to carbon 3 of glycerol. The fatty acids are either straight chained, monomethyl-branched, or unsaturated, ester linked to carbons 1 and 2. The glycerol ether lipids are mostly absent; tough trace amounts of alk-1-enylglycerols (plasmalogens), alkylglycerols (monoethers), and dialkylglycerols (diethers) can be found in few organisms.

The glycerolipids constituting the plasma membrane from archaea instead are mainly di- or tetraether (between 2% and 4% of the cell dry weight of halophiles and thermoacidophiles (*Sulfolobus, Thermoplasma) and* 2% and 6% of methanogens) [42]. The glycerol C2 from archaea glycerolipids is also asymmetric and optically active, resulting the specular images of its bacteria and eukaryote counterpart [38]. Fatty acids are practically absent in archaea; and rather than glycerol ester linked to fatty acids, the glycerol is ether-linked to phytanyl chains. These phytanyl chains are more hydrophobic [43], with a transverse section in the order of 100 Å2 (nearly twofold larger (40–60 Å2)), and packed loosely in comparison with the straight acyl chains [43].

Specific features of polar lipids, as a function of gender and habitat of each group of archaea, are detailed below.

3.3 Structural Features of Polar Archaeolipids and Membranes from Halophilic Archaea

The 100% of glycerolipids from halophilic archaea are acidic glycerol diethers. Such diethers were first isolated and studied long before the concept of archaea was formulated [44, 45]. The diethers are made of two C20 fully saturated phytanyl chains in ether linkage to glycerol (0-alkyl chains) ($C_{20}H_{42}$): 2,3-di-O-phytanyl-*sn*--glycerol (C20, C20), named archaeol ($C_{43}H_{88}0_3$, MW 652) [46] (Fig. 2). In some haloalkaliphile and *Halococcus* species, C20,C25- and C25,C25-diether variants of the diphytanylglycerol diether lipid core were also identified [47]. Archaeols are dextrorotary (L- or sn-2,3-glycerol stereoconfiguration), with polar head groups attached to carbon 1 of the glycerol molecule. Ordered in relative abundance, archaeols can be found as four analogues of phosphatidyl glycerosulfate > phosphatidylglycerol and phosphatidyl glycerosulfate, plus a triglycosyl diether glycolipid containing a terminal sulfate radical [46].

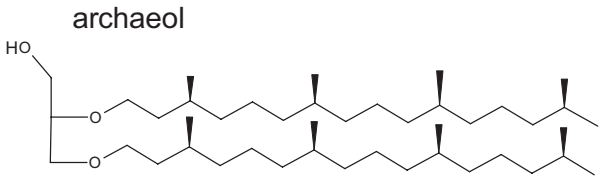

Fig. 2 Structure of a generic diether archaeolipid archaeol

The surface of the halophilic archaea displays a strong acidic character because of the presence of glycolipids, mainly acidic sulfated derivatives. These glycolipids are associated exclusively with the purple membrane fraction, which contains the photosensitive purple pigment bacteriorhodopsin. Such pigment has only been found in halophilic archaea and converts light into chemical energy to synthesize ATP. The purple membrane is a light-driven proton pump; its content increases in low O_2 tension and under UV light irradiation [48]; sulfated polar lipids are thought to be required as proton donors [46].

Halophilic archaea produce also cardiolipins, analogues to those from bacteria and eukaryote phospholipid dimers, mainly bisphosphatidylglycerol (BPG) (Fig. 3), with four identical C20 phytanyl chains, named sn-2,3-di-O-phytanyl-1--phosphoglycerol-3-phospho-*sn*- di-O-phytanylglycerol or archaeal BPG. The stereoconfiguration of archaeal BPG is S/S, whereas stereoconfiguration of those from bacterial or mitochondrial cardiolipin is in the R/R configuration. BPG is omnipresent in all genera of the family Halobacteriaceae [49]. Another glyco-cardiolipin analogue, the S-TGD-1-PA from halophilic archaea, is found strongly associated to the outer leaflet of purple membrane, probably accounting to the assembly of bacteriorhodopsin into the membrane [50]. Cardiolipins are found in high proportion, nearly 20% polar lipids or 4 × PG content, and were recently reported to display cytochrome c oxidase activity [51].

In contrast to the methanogenic and thermophilic archaea, no nitrogenous base-containing phospholipids, such as phosphatidylserine or phosphatidylethanolamine, are present in halophilic archaea [52].

A recent study on model halophilic archaea membrane analogues confirms that apolar polyisoprenoids, such as squalene (Fig. 1), are placed inside the bilayer midplane, extending their domain stability, reducing its permeability to protons but increasing that of water, and inducing a negative curvature in the membrane, allowing the transition to novel non-lamellar phases. The presence of squalane is thought to be related to the tolerance to temperature over 100 °C in halophilic adapted to hot environment archaea having a bilayer membrane [53].

Halophilic archaea thrive in extreme saline habitats, exposed to high ultraviolet radiation, conditions that preclude the viability of most living beings [54]. The survival of members of the family Halobacteriaceae depends on their distinctive metabolism that equilibrates the osmotic unbalance with the outer side with high intracellular K^+ levels and proteins rich in acidic amino acids [31]. The membrane of halophilic archaea is adaptable to sudden changes in environmental salinity,

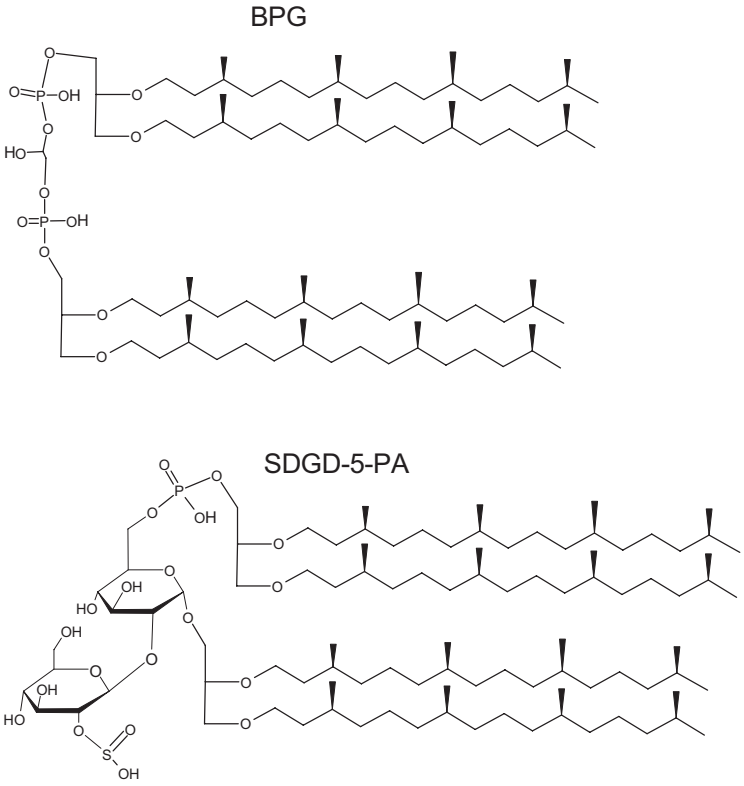

Fig. 3 Representative cardiolipins found in halophilic archaea

caused by rainfall, or water evaporation in dry periods. This is achieved at expenses of the phytanyl ether membranes, of highly stable membrane packing, due to the low degree of movement of the bulky branched methyl groups in the phytanyl chains, plus the van der Waals interactions between them [55]. Because of that, the membrane of halophilic archaea is impermeable to polyols such as glycerol, carboxyfluorescein, and NaCI and other salts, allowing the intracellular accumulation of Na^+ and K+ at concentrations higher than those in the outer side [56–58]. In addition, halophilic bilayers are rich in PGP-Me (Fig. 4), a double negatively charged archaeolipid of voluminous polar head. PGP-Me is thought to be responsible for steric repulsion between opposite bilayers or out of plane bilayer undulations; this prevents membrane agglomeration at high (3–4 M) NaCl concentration [59]. The chemical changes experienced by halophilic membranes against physical stress are minimal compared to those suffered by thermoacidophiles. Their content in carotenoids, for example, is modulated in response toward environmental stressors: chronically submitted to high doses of UV light, halophilic archaea can cope with severe UV-generated oxidative stress caused by oxidative radicals such as

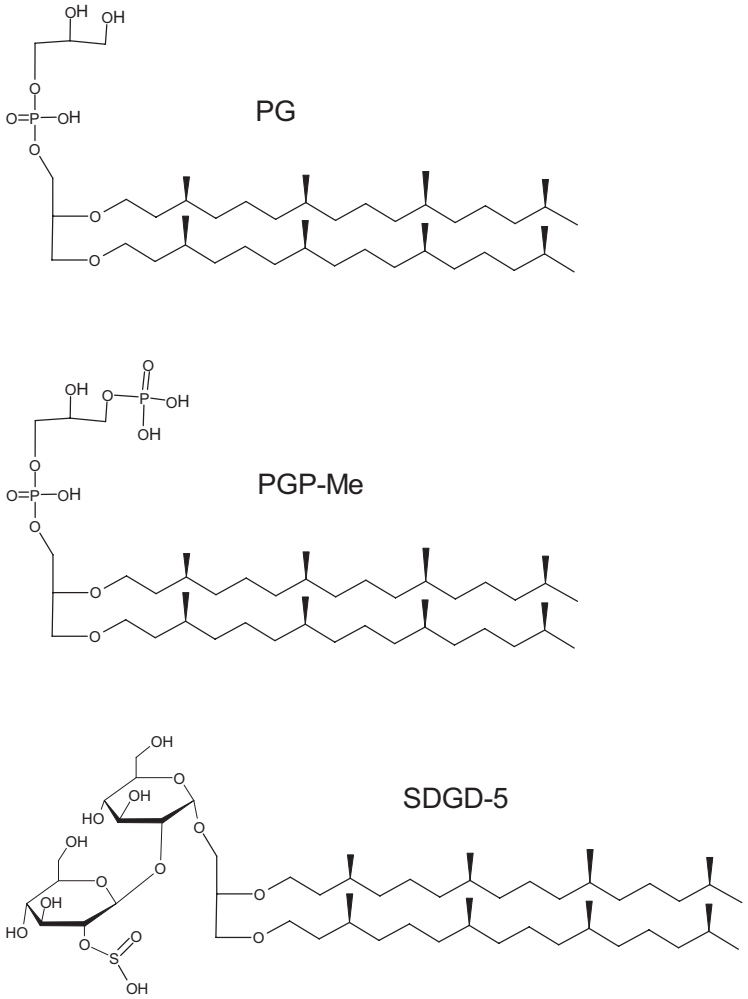

Fig. 4 Polar lipids from the halophilic archaea *Halorubrum tebenquichense*

superoxide (O_2^-). This is thought to be performed by increasing the bacterioruberin content, which is also increased under saline stress. It is hypothesized, therefore, that besides being photoprotector and antioxidant, bacterioruberin stabilizes the bilayer against decreased salinity [37, 60, 61]. The cardiolipin content seems to play a protective role as well: when submitted to hipoosmolarity, halophilic archaea increase their content in BPG and, in a glyco-cardiolipin analogue, the sulfodiglycosyl-diether-phosphatidic acid (S-DGD-5-PA) (Fig. 3), contents, at expenses of PG and the glycolipid SDGD-5, respectively. Cardiolipins, therefore, are regarded as osmoprotectants [51].

3.4 Structural Features of Polar Archaeolipids from Thermoacidophile Archaea

To perform vital functions, the bilayer of thermoacidophile, thermoanaerobic, and some methanogenic archaea needs to maintain the structural stability of its membranes against thermal variations, specifically by maintaining a reduced ion/proton permeability. This is fulfilled by the presence of diglycerol tetraethers, special lipids constituted by two fully saturated C40-biphytanyl chains ether-linked simultaneously to two opposite glycerol molecules. The $C_{40}H_{82}$ biphytane tetraether skeleton is made up of two C20-phytane units condensed at the 16,16′-gem-dimethyl ends [62–64], being the two diether molecules in antiparallel arrangement. The tetraether lipid is named caldarchaeol (2,3,2′3′-tetra-0-dibiphytanyl-di-*sn*-glycerol) ($C_{86}H_{172}0_6$, MW 1300) [65] (Fig. 5). Caldarchaeols are dextrorotary (both glycerols have the L- or *sn-2,*3-glycerol stereoconfiguration), and the primary -OH groups of the two opposed glycerols are in the *trans* configuration. Polar head groups, therefore, may be attached to glycerol at either or both carbon-1 or 1′ when present. *Sulfolobus* and *Thermoplasma* species produce the parallel isomer of the caldarchaeol known as isocaldarchaeol [17].

Caldarchaeols form monolayers which present a more compact and stable packaging than membranes made of two monolayers of diethers and preserve better the bilayer integrity against high temperatures [66, 67]. In addition, tetraethers may experience chemical modifications in response to increased temperature or acidity, displaying a higher number of structural variations than the dieters from halophilic or methanogenic archaea.

For example, thermoacidophile archaea avoid the excess of fluidity and of rotational freedom induced by high temperatures, introducing macrocyclic rings in up to 8 cyclopentyl rings/biphytanyl chain [62–64, 68–71]. Pentacycles are variable with species and are found above 85 °C and at pH 2–3, up to the highest temperature of 121 °C. The cyclization has been connected with the ability of growing in the presence of oxygen. Such strategy, together with the presence of hydrogen bonds between polar head groups, accounts for a higher membrane toughness and increases its tight packaging [72, 73]. Thus far, pentacyclic biphytanyl tetraethers have been found only in the thermoacidophilic archaea *Thermoplasma, Sulfolobus*, and *Thermoproteus.* At the highest temperatures and acidity, the membrane stability is reinforced by the introduction of covalent links between the tetraether hydrocarbon chains, giving rise to the H-shaped caldarchaeol [17] (Fig. 5).

Besides cyclization, 50–85% of *Sulfolobus* tetratethers present one glycerol substituted by a molecule of branched nine-carbon nonitol (C9H20O9), constituting the glycerol-alkylnonitol tetraether or calditol (Fig. 5), or glycerol-nonitol tetraether (C92H180_168O12, MW 1476–1464), of higher polarity than the ordinary glycerol dialkyl glycerol tetraether structure [63]. *Sulfolobales* tetratethers may also contain different chain lengths and include cyclohexanes, giving rise to structures known as crenarchaeol (Fig. 5).

Polar head groups of thermoacidophiles are restricted to either glucose, a glucosylgalactosyl disaccharide, phosphorylinositol, or sulfate.

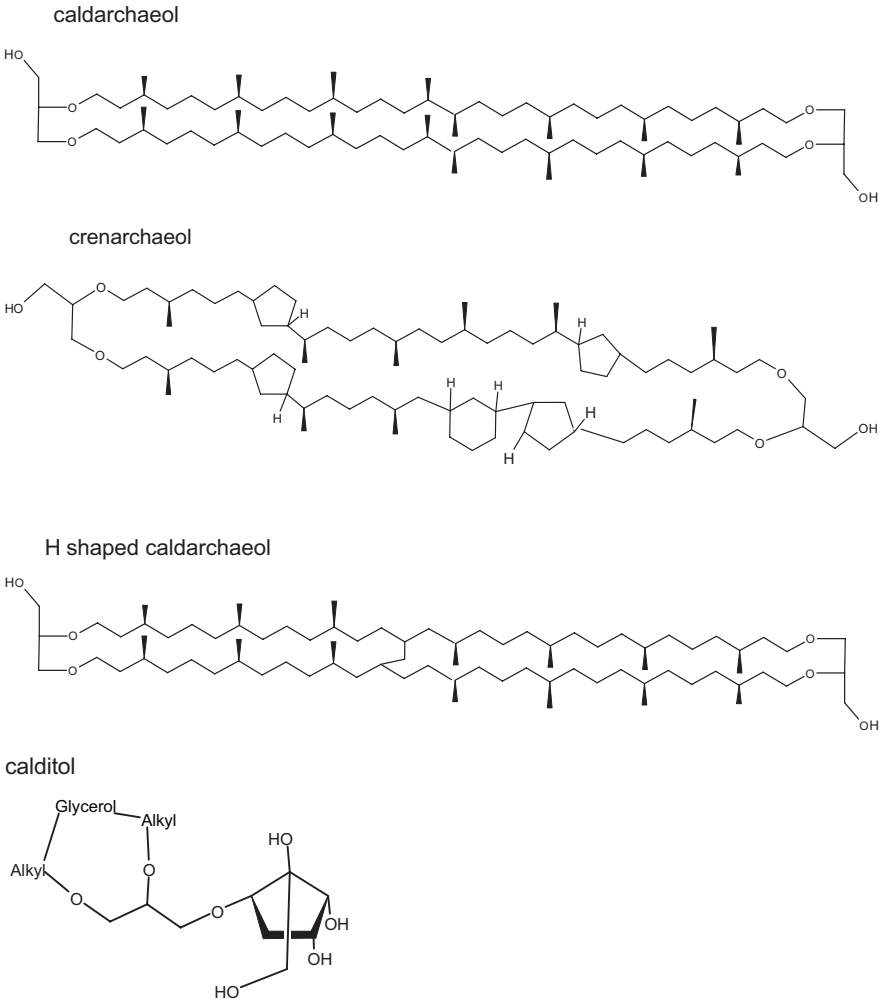

Fig. 5 Tetraether lipids found in thermoacidophile archaea

3.5 Structural Features of Polar Archaeolipids from Methanogens and Psychrophilic Archaea

Depending on the species, the plasma membrane of methanogens can be either made of 100% glycerodiethers, such as those of coccal morphology mostly, or contain a mixture of diethers and up to 60% tetraethers. The tetraethers are though to confer structural stability to the bilayer in the presence of huge concentration of methane gas. The thermophilic methanogens possess tetraethers whose biphytanyl chains are identical.

The presence of branched methyl groups in the isoprenoid chain is responsible for maintaining its liquid crystalline state at very low temperatures and across a wide range of temperatures (10–100 °C) [74]. At very the phase transition to gel-like phase (and subsequent reduced stability with increased permeability), by inducing selective unsaturation of isoprenoid chains [75, 76]. *Hrr. lacusprofundi*, for instance, has the ability (infrequent in halophilic) of synthesizing unsaturated diether lipids, as a crucial adaptation to life at low temperatures [77].

4 General Properties of Nanoarchaeosome Bilayers

In the absence of a rigorous definition of nanoarchaeosomes, we will describe them as nanovesicles exhibiting specific properties according to the mixture of polar and nonpolar lipids extracted from each species of archaea. The presence of archaeolipids in a membrane will always account for a higher resistance to phospholipases activity, oxidation, physical and chemical hydrolysis, thermal degradation, as well as lower permeability to Na^+, Cl^-, K^+, and polyols than a liposomal membrane. The term nanoarchaeosome, thus, encompasses different vesicular structures having in common a membrane fully made of archaeolipids, or combined in variable proportions with other amphiphiles such as natural or synthetic phospholipids or detergents. In the extreme, for example, the nanoarchaeosomes containing only complex tetraethers such as caldarchaeols and isocaldarchaeols extracted from thermoacidophiles, namely, *Sulfolobus acidocaldarius* [78] or *Thermoplasma acidophilum* [79], present a minimal H^+ permeability at high temperatures that can be further reduced by adding cyclopentane rings [80, 81] and polar heads having two or more sugar units [81]. On the other hand, if more permeable to protons than tetraether monolayers, diether bilayers offer higher structural endurance than liposomes. Besides, the z potential of halophilic nanoarchaeosomes is highly negative, because of the negatively charged nature of their archaeolipids; such type of nanoarchaeosomes spontaneously forms relatively small-sized, oligolamellar nanovesicles (below nearly 600 nm diameter) [82] (Fig. 6).

5 Preparation Methods of Nanoarchaeosomes

Liposomes are vesicles made of phospholipids extracted from eukaryote or bacteria or of synthetic origin, self-sealed in aqueous media upon the input of a small amount of energy, which were first described by Alec Bangham in the 1960s [83, 84]. In aqueous media, and upon a small input of energy, thin films of archaeolipids form sealed bilayers or monolayers, to form nanovesicles enclosing an inner aqueous space, the same as liposomes. Therefore, either at lab or industrial scale, the preparation of nanoarchaeosomes can be carried out employing the same methods used for liposomes. An updated review on lab- and industrial-scale preparation methods of liposomes has been recently published [85].

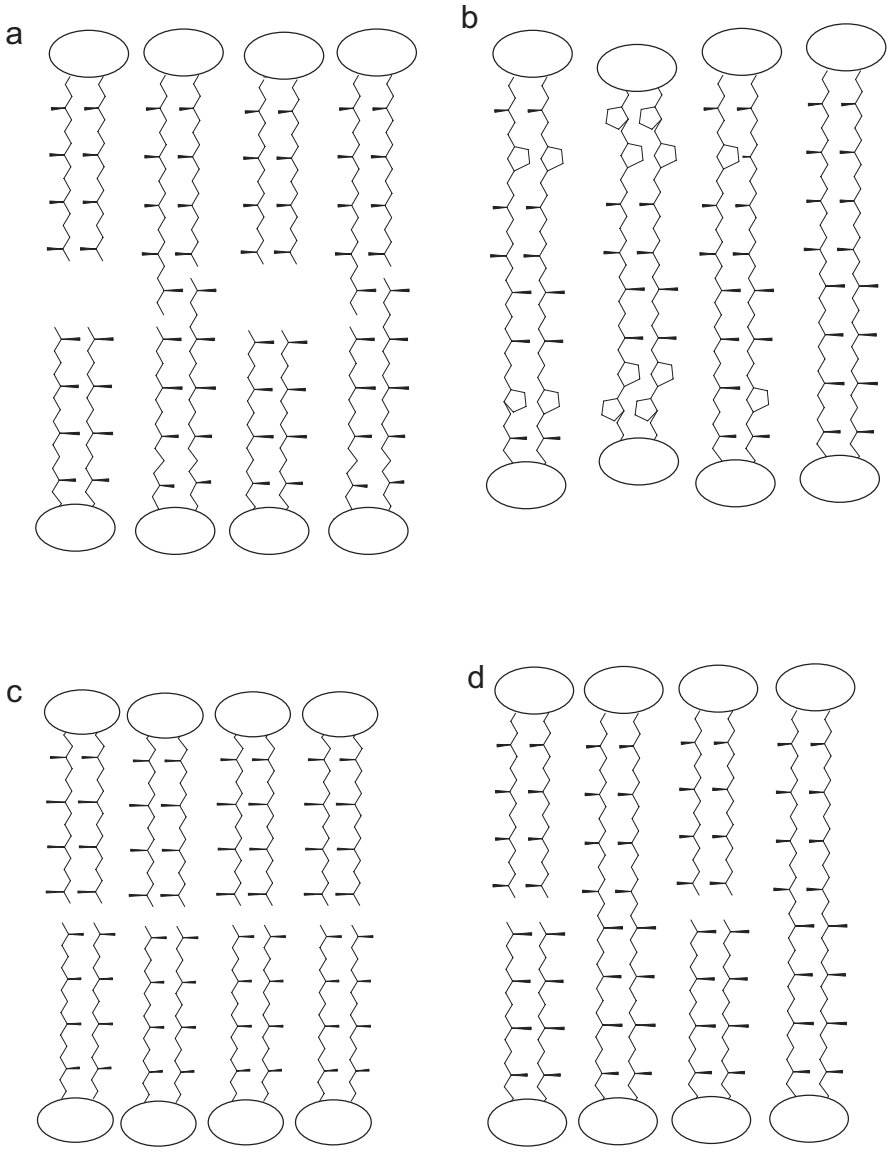

Fig. 6 Polar lipids forming different membranes in archaea. (**a**) Zip-type membrane made of C20C25 and C20C20 diethers from alkalophilic archaea; (**b**) rigid monolayers formed by C40C40 tetraethers found in thermoacidophile archaea; (**c**) C20C20 diether bilayers from halophilic archaea; (**d**) mixes of structures formed by C20C20 diethers and C40C40 tetraethers found in methanogen archaea

Archaeolipids, however, possess a remarkable property that simplifies the preparation of nanoarchaeosomes: their glass transition temperature is below 0 °C, an attribute that allows their handling at room temperature [86]. In contrast, high phase transition temperature (Tm) phospholipids such as HSPC or DSPC (53 and 55 °C Tm, respectively) used to prepare commercial liposomal formulations such as Doxil and AmBisome or DaunoXome and Vyxeos and that remain structurally stable avoiding drug release during circulation must be handled at temperatures above their Tm [87]. Nanoarchaeosomes employed in preclinical assays compiled in this chapter have been prepared by the thin lipid film hydration method, followed by extrusion or bath sonication and by dual asymmetric centrifugation (DAC) in lesser extent [88].

6 Nanoarchaeosomes in Drug Delivery

Both liposome and nanoarchaeosomes are lipid layered vesicles, but the latter, because of the unique chemical nature of archaeolipids, display a higher structural strength and, as described below, are distinguished by their peculiar pharmacodynamics. As discussed later, both features may positively influence the potential industrial manufacture of nanoarchaeosomes.

Because of their size, liposomes and nanoarchaeosomes are considered nanoparticulate material or nano-objects, constituting, when associated to different molecules of interest, new supramolecular entities known as non-biological complex drug or nanomedicines [89]. Between the approval of Doxil, the first nanomedicine in 1995, and 2020, the FDA approved nearly 60 more nanomedicines at a rate of 2 per year from 2010. Nanomedicines represent only the 6% of the pharmaceutical market of the USA (the world's largest) but constitute 14% of the FDA approval in the last 20 years, reflecting its fast growth in comparison to other well-established technologies [90]. An additional 12.8% growth by the year 2025 propelled by the evolution of vaccines against COVID-19 based on nanomedicines and the expected huge global demand for these products is expected [91]. The size of the global market of nanomedicines is nearly USD 142 billion, with a growth rate of 26.7% compared to 4 years earlier and more than a twofold growth compared to 2010 analytics [92], with nearly 50 more in advanced clinical trials as of 2020. Liposomal nanomedicines are top-ranked nanomedicines approved by the FDA [90], mostly because of their well-known low toxicity and feasibility of industrial scaling up [93]. In the pharmaceutical field, liposomal nanomedicines undoubtedly occupy the B-TRL 9.

The first preclinical application of liposomes was 44 years ago for intracellular delivery of the antileishmanial agent pentavalent antimonial [94]. The first preclinical application of nanoarchaeosomes instead was more recent – 1997 – and as vaccine adjuvant [95]. In the last 10 years, a sustainedly growing number of preclinical reports have been gathered on that subject. In this chapter however, the performance of nanoarchaeosomes as nanovaccination platform will not be addressed. Instead, we will exclusively be focused on their drug delivery uses.

Few research groups in the world are sustainedly focused in developing and testing nano drug delivery systems based on natural or synthetic nanoarchaeosomes:

- The group of Prof. Gert Fricker and colleagues (Institute of Pharmacy and Molecular Biotechnology, Department of Pharmaceutical Technology and Biopharmacy, University of Heidelberg, Germany) employs the tetraether lipids (GCTE) isolated from *S. acidocaldarius*, aimed to the oral delivery of peptides.
- The group of Prof. Udo Bakowsky and colleagues (Department of Pharmaceutics and Biopharmaceutics, University of Marburg, Marburg, Germany) employs tetratether lipids isolated from *S. acidocaldarius* to perform photodynamic therapy, including inhaled antitumoral photodynamic therapy.
- The group of Prof. Eder Lilia Romero and colleagues (Science and Technology Department, Nanomedicine Research and Development Centre (NARD), Quilmes National University, Buenos Aires, Argentina) employs diether lipids and nonpolar isoprenoids extracted from *H. tebenquichense* for oral delivery of peptides and proteins, for inhaled drug delivery, as anti-atherosclerotic agents and as vaccine adjuvants.
- The group of Prof. Thierry Benvegnu and colleagues (Ecole Nationale Superieure de Chimie de Rennes, Universite´Europeenne de Bretagne, Rennes, France) has shown that synthetic tetraether lipids could be used for the delivery of genes to cells in vitro and in vivo.
- The group of Prof. Jerry Yang and colleagues (Department of Chemistry and Biochemistry University of California San Diego La Jolla, USA) has synthesized a series of glycerol monoalkyl glycerol tetraether lipids to prepare antitumoral nanoarchaeosomes.

6.1 Nanoarchaeosomes in Oral Drug Delivery

Because of their high stability in acid media, presence of phospholipases, and bile salts [72, 96–98], nanoarchaeosomes are used to minimize peptides and protein lysis across the gastrointestinal (GI) tract and increase their bioavailability when administered by oral route (Table 1).

6.1.1 Nanoarchaeosomes Made of Thermoacidophile Tetraether Lipids

In an earliest approach, insulin was loaded within nanoarchaeosomes made of the polar lipid fraction E (PLFE; Fig. 7) from *Sulfolobus acidocaldarius* [99]. Such nanoarchaeosomes minimized the insulin release in simulated intragastric fluid (pH 2.0) and intraintestinal fluid (sodium taurocholate, pH 7.4), compared to liposomes made of egg phosphatidylcholine (EPC)/cholesterol (chol) at 3:2 molar ratio. They also enabled fluorescently labelled insulin to reside for longer periods in the GI tract, than conventional liposomes after oral administration. Finally, oral nanoarchaeosomal insulin induced hypoglycemia of diabetic mice, while free or liposomal insulin did not.

Octreotide, an eight-amino acid synthetic analogue of somatostatin, was loaded into nanoarchaeosomes made of glycerylcaldityl tetraether (GCTE) (Fig. 8) isolated

Table 1 Administration routes and detailed composition of nanoarchaeosomes-based drug delivery systems

Route of administration	Source of archaeolipids	Drug	Assays	Reference
Oral	Tetraether lipids (PLFE) from *Sulfolobus acidocaldarius*	Insulin	In vivo	[99]
	Tetraether lipid (GCTE) from *S. acidocaldarius*	Octreotide peptide 8 amino acids (1019 Da)	In vivo	[100]
	GCTE	Human growth hormone (hGH)	In vivo	[101]
	GCTE	Hepatitis B peptide drug Myrcludex B lipopeptide (5399 Da)	In vivo	[102]
	GCTE	Vancomycin	In vivo	[88]
	GCTE	Vancomycin	In vivo	[103]
	Synthetic tetraether lipids	Carboxyfluorescein (376 Da) hydrophilic	In vitro *drug release*	[104]
	Diether lipids from *Halorubrum tebenquichense*	^{99}Tc-DTPA	In vivo	[105]
	Diether lipids from *H. tebenquichense*	Superoxide dismutase (SOD)	In vitro Caco-2 cells	[107]
Inhalable	Diether lipids from *H. tebenquichense*	Dexamethasone phosphate	In vitro A549 and J774A.1 cells	[109, 110]
	Diether lipids from *H. tebenquichense*	Azithromycin	In vitro *Pseudomonas aeruginosa*	[111]
	Diether lipids from *H. tebenquichense*	Curcumin	In vitro A549 cells	[113]
	Tetraether lipids from *S. acidocaldarius*	Curcumin	In vitro A549 cells	[114]
Parenteral	Synthetic neutral and cationic tetraether lipids	pDNA	In vitro A549 cells	[115]
	Synthetic neutral tetraether lipids	pDNA	In vivo	[116]
	Synthetic diether and tetraether lipids-Peg 570-folic acid	pDNA	In vitro HeLa cells	[117]
	Synthetic Folic acid-Peg 5000-tetraether Peg 2000-tetraether	Peptide (17 amino acids, 2302 Da)	In vitro HeLa cells	[119]

(continued)

Table 1 (continued)

Route of administration	Source of archaeolipids	Drug	Assays	Reference
	Synthetic tetraether lipid (GMGTPC)	Cytarabine Vincristine Methotrexate	In vitro drug release	[120]
	GMGTPC derivative with 1 cyclohexane ring and 2 cholesterol (GcGTPC-CH)	Gemcitabine	In vitro drug release	[122]
	GcGTPC-CH derivative with 1 disulfuric linkage	Doxorubicin	In vitro drug release HeLa cells	[123]
	Tetraether lipids from *S. acidocaldarius*	Chlorin e6 Hydrophilic	In vitro Neuro-2a and SK-OV-3 cells CAM	[124]
	Tetraether lipids from *S. acidocaldarius*	Curcumin Hydrophobic	In vitro SK-OV-3 cell CAM	[125]
	Tetraether lipids from *S. acidocaldarius*	Hypericin Hydrophobic	In vitro SK-OV-3 cell CAM	[127]
	Tetraether lipids from *S. acidocaldarius*	Temoporfin Hydrophobic	In vitro SK-OV-3 cell CAM	[126]
	PLFE from *S. acidocaldarius*	Doxorubicin	MCF-7 breast cancer cells	[128]
	TPL from *Haloarcula 2TK2*	Isoniazid and rifampicin	In vitro drug release	[129]
	Diether lipids from *H. tebenquichense*	Alendronate	In vitro J774A.1 cells	[130]
Topical	Diether lipids from *Halobacterium salinarum*	Betamethasone dipropionate	Ex vivo pig skin	[131]
	Diether lipids from *H. salinarum*	Phenolic extract from olive mill waste	In vitro	[132]
	Tetraether lipids from *S. acidocaldarius*	Methylene blue	In vivo rat skin	[134]
	Diether lipids from *Aeropyrum pernix K1*	Calcein (fluorescent hydrophilic dye, 622 Da), listeriolysin (60 kDa), keratin 14, and plasmid DNA	In vitro HaCaT cells	[135]
	Diether lipids from *H. tebenquichense*	*Thymus vulgaris* essential oil	In vitro *Staphylococcus aureus*	[133]

Abbreviations: *CF* carboxyfluorescein, *DTPA* diethylene-triamine-pentaacetate, *GCTE* glyceryl-calditYl tetraether, *GMGTPC* glycerol monoalkyl glycerol tetraether lipids with phosphocholine head groups, *PLFE* polar lipid fraction E

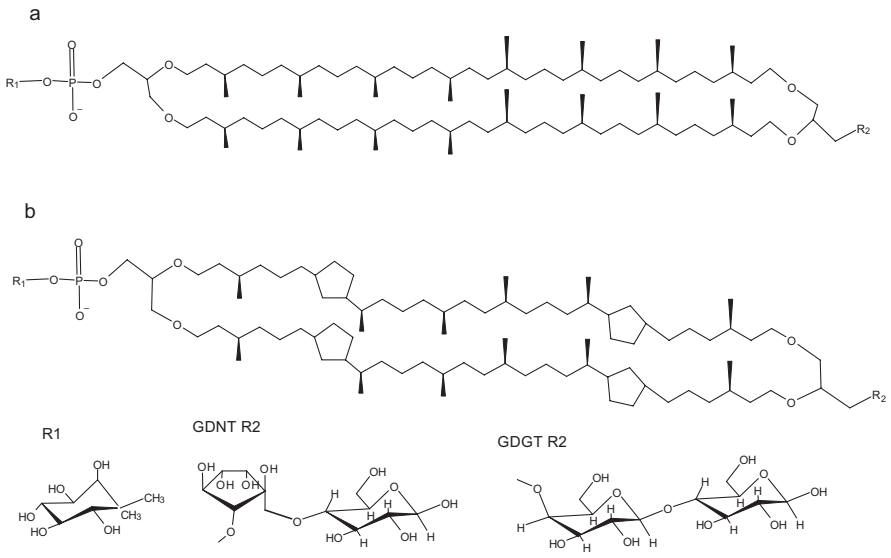

Fig. 7 Tetraether lipids in the polar fraction E (PLFE) of *S. acidocaldarius*. (**a**) Glycerol dialkyl glycerol tetraether (GDGT) (~10% of the extract) and (**b**) glycerol dialky calditol tetraether (GDNT) (~90% of the extract). In the hydrophobic region, PLFE lipids may contain 0–8 cyclopentene rings in each dibiphytanyl chain

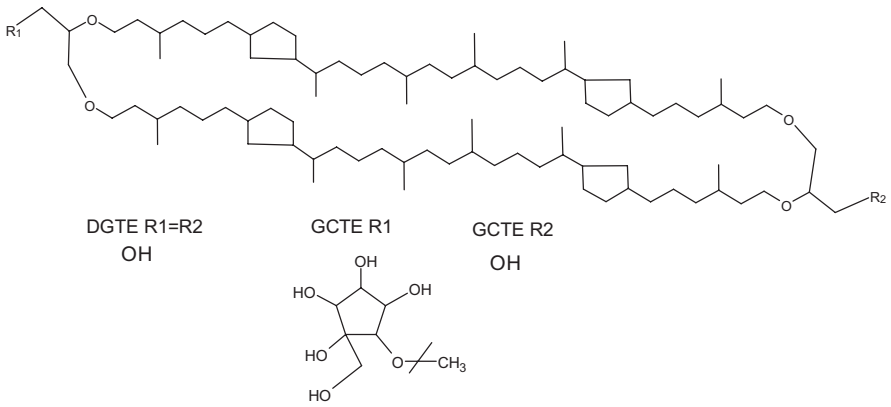

Fig. 8 Thermoacidophile archaeolipids glycerylcaldityl tetraether (GCTE) and diglyceryltetraether (DGTE) with an average number of four to six cyclopentyl rings

from *S. acidocaldarius* combined with variable amounts of dipalmitoylphosphatidylcholine (DPPC) ((up) DPPC/GCTE at 2:1 molar ratio), in order to increase its bioavailability [100]. Radiolabelled nanoarchaeosomes (0.75% ^{3}H-labelled DGTE added to EPC/GCTE at 4:1 molar ratio) were orally administered to rats, only a minor fraction of radioactivity (1.5–1.6%) was found in blood, while most of the radioactivity remained in the GI tract, suggesting a poor intestinal uptake of

nanovesicles or of radioactive label. In a subsequent study however, nanoarchaeosomes made of DPPC/GCTE at 3:1 molar ratio improved by 4.1-fold the oral bioavailability of octreotide compared with free octreotide. Authors hypothesized that nanoarchaeosomes protected octreotide along the GI tract, allowing its permeation into the blood after leak from enterocytes-adsorbed nanoarchaeosomes, with none or a minor fraction of intact nanoarchaeosomes being taken up. In line with these results, nanoarchaeosomes containing the bio-enhancer cetylpyridinium chloride (CpCl) and 0.9% mol GCTE (EPC/chol/GCTE/CpCl at 4:2.4:1:3.6 molar ratio) increased in 3.4% the bioavailability of human growth hormone, compared with s.c. administration. The bioavailability of orally administered free hormone on the other hand was only of 0.01% [101].

A substantial enhancement of liver uptake of I-^{131}-labelled hepatitis B peptide drug Myrcludex B after oral administration to rats of 3.5-fold increase compared to the free peptide was obtained upon loading in nanoarchaeosomes made of EPC/chol/GCTE at 8.5:1:0.5 molar ratio [102]. Opposite to Parmentier et al. [101] however, these authors reported that the inclusion of cetylpyridinium chloride in nanoarchaeosomes did not increase the oral bioavailability of Myrcludex B. In another study, the stability of the glycopeptide antibiotic vancomycin formulation loaded in the same nanoarchaeosomes was assessed, finding it more stable in simulated gastric and intestinal fluid, without significant changes in size and PDI along 5 h than in liposomes [88]. Blood concentration of vancomycin 1 h after vancomycin-loaded nanoarchaeosomes orally administered to rats was three- and twofold higher compared to free vancomycin and liposomal vancomycin, respectively. The increased oral bioavailability observed in this study, however, was considered not sufficiently high enough to grant a future clinical use [88]. Recently, Uhl et al. [103] modified the surface of nanoarchaeosomes with cell-penetrating peptides (CPP), aiming to increase its epithelial internalization and further vancomycin bioavailability. To that aim, cyclic arginine-rich CPP (known as R9K, more stable in simulated gastric and intestinal fluid than linear CPP)-phospholipid conjugates were synthetized and incorporated into nanoarchaeosomes (EPC/chol/GCTE/CPP conjugate at 8.4:1:0.5:0.1 molar ratio). Such CPP-nanoarchaeosomes rendered higher (in the order of fivefold) ^{125}I-vancomycin bioavailability compared to free, liposomal (EPC/chol at 9:1 molar ratio) vancomycin and nonconjugated nanoarchaeosome ^{125}I-vancomycin. The therapeutic efficacy of the formulation was assessed in a methicillin-resistant *Staphylococcus aureus* (MRSA) strain LAC* lux systemic infection mouse model. CPP-nanoarchaeosomes reduced the CFU in the kidneys of the infected mice in comparison to the negative control groups.

6.1.2 Nanoarchaeosomes Made of Synthetic Tetraether Lipids

Synthetic lipids allow a rigorous study of the effect caused by chain length, presence of rings, nature of polar heads, and conjugation with different molecules such as polyethylene glycol, folic acid, or cholesterol (see below) on the structure and performance of nanoarchaeosomes. Benvegnu et al. [104] synthesized analogues of

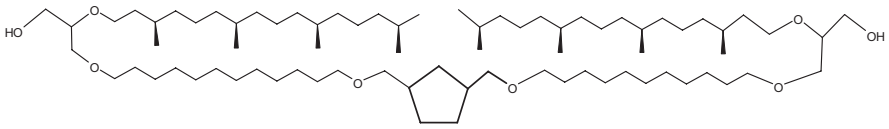

Fig. 9 Structure of a generic synthetic tetraether lipid. On this structural base, lipids with different polar head groups were synthesized including lactose, phosphatidylcholine, pegylated, and folic acid-pegylated

asymmetric tetraethers from thermoacidophile archaea, having one cyclopentane ring and different polar heads, and prepared pure or mixed nanoarchaeosomes by combining archaeolipids with EPC (Fig. 9). This work revealed that the polar heads were relevant to provide stability to the nanoarchaeosomes in terms of release of the low molecular weight fluorescent dye carboxyfluorescein. The presence of hydroxyl or lactosyl groups increased the membrane stability in the presence of detergents and serum lipoproteins, whereas the presence of phosphocholine head groups at both sides of the tetraether monolayer provided membrane integrity at low pH that resulted comparable to that of nanoarchaeosomes made of tetraether lipids extracted from *Thermoplasma acidophilum.*

6.1.3 Nanoarchaeosomes Made of Halophilic Diether Lipids

Nanoarchaeosomes made of diether lipids extracted from *Halorubrum tebenquichense* (Fig. 6) were first reported to be more extensively captured by M-like cells, compared with liposomes [105]. The uptake by M cells led to hypothesize that upon oral administration, nanoarchaeosomes could be transcytosed across the epithelia, to the lymphatic. When orally administered to rats, 22.3% (3.5-fold higher than liposomes) radiopharmaceutical ^{99m}Tc-DTPA loaded in nanoarchaeosomes was shuttled to the blood circulation. This study showed that not only tetraethers [106] but also diether lipids could be used to design nanoarchaeosomes to protect the structure of orally administered molecules. In a subsequent approach, the enzyme superoxide dismutase (SOD) loaded into nanoarchaeosomes made of diether lipids from *H. tebenquichense* was observed to retain its antioxidant and anti-inflammatory activity upon digestion in simulated gastrointestinal fluids and after 5 months of storage; in contrast, the activity of liposomal SOD (hydrogenated soybean phosphatidylcholine (HSPC)/chol at 3:2 molar ratio) was lost upon preparation, gastrointestinal digestion, and storage [107]. The colloidal stability of SOD in nanoarchaeosomes was higher at low pH and in presence of sodium cholate than that of liposomal SOD, retaining enzymatic activity upon 1 h incubation at pH 1.2.

6.2 *Inhaled Drug Delivery*

The use of nanoarchaeosomes for inhaled drug delivery is recent and relies upon their superior structural stability to the shear stress during nebulization and subsequent retention and protection of loaded drugs.

6.2.1 Nanoarchaeosomes Made of Halophilic Diether Lipids

The diether lipids from *H. tebenquichense* are rich in archaeol PGP-Me [108], which was shown to be a ligand for scavenger receptor A1 (SRA1) [109]. The SRA1 is highly expressed by macrophages and certain vascular endothelial cells and is responsible for the extensive internalization of *H. tebenquichense* nanoarchaeosomes. Such nanoarchaeosomes, thus, are naturally targeted to SRA1-expressing cells.

The first work in this field was done to deliver phosphate dexamethasone within pH-sensitive nanoarchaeosomes to the alveoli [109]. The pH-sensitive nanoarchaeosomes (*H. tebenquichense* diether lipids (Fig. 4)/dioleylphosphatidylethanolamine (DOPE)/cholesteryl hemisuccinate at 2.8:4.2:3 w:w) became more stable during storage and nebulization employing a NE-U22 vibrating mesh Omron nebulizer, than ordinary pH-sensitive liposomes. Nebulized on phagocytic cells stimulated with lipopolysaccharides (LPS), phosphate dexamethasone within pH-sensitive nanoarchaeosomes efficiently suppressed the production of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). Nanoarchaeosomes crossed the lung surfactant laying on the cell surface without inducing inactivation and upon endocytosis acceded to cell cytoplasm delivering higher amount of cargo than pH-sensitive liposomes [110]. The archaeolipids, thus, may be used to design structurally stable nanoparticles to provide efficient intracellular drug delivery, without damaging the compression-expansion dynamics of the surfactant.

The preparation of azithromycin solutions requires the addition of high salt concentrations, making difficult its efficient nebulization. In another approach, nanoarchaeosomes made of diether lipids from *H. tebenquichense* and loaded with the antibiotic azithromycin were observed to display lower MIC and minimal bactericidal concentration (MBC), higher disruptive activity on preformed biofilm, and higher anti-*Pseudomonas aeruginosa* (PAO1) activity than free azithromycin, without cytotoxicity on adenocarcinomic human alveolar basal epithelial cells (A549 cells) and human THP-1-derived macrophages [111]. Azithromycin was strongly trapped into the nanoarchaeosomes bilayer, by a combination of electrostatic attraction and mechanical immobilization by perpendicular methyl groups from polyisoprenoids. Such interaction was key to retain the azithromycin during storage and endure shear forces of nebulization.

The use of curcumin as therapeutic agent is limited by its poor aqueous solubility and by being prone to degradation [112]. To address these issues, a recent work showed that nanoarchaeosomes made of diether lipids from *H. tebenquichense*/Tween 80 at 1:0.4 w:w was efficiently trapped, increased aqueous solubility, and

retained curcumin against dilution, storage, and nebulization with vibrating mesh nebulizer. This time, the trapping of curcumin within the polyisoprenoid chains required the addition of Tween 80. Nebulized on an inflamed air-liquid interface of A549 cells, curcumin loaded in nanoarchaeosomes increased transepithelial electrical resistance (TEER), normalized the permeation of the low molecular weight marker Lucifer yellow, and decreased IL-6, TNF-α, and IL-8 levels, suggesting a promising use in epithelia reparation upon inflammatory damage [113].

6.2.2 Nanoarchaeosomes Made of Thermoacidophile Tetraether Lipids

Curcumin loaded into nanoarchaeosomes made of *S. acidocaldarius* tetraether lipids/DPPC at 1:9 molar ratio was used for photodynamic therapy (PDT) against lung cancer. Nanoarchaeosomes provided higher encapsulation efficiency and loading capacity of curcumin than liposomes made of DPPC/chol at 7:3 molar ratio or DPPC/DOPE at 7.5:2.5 molar ratio. As stated above, the curcumin stabilization in the archaeolipid membrane is a determinant factor during nebulization stress. After nebulized with a vibrating mesh nebulizer (PARI VELOX®, PARI GmbH, Starnberg, Germany), nanoarchaeosomes and DPPC/chol liposomes retained polydispersity index (PDI), z potential, and loading capacity, while fluid liposomes of DPPC/DOPE did not. In vitro irradiation experiments on A549 cells revealed an excellent cytotoxic response to both nebulized nanoarchaeosomes and liposomes, all showing significant differences between dark control and irradiated nebulized cells [114]. Nebulized curcumin-loaded nanoarchaeosomes can be considered as a promising approach for photodynamic therapy against lung cancer.

6.3 Parenteral Drug Delivery

To date, the parenteral route of administration of nanoarchaeosomes remains relatively unexplored. Most works in the field are focused on gene, drug delivery, or PDT, but their activities are shown in vitro only.

6.3.1 Nanoarchaeosomes Made of Synthetic Tetraether Lipids for Gene Delivery

Neutral and cationic archaeal tetraether-like analogues having a central cyclopentane ring were combined with bilayer-forming cationic aliphatic lipid glycine betaine derivative MM18 or synthetic cationic tetraether lipids with DOPE for in vitro gene transfection of A549 cells [115]. The nanoarchaeosome made of MM18/neutral archaeal tetraether-like lipid at 95.5 w/w was found to be as efficient as the widely used reagent Lipofectamine (at +8 charge ratio). In a further work, the in vivo transfection efficacy of complexes formed between a synthetic neutral

tetraether-like lipid with different cationic lipids and pDNA for luciferase was found to depend on factors such as the cationic lipid, the lipid/co-lipid molar ratio, and the administration route (intravenous or intranasal) [116]. Intravenously administered MM18/neutral archaeal tetraether-like lipid at 10:1 molar ratio provided efficient in vivo gene transfection, especially into the lungs, with some bioluminescence in the spleen. Such ratio seems to be ideal to ensure complex stability in plasma and to be able to disassemble, especially in endosomes, with subsequent release of pDNA. Besides, the complexes were also effective upon the addition of PE-Peg 500, after intranasal instillation.

Aiming to target tumoral cells overexpressing the folate receptor, there were synthesized diether and tetraether lipids, functionalized with a short poly(ethylene glycol) chain (Peg 570) and a folate group. These lipids, combined with conventional bilayer-forming glycine betaine-based cationic lipid at 5–10 w %, displayed significant transfection activity on HeLa cells that was inhibited in the presence of free folic acid. The diether derivative exhibited a very high transfection efficiency at neutral charge ratio that was much higher than that of Lipofectamine [117].

6.3.2 Nanoarchaeosomes Made of Synthetic Tetraether Lipids for Delivery of Antitumoral Drugs

Aiming to increase the circulation lifetime of the antitumoral peptide A1, it was loaded into nanoarchaeosomes made of synthetic tetraethers and functionalized with Peg 2000 linked to folic acid (EPC/Peg 2000-tetraether lipid (synthesized by Barbeau et al. [118])/folic acid-Peg 5000-tetraether lipid at 90:5:5 w:w) and its efficacy tested on HeLa cells, known to overexpress the folate receptor. The activity of the free peptide 1 h later was found to be higher than if loaded within nanoarchaeosomes. Upon increasing the incubation time, void nanoarchaeosomes or nanoarchaeosomes loaded with a non-active peptide induced comparable or of higher cytotoxicity that the A1 peptide loaded within nanoarchaeosomes [119]. The authors hypothesize that in vivo, the A1 peptide will be protected by nanoarchaeosomes.

More recently, cytarabine and vincristine were loaded within nanoarchaeosomes made of synthetic glycerol monoalkyl glycerol tetraether lipids, linked to phosphocholine head groups (GMGTPC) (Fig. 10). The nanoarchaeosomes retained these drugs more pronouncedly (up to ~ninefold decrease in drug leakage rate) compared to liposomes made of EPC. When loaded with methotrexate however, no appreciable differences in drug leakage were observed between nanoarchaeosomes or liposomes

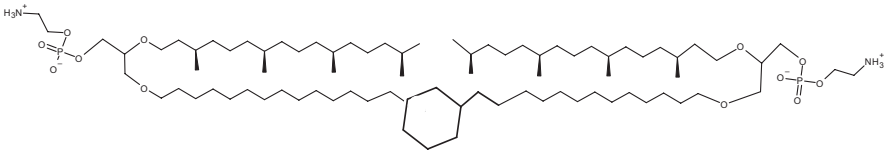

Fig. 10 Structure of the synthetic archaeolipid GMGTPC-CH

[120]. The addition of a cyclohexane ring to the isopranyl chain (GMGTPC-CH) is reported to form stable monolayers at room temperature with reduced leakage compared to EPC liposomes [121]. Nanoarchaeosomes prepared with a derivative of GMGTPC-CH by linking two cholesterols to the polyisoprenoid chains (GcGTPC-CH) [122] reduced by 30- and 10-fold the release of gemcitabine, compared to liposomes (palmitoyloleoylglycerophosphocholine (POPC)/chol at 6:4 molar ratio) and nanoarchaeosomes made of GMGTPC/chol at 6:4 molar ratio. These nanoarchaeosomes were stable in plasma, retaining 80% of loaded carboxyfluorescein along 5 days. Aiming to increase the nanoarchaeosomes stability in circulation, retaining doxorubicin but at the same time providing its intracellular release, a new derivative was synthesized, containing a disulfide linker near the polar head groups of the GcGTPC-CH lipid [123]. These nanoarchaeosomes showed an inhibitory concentration 50 (IC50) value similar to free doxorubicin and ~4- and 20-fold lower IC50 than nanoarchaeosomes without disulfide groups and Doxil, respectively, on HeLa cells.

6.3.3 Nanoarchaeosomes Made of Thermoacidophile Tetraether Lipids for Photodynamic Therapy

Different photosensitizers were loaded into nanoarchaeosomes made of tetraether lipids extracted from *S. acidocaldarius*. In a first work, the water-soluble photosensitizer chlorin e6 (Ce6) loaded into nanoarchaeosomes (tetraether lipids/dioleoyltrimethylammonium propane (DOTAP)/DPPC at 1.8:37.2:60.9 molar ratio) was reported to display phototoxic activity both in neuroblastoma (Neuro-2a cells) and in ovarian cell carcinoma (SK-OV-3) cells. The chick chorioallantoic membrane (CAM) model showed effective localized vascular destruction, without damaging the nonirradiated zones [124].

Curcumin loaded into nanoarchaeosomes made of tetraether lipids extracted from *S. acidocaldarius* at tetraether lipids/DSPC at 1:9 molar ratio resulted to be hemocompatible, with a coagulation time less than 50 s and a hemolytic potential below 40%. Their phototoxicity on SK-OV-3 ovarian carcinoma cells, however, was lower than that of liposomal curcumin (distearoylphosphatidylcholine (DSPC)/distearoylphosphatidylglycerol (DSPG) at 8:2 molar ratio). The authors explained that the tight trapping of curcumin within nanoarchaeosome monolayer made the release of curcumin difficult and thereby reduced its activity. Both curcumin-loaded nanoarchaeosomes and liposomes induced a complete destruction of the blood vessels at the site of irradiation, observed in a CAM model [125]. Comparable results were achieved with the second-generation photosensitizer temoporfin loaded into nanoarchaeosomes of composition identical to Duse (2017) that induced lower phototoxicity on SK-OV-3 carcinoma cells than liposomal (DPPC/chol at 9:1 molar ratio and DPPC/DPPE-Peg 5000 at 9.5:0.5 molar ratio) curcumin [126]. The highest reduction of IC50 was induced by the pegylated liposomes, with highest amount of reactive oxygen species (ROS) generated and blood vessel destruction in the CAM model at the irradiated area, without harming the developing embryo.

Nanoarchaeosomes of identical composition to Duse et al. [125] were loaded with hypericin or a hypericin-hydroxypropyl-β-cyclodextrin inclusion complex [127]. Nanoarchaeosomes showed to be hematocompatibles (hemolytic potential less than 20% and a coagulation time less than 50 s), whereas both formulations induced photocytotoxicity on SK-OV-3 cells in a therapeutic dosage range, and their effect on the CAM model was different: nanoarchaeosomes loaded with hypericin induced a substantial destruction of the microvasculature, while nanoarchaeosomes loaded with drug-in-cyclodextrin did not induce any effect. The authors suggest that nanoarchaeosomes loaded with hypericin would be suited for vascular targeting while hypericin-cyclodextrin-loaded nanoarchaeosomes could deliver the photosensitizer to the tumor site.

6.3.4 Nanoarchaeosomes Made of Thermoacidophile Tetraether Lipids Form Delivery of Antitumoral Drugs

Nanoarchaeosomes made of polar lipid fraction E (PLFE) isolated from *S. acidocaldarius* and DPPC at PLFE/DPPC at 3:7 molar ratio were found to be temperature-sensitive, experiencing an abrupt increase of the z potential when transitioning from 37 °C (−48 mV) to 44 °C (−16 mV) [128]. Authors hypothesize this is owed to the induction of DPPC domain melting and "flip-flop" of tetraether lipids. Loaded with doxorubicin, its release is induced when the temperature is raised from 37 to 42 °C, independently of the presence of serum proteins. A 15-min preincubation of doxorubicin loaded into nanoarchaeosomes with MCF-7 breast cancer cells at 42 °C caused a significant increase in the amount of doxorubicin entering the nuclei and an increase of cytotoxicity than at 37 °C. These results suggest that thermosensitive nanoarchaeosomes could be used for mild hyperthermia treatment of tumors triggering the drug release as temperature rises.

6.3.5 Nanoarchaeosomes Made of Halophilic Diether Lipids

The antituberculosis drugs rifampicin and isoniazid were loaded into nanoarchaeosomes made of diether lipids extracted from *Haloarcula 2TK2* strain at higher ratios than in liposomes (PC/chol at 2:1 molar ratio) and were released with different profiles [129].

The nitrogenate bisphosphonate alendronate was recently loaded within nanoarcheosomes made of diether lipids from *H. tebenquichense*. Such formulations extensively interacted with serum proteins but resulted refractory to phospholipases and constituted a better macrophage-targeted apoptotic inducers than alendronate in liposomes made of HSPC/chol at 7.5:2.5 w:w [130]. The addition of cholesterol to nanoarchaeosomes at diether lipids/chol 7:3 w:w decreased their cytotoxicity, making them pronouncedly anti-inflammatory on J774.1 cells, strongly reducing the production of reactive oxygen species (ROS) and IL-6 induced by LPS. Trapped in

nanoarchaeosomes, the alendronate pharmacodynamics was modified, potentially constituting a tool to target it to tissues other than the bone.

6.4 Topical Drug Delivery

The use of nanoarchaeosomes for topical drug delivery has been poorly addressed. A few manuscripts published nearly 10 years ago report the performance of tough nanoarchaeosomes used for drug delivery to the skin.

6.4.1 Nanoarchaeosomes Made of Halophilic Diether Lipids

The hydrophobic and moderate to potent glucocorticoid steroid betamethasone dipropionate loaded into nanoarchaeosomes made of diether lipids from the halophilic *Halobacterium salinarum* (diether lipids/chol at 15:2 w:w) was reported to achieve a slightly higher penetration and accumulation in pig epidermis, than in soybean phosphatidylcholine (SPC)/chol at 15:2 w:w [131]. In a subsequent work, these nanoarchaeosomes showed to be better suited than liposomes for topical delivery of natural antioxidant phenolic compounds recovered from olive mill waste, in terms of stability, percentage of loading, antioxidant activity, and feasibility of incorporation into gels [132].

Bacterial infections of the skin are highly common and involve mostly Gram-positive bacteria; those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) comprise nearly 50% of the isolates from *S. aureus*. In such context, Perez et al. [133] showed that *Thymus vulgaris* essential oil loaded into nanoarchaeosomes (*H. tebenquichense* diether lipids/SPC/Tween 80/*Thymus vulgaris* essential oil at 0.5:0.5:0.75:0.7 w:w) displayed better antibacterial activity (lower minimal inhibitory concentration 90 (MIC90) against planktonic *S. aureus* ATCC 25923 and four MRSA clinical strains, higher antibiofilm formation capacity) and stability during storage, than *Thymus vulgaris* essential oil in Tween 80 emulsions and in liposomes (SPC/Tween 80/*Thymus vulgaris* essential oil at 1:0.75:0.3 w:w). Besides essential oils being currently accepted as food additive, their loading in nanoarchaeosomes opened new avenues to explore their performance as intracellular antimicrobials.

6.4.2 Nanoarchaeosomes Made of Thermoacidophile Tetraether and Thermophilic Diether Lipids

Nanoarchaeosomes made of tetraether lipids extracted from *S. acidocaldarius* were shown to increase the permeability of loaded methylene blue across rat skin, compared with free methylene blue. The report, however, did not compare the permeability with that of ultradeformable liposomes, specially designed to increase the skin penetration [134]. On the other hand, nanoarchaeosomes made of diether lipids

extracted from hyperthermophile *Aeropyrum pernix K1* were shown to provide intracellular delivery of small molecules, small and large proteins, and plasmid DNA, into epithelial cells grown in culture (HaCaT cells), without noticeable cytotoxicity up to 500 μg/ml [135].

7 Preparation Methods of Solid Archaeolipid Nanoparticles and Nanostructured Lipid Carriers

Diether and tetraether archaeolipids as well as neutral lipids can be used to prepare solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), the polar lipids in the outer shell and the neutral within the hydrophobic core of the nanoparticles. Briefly, SLN are particles containing a core made of safe and biodegradable lipids which are solid at body temperature, whereas the core of NLC is a combination of solid and liquid lipids; both are covered by a shell of surfactants and cosurfactants. The NLC are mainly used to load hydrophobic drugs, but hydrophilic drugs, large biomacromolecules (polysaccharides, etc.), genetic material (DNA/siRNA), and vaccine antigens are also admitted as cargo [136]. The preparation methods of SLN and NLC have been recently reviewed by Duan et al. [137]. The nanoparticles described below were prepared by the emulsion-ultrasonication method.

8 Solid Archaeolipid Nanoparticles and Nanostructured Lipid Carriers in Drug Delivery

Two recent works employed diethers from halophilic archaea as part of the structure of SLN and NLC. The first of them loaded dexamethasone into SLN made of Compritol/*H. tebenquichense* diether lipids/SPC/Tween 80 at 4:0.9:0.3:3% w/w for oral targeted delivery to macrophages of inflamed mucosa [138]. The diethers-containing SLN strongly reduced the levels of TNF-α, IL-6, and IL-12 on J774A1 cells stimulated with LPS as compared with free dexamethasone or dexamethasone loaded in SLN without diether lipids. After in vitro digestion, the anti-inflammatory activity of diethers-containing SLN was retained, while that of dexamethasone loaded in SLN without diether lipids was lost. In the following, a core was made of bacterioruberin, Compritol, and dexamethasone and covered with *H. tebenquichense* diether lipids and Tween 80 (2:2:1.2:3% w/w) [139]. These NLC were extensively captured by macrophages and Caco-2 cells and showed high anti-inflammatory (reduced 65% and 55% TNF-α and IL-8 release) and antioxidant activities (reduced by 60% ROS production) on a gut inflammation model made of Caco-2 cells and LPS-stimulated THP-1-derived macrophages. The NLC reversed the morphological changes induced by inflammation and increased the transepithelial electrical resistance, partly

reconstituting the barrier function. The activity of bacterioruberin and dexamethasone in NLC was partially protected after simulated gastrointestinal digestion.

9 Conclusions

Today, the therapeutic potentialities of nanoarchaeosomes in drug delivery remain confined to the preclinical field. The production of their building blocks, the natural polar archaeolipids, is still in the B-TRL 3, being manufactured in very low amounts compared to ordinary phospholipids. A successful scale-up of nanomedicines requires nanostructures of maximal simplicity and reduced preparation steps. The use of archaeolipids offers the opportunity for a rational design of nanomedicines to better deal with such critical aspects.

For instance, one typical issue of liposomal nanomedicines is their high lability. Their production methods and storage conditions must be tailored in order to maximize their preservation as longest as possible [140]. Nanoarchaeosomes instead exhibit higher structural strength against physical, chemical, and enzymatic attacks than liposomes. Other properties, such as the ability to remain in liquid crystalline phase at low temperatures, are partly responsible for their increased shelf life. The avoidance of phase transition (which leads to membrane discontinuities), together with the fully saturated isoprenoid chains, makes nanoarchaeosomes less sensitive to temperature fluctuations that may induce loss of inner cargo, or oxidation. Their manipulation can be performed at room temperature, eventually making their industrial production easier.

Another challenge that makes difficult the industrial nanomedicines to scale up is the obtention of reproducible batches of nanoparticles derivatized with surface ligands exhibiting complex nanostructures. Nanoarchaeosomes offer a collection of different anionic and oligosaccharide head groups that vary according to the archaea gender and species. Selected head groups thus can be combined to provide surfaces exhibiting ligands of specific interest, reducing the need for derivatization. In other words, some nanoarchaeosomes are naturally targeted, being their structure simpler than that of artificially derivatized liposomes, a critical point that may simplify their industrial scale-up [141]. Despite its significance, however, the search for receptors for different head groups is still poorly explored.

The hydrophobic polyisoprenoid core from nanoarchaeosomes is a disorganized but much more viscous environment, of lower lateral mobility, than the liner acyl chains of liposomes. To increase the acyl chain viscosity and reduce the lateral viscosity of liposomes, artificially hydrogenated lipids and cholesterol must be added. Remarkably, the methyl groups perpendicular to the polyisoprenoid chains from nanoarchaeosomes may tightly trap low molecular weight drugs, which are difficult to dissolve in ordinary organic solvents. Such ability aids to simplify their membrane composition and/or avoid the use of high ionic strength or extreme pHs to dissolve the cargo.

Overall, the experimental evidence gathered so far shows that nanoarchaeosomas may well be used to design nanomedicines for drug delivery with abilities equal or superior to those from liposomes. Besides, the industrial fabrication of nanoarchaeosomes-based nanomedicines would not differ or, as discussed earlier, is expected to be less challenging than that of liposomes. Nanoarchaeosomes, however, should transit an uncertain pathway before gaining the acceptance of pharmaceutical regulatory organisms. Different from liposomes, critical aspects of their in vivo performance, such as pharmacodynamics and toxicology, that will vary according to the type of archaeolipid, source, and manufacture process remain poorly explored by the academic community.

Recent works show that, different from liposomes, upon being extensively endocytosed, *H. tebenquichense* nanoarchaeosomes reduce the mitochondrial membrane potential and would induce autophagy in macrophages [130]. A recently discovered aspect is that such nanoarchaeosomes display anti-inflammatory activity on human vascular endothelia activated by TLR 2 and 4 agonists, probably owed to changes induced in cell membrane fluidity. The additional ability to reduce the release of von Willebrand factor suggests that in vivo these nanoarchaeosomes may display antithrombotic activity [142]. The pharmacological activity of nanoarchaeosomes, thus, is emerging as another feature that, together with their structure, differentiates them from liposomes. Moreover, the effects of nanoarchaeosomes on clinically relevant models are, for the moment, completely unknown.

As described above, synthetic archaeolipids are so far prepared mainly ad hoc at lab scale. In such sense, neither egg-derived (require additional testing for viral contamination) nor bovine-derived ordinary phospholipids are suitable for clinical applications, due to stability problems and the possibility of viral or protein (bovine spongiform encephalopathy (BSE)) contamination. Besides, the quality control of synthetic products may be complicated by the presence of stereochemical impurities, as occurs with semisynthetic lipids prepared from glycerol or glycero-3-phosphocholine (GPC) derived from a plant or animal source [143]. In case of nanomedicines, this magnifies the difficulties of preparing reproducible batches [144]. The problem of the origin or the enantiomeric impurities is absent for natural archaeolipids. On the other hand, reproducibly harvesting massive quantities of specific lipid mixtures from cultured archaeal species can be challenging, especially from thermoacidophiles that require high temperatures (80–100 °C) and low pH (2–3). The consistence and reproducibility of the lipid mixtures must be granted, by controlling the growth parameters in a fermenter environment. The extraction/purification of the polar lipids needs to be scaled up to obtain a reproducible product of defined composition, with the use of a minimum volume of solvents. To that aim, methodologies that do not require or minimize the use of solvents, such as the supercritical fluids, can be used.

Overall, the popularization of the abilities of the diverse types of nanoarchaeosomes through the academic community, together with the aid of biotechnology and of molecular biology tools to increase the speed of biomass growth and product yield, will be critical to fill the main gaps toward translation: their suboptimal availability and their poorly known biocompatibility.

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Bioengineered Metallic Nanomaterials for Nanoscale Drug Delivery Systems

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1 Introduction

Drug delivery systems (DDSs) are known as effective carriers for active pharmaceutical ingredients (APIs), which are responsible for providing therapeutic response, targeting, stability, and safety. The pharmacokinetic behavior of APIs could be manipulated by formulating various DDSs [1].

Conventional DDSs (CDDSs) have been mostly formulated as oral dosage forms with immediate-release profiles which lead to multiple dosing. The risk of fluctuation would be high, and maintenance of plasma concentration in the range of therapeutic window is one of the challenges against the administration. Therefore, controlled DDSs as one of the branches of novel DDSs were developed [2]. Other limitations of conventional dosage forms include poor patient compliance, risk of

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missing dose, poor absorption and bioavailability, targeting, premature metabolism, and elimination [3, 4].

Generally, the novel DDSs could be classified into three major categories including macroscale, microscale, and nanoscale. The formulation of macroscale DDSs was initiated by utilizing nonbiodegradable polymers like polyurethane or silicone rubber [5]. Norplant® [6] and Progestasert® as contraceptives [7], Ocusert® in glaucoma therapy [8], Viadur® implant for prostate tumors [9], and Geo-Matrix-based tablets loaded with paroxetine, ropinirole, or diltiazem [10–12] have been approved by FDA and belong to this category. Microscale DDSs evolution was triggered by the study of Robert Langer and Judah Folkman in 1970, which formulated the controlled DDSs of macromolecules by using hydrophobic polymers [13]. Polymers like PLA, PLGA, polycyanoacrylate, and polyanhydrides have been utilized for microscale DDSs [14]. A wide range of different molecules could be loaded in microstructures. Nafarelin as a contraceptive, LHRH, triptorelin for prostate cancer, and even atenolol for hypertension are some representative drugs that were formulated in microparticles [15, 16]. The difficulty of formulating monodisperse microparticles is one of the limiting obstacles against microscale DDSs [17]. Nanoscale DDSs with higher encapsulation efficiency, smaller size, the potential for target therapy, higher bioavailability, and manipulated release kinetic have been introduced as efficient DDSs [18]. As nanomaterials are known as materials with particle sizes under 100 nm, they bear specific physicochemical, electromagnetic, mechanical, optical, and biological characteristics [19, 20]. Nanomaterials are in various sizes, appearances, constructions, and sources. The reported shapes are spherical, conical, rod, spiral, tubular, porous, etc. They could be organic, inorganic, carbonic, or composite [21]. Nanodiamonds, carbon nanotubes, fullerene, and graphene are some examples of carbonic nanomaterials [22]. Inorganic nanomaterials are consisting of metals and metal oxide nanostructures. Silver, iron, gold, palladium, copper, and zinc are some examples of this group [23]. Organic nanomaterials could be originated from nature or could be synthesized. Liposomes, polymersomes, and chitosan-based nanoparticles (NPs) are some typical examples of this group. Apart from the components, one of the main differences between organic NPs and inorganic NPs is the basis of the fabrication process. The reduction of ions and precipitation of inorganic salts leads to the production of inorganic NPs. The covalent or metallic binding is responsible for making a 3D structure of inorganic NPs. However, the 3D structure of organic NPs is formed by the self-assembling or chemical binding of organic molecules [24]. The composite nanomaterials as the last group are materials that were incorporated with any kinds of nanomaterials including metallic, carbonic NPs, or even organic nanomaterials [25, 26]. The advantages of inorganic nanomaterials compared with other groups include their specific electrical, magnetic, optical, and colloidal properties [27]. The physicochemical characteristics of metal NPs (MNPs) are related to their shapes and sizes which makes them desirable to be utilized as novel drug delivery systems. The attitude of the researches has been oriented to develop economical, environmentally safe, and stable MNPs [28]. The applied methods for synthesis of MNPs have been categorized in three main groups as physical, chemical, and biological methods. Each of these methods

has been considered based on the provided conditions and utilized resources. Biological methods use natural origins as reducing agents for production of MNPs from metal ions. Green synthesis which is known as the mentioned biological methods has been recently noted in most of the researches as green nanotechnology. This preference is originated from advantages like nontoxic condition, stability, and monodispersity of MNPs and cost-benefit procedures [29]. Evaporation-condensation, laser ablation, thermolysis, and spluttering are some representatives of physical approaches. Chemical methods include UV-initiated photoreduction, sono-electrochemistry, microemulsion techniques, electrochemical synthesis method, irradiation methods, solgel method, and hydrothermal synthesis. Finally, biological methods are consisting of utilizing plant, microbial, and marine sources [30, 31]. In this chapter, various kinds of nanomaterials would be explained and classified based on their application as DDSs. The metal-based nanomaterials are fully discussed as DDSs with emphasis on biogenic fabrication of MNPs. The various resources for biofabrication of MNPs are separately provided and explained. Next, recent advances in application of biogenic metal-based nanomaterials for drug delivery systems would be presented, and it ends with a conclusion with a future outlook.

2 Nanomaterials for Drug Delivery Systems: An Overview

Nanometer scale has exhibited unique physicochemical and biological properties in materials and pharmaceutical sciences. The extraordinary characteristics which have been provided by nanotechnology lead to an improvement of the functions of materials by manipulation at the levels of atoms and molecules [32, 33]. The combination of nanotechnology and materials sciences with a biological approach has provided a great opportunity in medicine including diagnosing, drug delivery, gene delivery, bioimaging, etc. DDSs have been developed for prevention, management, and therapy. Increasing therapeutic efficacy, drug targeting, and decreasing the side effects are the main goals in this field [34].

It could be expressed that the revolution of DDSs was initiated by the entrance of nanomaterials which have been making a significant improvement in the efficacy of the DDSs [35]. Nanotechnology has provided an opportunity for biodegradable and biocompatible materials to be optimized based on physicochemical parameters and formulate more effective carriers for drugs which could bring better pharmacokinetic properties and delivery to cells. Formulating biosimilar and biocompatible DDSs could lead to improved cell attachment, entrance, and drug release at the site [36]. Nanotechnology could also manipulate the sensitivity of DDSs to various factors which control the release of drug by changing pH, magnetic field, temperature, ultrasonic waves, and other stimulants [37]. Nano drug delivery systems (NDDSs) could inhibit some physiological obstacles to the administration of conventional DDSs like rapid plasma elimination, high immunogenicity, lack of stability, low therapeutic activity, and vital adverse effects [38, 39]. NDDSs have the opportunity

of delivering the drug molecules straightly to the cells in contrast to CDDSs which only target the tissue [40]. NDDSs could improve drug delivery by active and passive targeting and also improve the duration of drug residence in plasma and decrease toxic side effects [41, 42].

The prominent advantages of NDDSs compared with CDDSs include enhanced residence time in plasma, improved permeation to tissues and retention in organs, better cellular attachment and uptake, increased therapeutic effectiveness by target delivery, and prolonged administration intervals by sustaining drug release [43]. There are critical parameters that should be considered for developing efficient NDDSs like hydrophilicity of the structure, distribution, biocompatibility, biodegradability, release profile, entrapment, size, and shape [44]. NDDSs could be formulated for various routes of administration like oral, parenteral, topical, transdermal, ocular, etc. [45]. Some NPs could act as a contrast agent and would be used as a bioimaging agent for tumor cell diagnosis. Iron oxide NPs have been widely utilized for tumor detection because of their rapid and long effectiveness [46]. Some nanomaterials demonstrate a mucoadhesive property which provides a remarkable depot of the drug at the targets. NPs as drug carriers also have shown an improved bioavailability as ocular DDSs [47]. Achieving efficient and safe DDSs to CNS has been reached by NDDSs. The characteristics of NPs have provided this opportunity to be able to cross the blood-brain barrier [48]. The new studies on cancers also revealed that NDDSs play an impressive role in novel chemotherapy management. The therapeutic results of applying CDDSs in chemotherapy protocols showed that only a small proportion of chemotherapeutic agents would be delivered to the tumor tissue which refers to general biodistribution without any cellular selectivity, while NDDSs could provide a better concentration in the cancerous cells based on the EPR theory and also reduce the toxic effect of the agents on normal cells. Moreover, they could have a slower plasma elimination which enhances the residence time of the drug in blood circulation. In addition to passive targeting, the NDDSs could be engineered to be utilized for active targeting of the tumor cells. The explained properties of NDDSs could help the physicians to overcome the current multiresistance of chemotherapies [49]. Therefore, the nanoscale size, less side effects, release sustainability, and pharmacokinetic adjustment are main responsible factors for unique effectiveness of NDDSs [50].

Based on the definition of the International Organization for Standardization (ISO), nanomaterials are materials with at least one dimension on the scale of a nanometer. The nanoscale is defined as 1–100 nm [51]. The European Commission also adds more details and notices no limitation on the structures like unbounded, agglomerated, or even aggregated particles [52]. Generally, nanomaterials are classified based on dimension, size, shape, composition, and source. The priority of each categorization is related to its beneficial capability to predict the special properties of nanomaterials. Dimensionally, nanomaterials with all three dimensions in the scale of nanometers belong to zero-dimensional classification. Quantum dots are examples of this group. One-dimensional nanomaterials like nanofibers have two dimensions on the nanometer scale. Two-dimension nanomaterials bear one dimension in the scale of nanometers. Graphene which has a sheet structure with a

thin layer of atoms at the scale of a nanometer is a 2D nanomaterial. 3D nanomaterials do not externally have any dimensions in nanometers and are named bulk nanomaterials. However, they consist of nanostructures. Box-shaped graphene could be mentioned as an example of this group [53]. Considering the shapes, they could be classified as spherical, sheet, fiber, cylindrical, tubular, hollow, irregular, etc. In terms of size, it could be in any size under 100 nm. Based on composition and source, the nanomaterials could be classified into four major groups organic, inorganic, composite, and carbonic.

2.1 Carbonic Nanomaterials

Carbonic nanomaterial progression has been initiated after the introduction of the C60 because of their distinct structure and activity compared with similar macroscopic scale. Fullerenes (C60, C80, and C240), graphene, carbonic nanotubes (CNTs), diamonds, carbonic-based NPs, nanofibers, nanobubbles, and any composite nanomaterials are merged in this group [54, 55]. The reason for paying great attention to this group is related to their particular structure, small size under 50 nm, great surface area, amorphous nature, and geometrical structure [55]. The size of these nanomaterials is near the size of the organelles, and also their special surface areas help better cellular attachment and intake. Moreover, they have depicted a desirable characteristic in providing a controlled release profile of the drug. Considering all these parameters makes them an adorable choice for developing NDDSs. The capability of carbonic nanomaterials for conjugation with DNA molecules, proteins, and peptides provides modification opportunities which makes them more efficient as NDDSs [56]. The main obstacle against utilizing carbonic nanomaterials as DDSs is the reported toxicity. Based on the cellular toxicity studies, carbonic nanofibers and NPs are more toxic than carbonic nanotubes. Moreover, it was reported that the toxicity of carbonic nanotubes with carbonyl, hydroxyl, and carboxyl groups was higher [57, 58]. Therefore, utilizing carbonic nanomaterials for drug delivery is still in doubt. Despite this, it was believed that the functionalization of carbonic nanomaterials could reduce their toxicity but there is uncertainty about guaranteeing the maintenance of their safety in a biological system [59].

2.1.1 Fullerene

Fullerenes (C60, C70, C80, C90, etc.) are spherical carbon molecules that consist of carbon atoms. The diameter of single-layer fullerenes is under 8.2 nm, while the multilayer ones are under 36 nm [60]. The drug molecules could be conjugated to hydrophilic functional groups of the modified fullerenes [61]. There are many studies on the evaluation of the toxicity of fullerenes, but it could not be certainly concluded that they are toxic DDSs [62–64].

2.1.2 Carbon Nanotubes

Carbon nanotubes are consisting of carbons in a tubular morphology which could be arranged as a single-wall or multiwall constructions. Their diameter ranges from 0.7 nm for single walls up to 100 nm for multiwalls [65]. Drug molecules including antifungal peptides, folic acid, antibodies, and various chemotherapeutic agents have been conjugated to carbon nanotubes as NDDSs [66]. As a representative, the conjugation of paclitaxel to carbonic nanotubes exhibited better efficacy toward tumor cells compared with the paclitaxel [67, 68]. Cellular toxicity, blood coagulation disorders, pulmonary inflammations like foreign-body granuloma, or other inflammatory reactions such as interstitial lung fibrosis are reported toxicity for carbonic nanotubes [69].

2.1.3 Graphene

Graphene as a sheet construction of carbon atoms with a single atomic diameter dimensionally belongs to the 2D nanomaterial group [70]. Graphene oxide and reduced graphene oxide were also utilized for biopharmaceutical purposes. Significant adsorption of the drug on graphene oxide nanomaterials was exhibited in comparison with all kinds of carbonic nanomaterials [71]. Besides, the hydrophilic polymers and functional groups have been working to improve the characteristics of graphene oxide [72]. Graphene, having the capability to permeate the cells, is responsible for the efficient delivery of carried drug molecules within the cellular targets [73]. Moreover, diverse materials like metals, proteins, polymers, quantum dots, etc. have been linked to graphene to improve the physicochemical characteristics of graphene molecules for chemotherapeutic purposes [74]. However, based on the intrinsic optical properties of graphene, they have been utilized for photothermal chemotherapy of cancerous cells too [75]. Considering the cellular and in vivo studies, the graphene oxide individually demonstrates toxicity, but the surface functionalization of them with biocompatible molecules omits the toxicity [76].

2.1.4 Nanodiamonds

Nanodiamonds are diamond-shaped structures of saturated carbon atoms on nanometer scale which have the capacity of being loaded with active pharmaceutical agents [77]. They have been utilized as a DDS in parenteral and topical dosage forms. However, they have the potential of being fabricated for other administration routes with various properties like controlled release or targeted formulations too [78].

2.2 *Organic Nanomaterials*

The most utilized nanomaterials as DDSs belong to organic nanostructures. Polymeric NPs, dendrimers, liposomes, and micelles are some examples of this group. Safety, biodegradability, biocompatibility, encapsulation capability, etc. are some properties of these kinds of nanomaterials that make them desirable for formulating efficient DDSs.

2.2.1 Polymeric NPs

Polymeric-based DDSs can be formulated by conjugation of drug molecules to the polymeric chains or by encapsulation of the drug molecules. The linkage of the drug molecules could be done by physical adsorption or chemical reaction over the surface of the polymeric NPs. The encapsulation approach is divided into two main methods including polymerization and desolvation of macromolecules which directly influence their physical characteristics and release profile [79, 80]. They are in a colloidal manner with a particle size of less than 1000 nm which could be formulated by natural or synthetic biocompatible polymers [81]. The smart polymeric NDDSs were also formulated based on sensitivity to a magnetic field, pH, heat, etc. [72]. Generally, polymeric NDDSs are safe, and various nanostructures like NPs, nanocapsules, and nanoemulsions have been studied, and some of them even progressed as far as clinical trials [82].

2.2.2 Dendrimers

Dendrimers are polymeric NPs that consist of a core with many symmetric branches around them. Drug molecules could be loaded within the core or be linked to the branches. The main advantages of dendrimers that make them desirable for being utilized as DDSs include surface manipulation capability, low polydispersity, weight predetermination, controlled pharmacokinetics, enhanced cellular attachment and permeation, safety, and non-immunogenicity [83]. Dendrimer-based DDSs have been formulated for both active and passive targeting of drugs, DNA molecules, proteins, peptides, etc. No limitations were reported for the loading of hydrophilic and hydrophobic active agents [84, 85].

2.2.3 Nanofibers

Nanofibers (NF) are fibers with diameters on a nanometer scale that have been utilized as NDDSs for oral, topical, transdermal, ocular, transmucosal, rectal, and other administration routes. Electrospinning is the most applied method. However, self-assembling, phase separation, and template synthesis are other production

methods. High surface-volume ratio, high encapsulation yield, flexibility, porosity, production simplicity, monodispersity, biocompatibility, drug release control, capability to be functionalized, and cellular attachment are the main advantages of utilizing nanofibers for DDSs [86, 87].

2.2.4 Liposomes

Liposomes are small unilamellar (SUV), large unilamellar (LUV), multilamellar (MLV), and oligolamellar (OLV) phospholipid-based structures. The type of utilized phospholipid, zeta potential, particle size, and production method are the key parameters that affect their properties [88]. These organic nanomaterials have been largely applied as NDDSs with a majority in cosmeceuticals. The characteristics which make them a suitable carrier for drugs are the structure of the double-layer phospholipid which is similar to cell membrane, nanoscale size, flexibility, stability against drug decomposition, zeta potential determination for targeted delivery, capability to be functionalized, drug loading capacity, improvement in drug solubility, pharmacokinetic parameters and drug potency, reduction in cytotoxicity, and potential of being used in active or passive targeting of the tumor cells [89, 90]. The release profile of the drug is related to the lipid composition, acidity, gradient, and the exposed medium [91]. The main obstacles against utilizing liposomes as NDDSs are their limited entrapment efficiency, leakage, rapid burst release, and low stability [92]. Exosomes, niosomes, ethosomes, transferosomes, marinosomes, DNAsomes, etc. have similar vesicular morphology with different compositions from surfactants up to protein and DNA [93–95].

2.2.5 Solid Lipid NPs (SLNs), Nanostructured Lipid Carriers (NLCs), and Lipid Drug Conjugates (LDCs)

SLN and NLC are lipid-based structures that consist of solid lipids and combined liquid-solid lipids which could be loaded by drug molecules, while LDCs are made by covalent conjugation of drug molecules with lipids [96, 97]. These lipid-based nanocarriers are preferred to liposomes for preparing NDDSs in case of their biological stability [98]. NLCs have been widely used as transdermal DDSs [99]. LDCs have been utilized for delivering lyophobic active agents [100]. The toxicity of lipid NPs is less than other nanostructures [101]. SLNs have been widely formulated in topical, oral, and ocular routes of administration. They have been also used in inhalers, suppositories, and injection dosage forms [102]. The three main types of SLNs as DDSs are homogenous dispersion of drugs in the matrix, shell deposition, or core deposition. Although SLNs could provide a stable controlled release formulation, their encapsulation efficiency is low, while the NLCs have more capacity for drug entrapment with higher stability. The three main types of NLC DDSs are imperfect kind with adequate spaces for drug loading, multiple kinds in which drug molecules would be dispersed in oil and then incorporated within the lipid nanocarrier, and

amorphous kind which inhibits drug crystallization. Obviously, the production method and the utilized surfactants are responsible for the release behavior of the lipid-based NDDSs [103].

2.2.6 Micelles

Micelles are spherical NPs consisting of amphiphilic building blocks with a hydrophobic core and hydrophilic shell which have been utilized as a DDS for lipophilic and hydrophilic drugs [104]. Their desirable stability in biological systems is related to their low critical micellar concentration which makes them a great choice to be used as NDDSs [105]. Considering the various building blocks, copolymers are more stable than surfactants in physiological medium [106]. Different modifications could be done over micelles from conjugation by ligands like antibodies, affibodies, etc. up to smart groups like pH-sensitive or thermosensitive ligands. Chemotherapeutic agents like docetaxel were loaded in micelles as NDDSs [107]. Active targeting was also investigated by linkage of special ligands like transferrin [108]. MNPs were incorporated in micelles for bioimaging and therapeutic goals in tumor cells [109]. Ocular DDS was prepared by micelles for delivery of cyclosporine A [110]. The brain delivery by micelles was accomplished by loading rhodamine PE [111]. The polymeric-based micelles have been significantly noticed to be used as NDDSs because of the special characteristics of these nanomaterials like the capability of being formed by self-assembly, biodegradability, and biocompatibility of the polymers, stability, long presence in blood circulation, etc. [112].

2.2.7 Nanowire, Nanoflower, and Nanocoil Nanomaterials

Nanowires are wires with a diameter on the scale of a nanometer. Nanoflowers are flower-like assemblies of materials in nanometer dimensions. Nanocoils or nanosprings are helical assembling morphology of materials on the nanometer scale. There are limited reports about utilizing these nanomaterials as DDSs.

2.2.8 DNA-Based Nanomaterials

DNAsomes, DNA nanotubes, and cloverleaf-like DNA nanostructures are some examples of DNA-based nanostructures. Some studies showed the valuable potential of these particles for drug delivery purposes [113].

2.2.9 Virus-Like Particles (VLPs)

VLPs are derived from viruses through viral replication which is similar in size to viruses. They are known to have immunogenic characteristics, but they have been studied to be utilized as NDDSs too [114].

2.3 Inorganic Nanomaterials

Metallic and metal oxide materials with at least one dimension on the nanometer scale belong to this group. Despite a bit of complication in the synthesis of inorganic NPs, small sizes with various shapes could be produced which provides special physiochemical and biological properties for them. Au, Ag, Fe, and mesoporous silica are some examples of this group that have been used as NDDSs and also in bioimaging for diagnosis [115].

2.3.1 Ceramic NPs

Ceramic NPs, like silica, titanium, and alumina, are porous NPs that have been mostly utilized as a stabilizer in dental and bone scaffolds and implants [116]. However, they have been used as NDDSs because of their ability to provide more stability for entrapped drug molecules and prevent them from thermal and pH tensions. The main characteristics of ceramic NPs which make them a great option to be used as NDDSs include biocompatibility; safe carrier for biomolecules like peptides; the capability of manipulation in their size, shape, and porosity for enhancing their presence in blood circulation; and potential of being modified by targeting agents like antibodies for active targeting of the tumor cells [117].

2.3.2 Silica NPs

Silica NPs' characteristics like biocompatibility, small size, the hydrophilicity of the surface, amorphous manner, simple synthesis, high stability, and porous structures which not only provide high surface-area volume but also could lead to efficient drug encapsulation with controlled release capability, drug protection from the immune system, and surface manipulation potential make them a great choice for NDDSs. Generally, solid, nonporous, and mesoporous are three kinds of silica NPs. The monodispersity, homogenous morphology, capacity for even large molecules, and greater surface-to-volume ratio of mesoporous silica NPs make them a better choice for DDSs than two other types. Based on in vivo studies, only NPs larger than 100 nm may cause liver inflammation. The cellular studies also confirmed that silica NPs with particle size less than 100 nm and concentrations less than 100 mg/mL are safe [118–120].

2.3.3 Metal-Based NPs

Gold, silver, palladium, nickel, copper, iron, and gadolinium are some main MNPs that have been studied as NDDSs so far [27]. Magnetic NPs are great options for delivering drugs and also contrast-making agents in magnetic resonance imaging. Iron oxide NPs possess superparamagnetic properties which make them a desirable choice for target drug delivery which not only selectively would target the tumors but also could improve the permeability of drug molecules [121]. The thermosensitive metal-based NDDSs could be also prepared which provide thermal-dependent release formulations [122]. Gold nanoparticles (AuNPs) are famous in DDSs, bioimaging, and biosensors. They have a size range of 1 nm to 8 μm with spherical, decahedral, icosahedral, hexagonal, rod, triangular, prismatic, and irregular shapes. MNPs could be widely formulated as smart NDDSs like pH-sensitive, thermosensitive, and light-sensitive and also for active and passive targeting of the tumor cells [123]. The conjugation of specific ligands like amino acids could prevent cytotoxicity of MNPs [124].

2.3.4 Quantum Dots (QDs)

QDs are combined inorganic-organic NPs. The core part consists of metals and the shell is responsible for the optical properties. Their high surface-area-to-volume ratio and optical activity are responsible for utilizing them as NDDSs and in diagnosis imaging and conjugation of QDs with macromolecules like DNA, peptides, etc, which have made them a desirable choice for cellular and subcellular labeling. The QD utilization as NDDS could be done by two main methods including direct conjugation of drug molecules to QDs and the drug and QDs co-encapsulated in other nonmaterial like lipid or polymeric NPs [125, 126].

2.3.5 Nanocrystalline Materials

Nanocrystalline materials have a crystallite size under 100 nm. Both hydrophilic and lipophilic drugs could be incorporated into nanocrystalline materials. Doxorubicin (DOX), tetracycline, paclitaxel, and docetaxel are some examples that have been loaded into nanocrystalline materials [127].

2.4 Nanocomposites

Nanocomposites are materials loaded with any kinds of NPs like metallic, carbonic, or organic types. They have been widely used for topical and transdermal DDSs. Chitosan-based nanocomposites in hydrogels and biofilms are well studied. Magnetic NPs, hydroxyapatite NPs, metals, carbon nanotubes, and graphene are

examples of nanostructures that were incorporated into bulk materials for the production of nanocomposite-based DDSs [25, 26, 128].

3 Bioengineered Metal-Based Nanomaterials: An Overview of Biofabrication

The metals which possess special electrical and thermal characteristics have been noticed in nanotechnology. Considering all types of nanomaterials, the synthesis of MNPs has been more attractive for scientists because of their unique features like increased reactivity which relates to their high surface area. Silver, gold, copper, iron, and zinc are examples of MNPs [129]. MNPs as inorganic nanomaterials bear priority over other nanomaterials to formulate DDSs. These characteristics include biological compatibility, stability, size-related performance, non-immunogenicity, desirable monodispersity, conjugation potential, catalyzing property, renewability, productibility, and potential of manipulating the charge, size, encapsulation, and structure with color change detectability [27]. The synthesis of MNPs by biological resources belongs to green nanotechnology as a bottom-up approach to engineering nanomaterials. The priority of this approach compared with other methods including chemical and physical methods is related to biological compatibility, safety, high efficacy, financial acceptability, fewer energy resource consumption, and less pollution making which protects our planet. Plants, bacteria, fungi, yeast, virus, and algae are main green resources for biofabrication of MNPs [130]. Figure 1 exhibited a schematic description of biogenic resources and involved compounds in synthesis of silver nanoparticles (AgNPs) as a representative of MNPs. The reducing compounds like alkaloids, terpenoids, polysaccharides, proteins, enzymes, coenzymes, bio-surfactants, etc. are responsible for reducing the metal ions to MNPs. Microorganisms, strictly bacteria, have shown higher fabrication yield and also less medium acidity and thermal and pressure control compared with other green resources. However, plants and algae are more available and safe compared to microorganisms [131, 132].

3.1 Plant-Mediated Synthesis of Metal-Based Nanomaterials

Biofabrication of metal-based nanomaterials by plants provided ecological benefits which also possess detoxification features. Two main stages are known for the production of biogenic metal-based nanomaterials including reduction and stabilization procedures which would be conducted by reducing and capping agents, respectively. Various components in plant extract are responsible for acting as reducing and capping agents including polyphenolic compounds, alkaloids, sugars, isoprenoids, etc. The procedure is consisting of exposing the solution of metal salts

to the plant extract at room temperature and leaving it until the color changes as indicating criteria to prove the bioformation of MNPs [133, 134]. Some parameters could be modified during the procedure including the reaction period, the plant extract concentration, the metal solution concentration, acidity of the medium, and the room temperature. Changing any of these parameters could alter the particle size, structure, and physicochemical properties of the MNPs. Thus, these parameters could be utilized for controlling the above characteristics of the MNPs [135]. AgNPs were also produced by seed extract of *Medicago sativa* in nanoflower, triangular and spherical morphologies with 5–108 nm sizes which demonstrate the high manipulation potential of MNPs by plant-mediated method [136].

The priority of this approach compared with other methods is related to properties like the non-pathogenicity, non-immunogenicity, being available, large-scale production capacity, providing capping agents, and less dedicated time. The energy consumption for heating in the extraction process and polydispersity could be considered disadvantages of this method. The demonstrated polydispersity could be related to the action of utilizing various reducing compounds in the plant extracts or the different levels of each of these compounds in the plants which grow in diverse climates. Based on the compound deposition, any parts of the plant could be utilized for extraction like aerial sections, leaves, seeds, rhizomes, peels, petals, fruits, or the whole body. Boiling or Soxhlet apparatus is utilized for extraction. Generally, the biosynthesis procedures of MNPs include two important stages [137, 138]. The first stage consists of reducing the chain of metal ions by nucleation followed by a growing step. The second stage is stabilization which would be conducted by capping agents. Gold, silver, copper, copper oxide, iron, palladium, zinc, selenium, platinum, and indium are examples of metals that have been fabricated as MNPs by plant-mediated method [139]. Some conducted studies for plant-mediated synthesis of MNPs are listed in Table 1.

3.2 Fungal-Mediated Synthesis of Metal-Based Nanomaterials

Fungi are beneficial bioreactors for the synthesis of metal-based nanomaterials with a bottom-up approach. The production procedures could be in extracellular or intracellular pathways [140]. The function of fungus in an extracellular pathway for producing MNPs includes the excretion of enzymes when exposed to metallic salts to defend itself from unknown compounds in the medium around. This action would provide the opportunity for the metal ions to be reduced by the secreted enzymes [141]. The naphthoquinones and anthraquinones are also excreted by fungi which could act as reducing agents too.

Fungi have some superiorities over bacteria and other organisms which relate to specific characteristics like high biofabrication yield. Their excessive yield of extracellular MNP biofabrication is significant. The mycelia which improve protein secretion are responsible for this phenomenon. On the other hand, some fungi have a great potential for entering extra amounts of metal ions which leads to fabricating

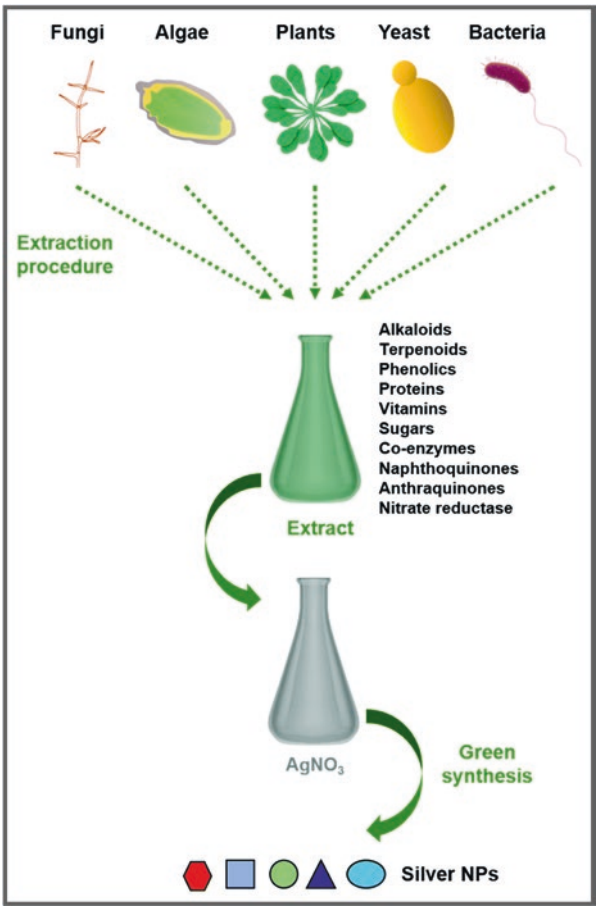

Fig. 1 Schematic description of biogenic resources and involved compounds in the synthesis of AgNPs as a representative of MNPs [221]. (Reprinted from open-access article under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence (CC BY-NC 3.0); Royal Society of Chemistry, 2019)

MNPs in a smaller size. Other advantages of fungal resources include facile production on large scales, excessive excretion of enzymes which promotes the biofabrication yield in an extracellular pathway, financial acceptability, and easy handling of biomass harvest and purification. Based on previous studies on using the fungus *Verticillium* for the production of AgNPs with intracellular pathway, it was revealed that the MNPs were formed beneath the cell wall and declared that the enzymes exist in the cell wall. Since the intracellular pathway imposes a financial burden on the purification process, the extracellular pathway is more acceptable [142, 143]. The extracellular biosynthesis of AgNPs by *Aspergillus fumigatus* leads to a fast production period and stable MNPs. The production of MNPs by *Aspergillus* strains has a pathological limitation. The downstream processing for MNPs produced by

Table 1 Some examples of biosynthesized MNPs with plants, bacteria, fungi, and algae resources

Microorganism	MNPs	Pathway	Particle size (nm)	Reference
Plants				
Tinospora cordifolia	Silver	Extracellular	50–70	[161]
Medicago sativa	Silver	Extracellular	5–108	[136]
Geranium leaves extract	Sliver	–	16–40	[162]
Alfalfa sprouts	Silver	Intracellular	2–20	[163]
Ziziphus zizyphus	Gold	Extracellular	40–50	[164]
Lemongrass extract	Gold	–	200–500	[165]
Aloe vera	Gold	Extracellular	50–350	[166]
Avena sativa	Gold	Extracellular	5–85	[167]
Euphrasia officinalis	Silver and gold	Extracellular	38.57–42.17 for AgNPs 48.52–50.92 for AuNPs	[168]
Indigofera tinctoria	Gold and silver	Extracellular	9–26 for AgNPs 6–29 for AuNPs	[169]
Cinnamomum camphora	Gold and silver	Extracellular	5–80	[170]
Azadirachta indica	Gold, silver, and gold/silver	Extracellular	5–100	[171]
Bacteria				
Bacillus cereus	Silver	Extracellular	20–40	[172]
Plectonema boryanum	Silver	Intracellular	1–10	[173]
Morganella sp.	Silver	Extracellular	20–30	[174]
Klebsiella pneumoniae	Silver	Extracellular	5–32	[175]
Pseudomonas stutzeri	Silver	Intracellular	About 200	[176]
Lactobacillus spp.	Silver and gold	Intracellular	–	[177]
Rhodopseudomonas capsulate	Gold	Extracellular (pH = 7) Extracellular (pH = 4)	10–20 50–400	[178]
Escherichia coli	Gold	Intracellular	25–33	[179]
Rhodococcus sp.	Gold	Intracellular	5–15	[180]
Thermomonospora sp.	Gold	Extracellular	8	[181]
Pseudomonas aeruginosa	Gold	Extracellular	15–30	[182]
Kocuria flava	Copper	Extracellular	5–30	[183]
Acinetobacter spp.	Magnetite	Extracellular	10–40	[157]
Clostridium thermoaceticum	Cadmium	Intracellular and extracellular	–	[184]
Escherichia coli	Cadmium	Intracellular	2–5	[185]
Shewanella oneidensis	Uranium	Extracellular	–	[186]
Fungi				
Rhizopus oryzae	Gold	Extracellular	16–43	[187]

(continued)

Table 1 (continued)

Microorganism	MNPs	Pathway	Particle size (nm)	Reference
Penicillium diversum	Silver	Extracellular	10–50	[188]
Penicillium waksmanii	Iron oxide	Extracellular	71–151	[189]
Chrysosporium indicum	Silver	Extracellular	10–31	[190]
Aspergillus fumigatus	Silver	Extracellular	5–25	[191]
Trichoderma asperellum	Silver	Extracellular	13–18	[192]
Verticillium	Silver	Intracellular	13–37	[193]
Phanerochaete chrysosporium	Silver	Extracellular	50–200	[194]
Phoma sp.	Silver	Extracellular	71–74	[195]
Fusarium oxysporum	Silver	Extracellular	20–40	[196]
Fusarium oxysporum and *Verticillium* sp.	Magnetite	Extracellular	20–50	[197]
Algae				
Oscillatoria willei NTDM01	Silver	Extracellular	10–25	[198]
Chlorococcum humicola	Silver	Intracellular	16	[199]
Oscillatoria sp.	Silver	Extracellular	14–48	[153]
Nostoc ellipsosporum	Gold	Intracellular	20–40	[200]
Spirulina platensis	Gold	Extracellular	20–30	[201]
Sargassum wightii	Gold	Extracellular	8–12	[202]

these fungi not only bears risks but also is hard [144]. On the other hand, some nonpathogenic fungi could be utilized for this purpose. As an instance, in a study AgNPs were synthesized by *Trichoderma asperellum* with nanocrystalline morphology [145]. Various MNPs like gold, silica, magnetite, titania, and zirconia were fabricated by different fungi including *Penicillium* sp., *Fusarium* sp., etc. [143].

3.3 Bacterial-Mediated Synthesis of Metal-Based Nanomaterials

The most utilized microorganisms for biogenic fabrication of metal-based nanomaterials are bacteria [146]. *Staphylococcus aureus*, *Escherichia coli*, *Thiobacillus ferrooxidans*, *Bacillus subtilis*, *Bacillus megaterium*, and *Pseudomonas aeruginosa* are some examples of bacteria used in the biosynthesis of metal-based nanomaterials [147]. Actinomycetes also were utilized for this purpose because of the high contents of proteins and bio-reductant compounds [148]. Figure 2 illustrated the production process of AgNPs as a representative which was conducted by extracellular NADH-dependent nitrate reductase of actinomycetes [149]. Silver, gold, palladium, iron, and cadmium are some metals that have been fabricated to MNPs by bacteria. There are some advantages to using bacteria as reducing bioreactors which include controllable changes in size, structure, and shape of the particles,

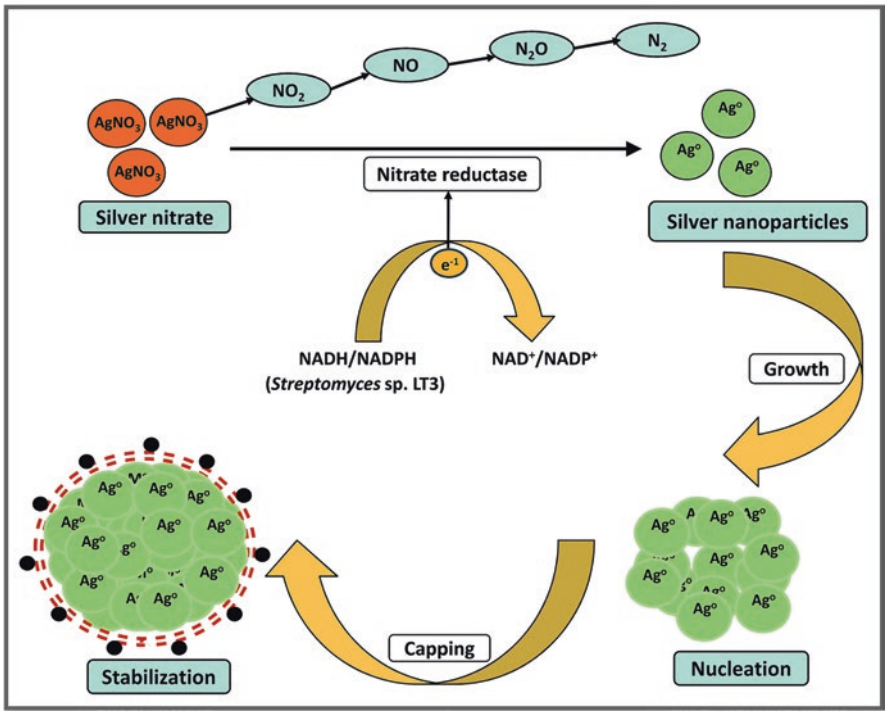

Fig. 2 Biofabrication procedures of AgNPs by extracellular NADH-dependent nitrate reductase of actinomycetes [155]. (Reprinted from open-access article under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence (CC BY-NC 3.0); Royal Society of Chemistry, 2019)

biocompatibility, non-immunogenicity, high production yield, and financial benefits. Studies showed the concentration of metal ions is in direct relationship with the achieved particle size [150].

Bacteria could also bear both two pathways – extracellular and intracellular. AuNPs were firstly biofabricated with an extracellular pathway in 1980 by using *B. subtilis* [151]. On the other hand, silver, iron, cobalt, rhodium, palladium, nickel, and lithium NPs were produced intracellularly by *P. aeruginosa* [152]. There are two main factors affecting the reduction process of producing MNPs from metal ions including the mineralization-inducing compounds in the cell wall and the medium conditions. The concentration of metal ions, pH, compounds present in the environment, and temperature are conditions that should be considered. These parameters are responsible for the size, shape, structure, and morphology of the attained MNPs. Therefore, the optimization of these parameters through the biofabrication process is essential [153, 154]. For instance, changing the mentioned parameters leads to biosynthesis of various shapes of AgNPs and AuNPs including spherical, cubic, and even plate forms with diverse sizes [155]. The polydispersity of AuNPs was reduced by increasing the temperature accompanied by pH

enhancement. Moreover, the pH enhancement could increase the particle size of MNPs. Preserving the pH value at 3 could form particles under 10 nm [155, 156]. The magnetic NPs could be produced by sulfate-reductant bacteria in an anaerobic situation and also by *Actinobacteria* in an aerobic situation [157, 158]. Other than the described parameters, the bacterial strain, reductant exposure time, and radiation could affect the size, structure, and polydispersity. More reductant exposure time could produce larger particles with a lower polydispersity index. The biofabrication rate efficiency of MNPs would be improved by temperature elevation [159].

3.4 Algal-Mediated Synthesis of Metal-Based Nanomaterials

Algae mainly grow in watery conditions and are known as photosynthetic eukaryotes with no specified application parts like leaves, roots, or stems. Algae are estimated to be more than one million species. Cyanophyta, Phaeophyta, Chrysophyta, Chlorophyta, Bacillariophyta, Rhodophyta, and Dinophyta are some examples of this organism. The algae have shown various properties including anti-inflammation, anticancer, and antioxidant effects. Besides, the bioreducing agents of algae could provide the opportunity for biogenic synthesis of MNPs. Their high capacity for taking metal ions and availability and being cheap make them a desirable choice for bioengineering the MNPs. Similar to plants, these organisms have special compounds like alkaloids, terpenoids, phenols, amines, amides, flavonoids, and even proteins which could act as reductant and capping agents. Various MNPs like silver, palladium, gold, iron, cadmium, zinc, and platinum have been fabricated by algae-mediated method [160]. Algae do not need an expensive culture medium for growing and have a low cost of MNP production. Less required bioengineering time, no chemical byproducts, and no environmental pollution are significant characteristics of using algae in the biofabrication of MNPs. *Sargassum* spp. compared with other algae demonstrated a great ability to synthesize various types of MNPs like gold, silver, zinc oxide, and titanium dioxide. Important parameters like acidity, ion concentration, temperature, exposure time, and medium condition should be optimized for achieving specified shapes, sizes, and structures. Despite the declared potential of using algae, the limited information about related biosynthesis mechanisms would be an obstacle to utilizing all types of algae for this approach [159].

4 Biogenic Metal-Based Nanomaterials for Drug Delivery Systems: Recent Advances

The core issue in designing and developing novel drug delivery platforms is the precise and effective delivery of drugs to their designated locations at the appropriate time [203]. An ideal drug delivery system would supply the medical agent

throughout a defined treatment duration while achieving the most significant therapeutic impact possible, and it would be enhanced with a carrier system such as NPs [204]. Targeted nanocarriers must navigate blood tissue barriers to reach target cells [205]. Furthermore, to reach targeted cells, specifically, aimed nanocarriers must make contact with cytoplasmic recipients via unique endocytosis and transcytosis transfer pathways [206].

It is expected that nanoparticle-interceded targeted delivery of medications with improved properties, efficiency, and low toxicity would significantly reduce the number of anticancer treatments available [203]. This is important since many anticancer medicines' hydrophobicity is a hindrance to their practical usage. To reach cells in conventional treatment, the molecules must be dissolved in hazardous solvents, penetrating less deeply than expected. Therefore, various conjugated and encapsulated nanoparticle formulations have indeed been developed to improve the solubility of these drugs [207]. It is expected that the usage of nanotechnology-based therapies and diagnostics in clinics will increase in the next years. Individual medicine is another important sector in which nanotechnology can play a critical role. Because of cancer heterogeneity and the increase of drug persistence, every especially targeted therapy may not work for every sick population [203].

In a related context, MNPs have been developed as prospective structure for drug and gene delivery, with roles that complement more traditional delivery carriers [208]. The novel targeted delivery of anticancer drugs to malignant cells without damaging normal cells improves pharmacokinetics and biodistribution of therapeutic components, reducing the adverse effects of standard chemotherapeutic treatments [209]. Because of its capacity to bind strongly to thiols (–SH) and amines (–NH2), gold, for example, could well be easily modified. Covering the AuNP surface with biomolecules that function as chemotherapeutic and targeting agents is thus quite straightforward [210]. In line with the goals of this section of the book, functionalized bioderived MNPs with unique physicochemical characteristics are stated to be effectively used as nanocarriers to treat endocellular diseases through targeted delivery of drugs [204]. Bioconjugated MNPs could be synthesized with a combination of reduced innate toxicity, increased surface area to volume proportion, stability, and tunability, providing them with different properties [210]. Therefore, attaching the required quantity of drugs to the MNPs, as well as controlling the release of chemotherapeutic drugs to the afflicted region, enables the use of low-dose chemotherapy treatments [204, 211]. Because of their enormous surface area, biomediated nano assemblies can form covalent or ionic interactions with medicines, proteins, and other chemical substances [204]. Eventually, the cytotoxic action of MNPs can be exploited for both medication delivery and cancer cell targeting by using various capping agents. Whether the targeted NPs themselves act as therapeutic or the NPs serve as carriers for another drug, biosynthesized NPs are becoming increasingly important in nanomedicine [209, 210].

As mentioned earlier, the targeted drug delivery approach has received much interest in cancer treatment during the last few decades. When it comes to nanocarrier-based targeted delivery systems, AuNPs stand out due to their exceptional physicochemical features, photothermal properties, controlled size, and

biocompatibility. Further, AuNPs may be concentrated in target cells via endocytosis and have been employed as a targeted vehicle for delivering anticancer drugs [212, 213]. For example, a study reported exploiting extracts of *Mappia foetida* leaves as bioreducing agents to synthesize AuNPs and their use in the effective delivery of chemotherapeutic medicines to human cancerous cells. The authors conjugated activated folic acid (FA) as a navigational component for targeted delivery of DOX to form spherical nanoconjugated DOX-loaded AuNPs-FA with a 20–50 nm size range. MTT assay was used to assess the cytotoxic effect of bioconjugated DOX-loaded AuNPs-FA complex on several cell lines, including HeLa, SiHa, MDA-MB-231, and Hep-G2 (human cancer cells) and epithelial Vero cell lines (normal cells). The cell viability followed by DOX-loaded AuNPs-FA treatment was ~50% for normal cells and ~7% for cancer cells at 50 μg/mL concentration. In comparison, with 50 μg/mL of DOX, cell survival is reported to be around 40% for normal cells and 35% for malignant cells. In addition, the IC_{50} values of DOX-loaded AuNPs-FA on normal and cancer cells were 50 and 20 μg/mL, correspondingly. The cytotoxic effect of DOX-loaded AuNPs-FA was shown to be lower on normal Vero cells and higher on cancer cell lines when compared to standard DOX. For the sustained drug delivery to the tumor environment, DOX-loaded AuNPs-FA were evaluated for drug release utilizing a dialysis tube in phosphate buffer solution at various pH levels. The quantity of medication released was greatest at pH 5.3 (the ambient pH for I.V. cancer medications), followed by pH 7.2 and 6.8. At a slightly acidic pH, the non-covalent chemical interaction between AuNPs and DOX may be hydrolyzed. As a result, the authors concluded that the DOX-loaded AuNPs-FA complex is fairly biocompatible for normal cells, most likely due to the influence of potential cancer cell targeting [214].

Furthermore, a study explored porphyran, a marine red algal-derived sulfated polysaccharide (*Porphyra vietnamensis*) used as both a reducing agent and a capping agent in a one-pot size-controlled biosynthesis of AuNPs. These AuNPs have been used as carriers for DOX. Based on their TEM study, DOX-loaded AuNPs had a consistently spherical 14 ± 3 nm morphology. The following diagram depicts a schematic illustration of porphyran-reduced AuNPs and subsequent loading of cationic DOX on porphyran-capped AuNPs (Fig. 3). The percent loading of DOX onto AuNPs was measured using the DOX concentration in the supernatant and the resulting pellet after centrifugation, and it was found to be 38% and 60%, respectively. Additionally, the cytotoxic effect of the DOX-loaded AuNPs was determined utilizing an in vitro MTT assay method to prove the capabilities of the biofabricated AuNPs as an effective drug carrier. With increasing DOX concentration, both native DOX and DOX-loaded AuNPs reduce the viability of human glioma cells (LN-229). But it was shown that the cytotoxic impact of DOX-loaded AuNPs was statistically superior to that of native DOX. After 48 h, the reduction in cell viability with natural DOX and DOX-loaded AuNPs in the examined concentration range (1.0–20 μg/mL) was determined to be between 60–35% and 45–15%, correspondingly. The authors ascribed the increased cytotoxicity of drug-loaded NPs to an increase in the internalization of DOX-loaded AuNPs via an endocytosis process as opposed to the passive diffusion method of native DOX into cells [215].

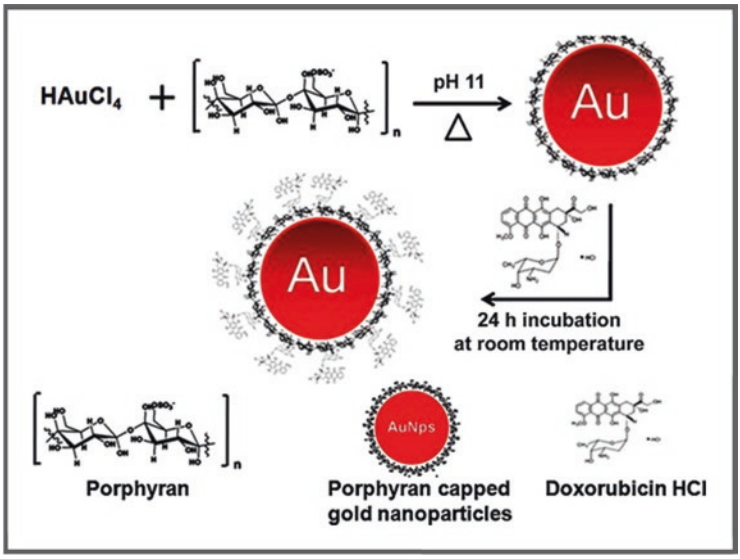

Fig. 3 Porphyran-reduced AuNPs are shown schematically, followed by the loading of cationic DOX-HCl on porphyran-capped AuNPs. (Reprinted with permission from Venkatpurwar et al. [215])

Moreover, another study approached the biogenesis of AuNPs employing leaf extract of *Peltophorum pterocarpum* as a vehicle for a DOX delivery system. As described in the study, an exact concentration of the mentioned extract was adequate to decrease all Au ions ($HAuCl_4$), resulting in optimal, stable, spherical, and 5–15 nm AuNPs as determined by TEM. The surface plasmon resonance wavelength of the AuNPs was shifted toward a higher wavelength when they were conjugated with DOX, confirming the attachment of DOX to the nanoconjugate. As DOX-loaded AuNPs were administered, they substantially suppressed the proliferation of lung cancer cells (A549) and melanoma cells (B16F10) in vitro, as well as tumor growth in an in vivo model, when compared to DOX individually. Besides, cellular uptake and release of nanoconjugated DOX were quicker than unconjugated DOX uptake and release. Additionally, to investigate the mechanism of the enhanced anticancer activity of DOX-loaded AuNPs toward the B16F10 cells, Western blotting analysis was executed to show the upregulation of p53 protein (a cell cycle regulator and tumor suppressor protein) in the mentioned cells treated with the DOX-loaded AuNPs for 24 h compared with untreated B16F10 cells and cells treated with free DOX. Moreover, the in vivo toxicity investigation found no significant alterations in the hematology, serum clinical biochemistry, or histopathology of important organs in melanoma model mice following 7 days of successive intraperitoneal (IP) injections of nanoconjugated particles [216].

Additionally, using the plant pathogenic fungus *Helminthosporium solani*, researchers set out to investigate the possibility of generating and separating morphologically distinct AuNPs. The abovementioned fungi's mycelia were incubated

with a solution of chloroaurate ions, yielding a heterogeneous assortment of extracellular Au nanostructures ranging in size from 2 to 70 nm. The majority of biosynthesized particles were polydisperse spheres, although TEM investigation reveals a large number are homogeneously sized rods, pentagons, stars, triangles, and pyramids. The mixture of biosynthesized AuNPs was sorted by size and shape via a microcentrifuge using the sucrose density gradient approach. The separated AuNPs appeared to be hydrophilic, and the researchers indicated bioconjugation through carbodiimide-mediated cross-linking of the smallest size fractions (2–5 nm) to DOX. The results of their DOX-loaded AuNP labeling pattern in HEK293 cells obtained by epifluorescence microscopy were markedly different from those obtained with DOX alone. Most fluorescence occurred in the nucleus in DOX-only cells, and cells under phase contrast look smooth-edged and healthy. DOX-loaded AuNPs, on the other hand, did not penetrate the nucleus and fluoresced brightly throughout the cytoplasm, and phase-contrast imaging showed membrane damage in the shape of blebbing. Additionally, the findings of the pseudocolor confocal images of DOX alone and DOX-loaded AuNPs in HEK293 cells confirmed that the conjugates stayed at the cytoplasmic phase, a problem that, as indicated by the authors, may affect the genotoxicity of the drug [217]. Moreover, carrageenan oligosaccharide (CAO) was employed as a biocompatible reducing agent in the green fabrication of CAO-AuNPs, and the resulting CAO-AuNPs were then used as a delivery vehicle for pH-triggered delivery of epirubicin (EPI). Carrageenans are sulfated linear polysaccharides derived from marine red algae. Using electron microscopy and DLS analysis, the biogenic EPI-loaded CAO-AuNPs were shown to be spherical and homogenous, with an average diameter of 141 ± 6 nm. Figure 4a depicted a schematic representation of the EPI-loaded CAO-AuNP drug delivery platform and subsequent pH-triggered drug release under intracellular endo-/lysosomal environments. The cytotoxicity of EPI-loaded CAO-AuNPs on normal human umbilical vein endothelial cells (HUVEC) and malignant human hepatoma cells (HepG2) was assessed using a colorimetric test using sulforhodamine B (SRB). The results indicated that the EPI-loaded CAO-AuNPs had lesser cytotoxic activity on normal cells than free EPI, signifying that EPI-loaded CAO-AuNPs decreased the cytotoxicity of free EPI on normal cells. Both EPI and EPI-loaded CAO-AuNPs inhibited cell viability against HepG2 cells in a dose-dependent manner; however, the inhibition of EPI-loaded CAO-AuNPs was greater than that of EPI at the same dose. The scientists ascribed the viability assay results in AuNP specificity to improve cellular uptake and increase drug bioavailability. Furthermore, CAO with a certain amount of anticancer action may have a synergistic impact when combined with EPI. Besides, confocal laser scanning microscopy with DAPI staining (Fig. 4b) and flow cytometry analysis with Annexin V-FITC, and propidium iodide staining revealed that EPI-loaded CAO-AuNPs were confined in the cellular nucleus and reported to induce more apoptosis in HCT-116 and HepG2 cells than free EPI in a concentration-dependent manner. The results of an in vitro drug release test revealed that the release of EPI from NPs was considerable under acidic environment resembling a cancer condition. At the same time, it was minimal at physiological pH,

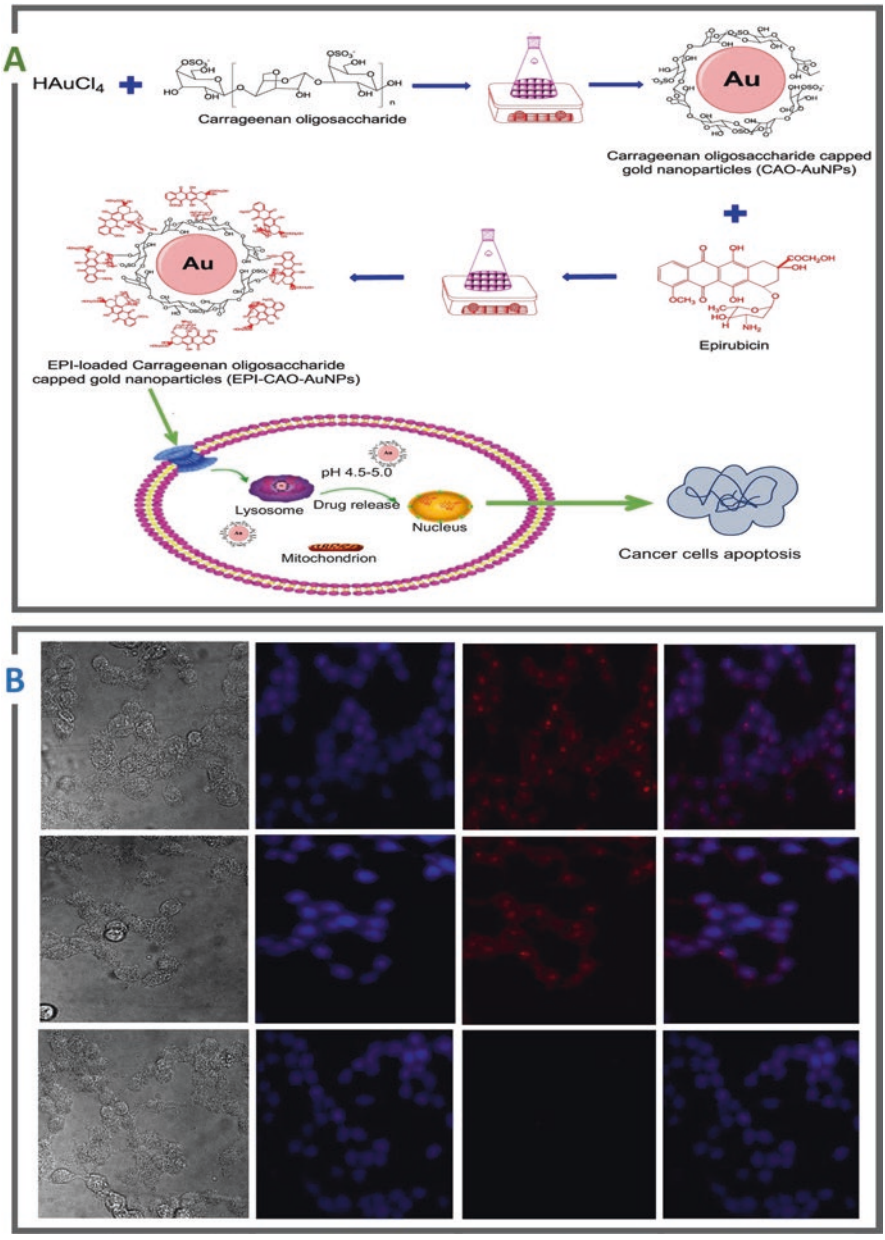

Fig. 4 (**a**) The EPI-loaded CAO-AuNP drug delivery system and subsequent pH-triggered drug release under intracellular endo-/lysosomal conditions are depicted schematically; (**b**) CLSM pictures of HCT116 cells treated with EPI-loaded CAO-AuNPs and free EPI for 4 h at 37 °C and DAPI for 10 min at room temperature [212]. (Reprinted from open-access article under a Creative Commons Attribution 4.0 International License (CC BY 4.0); Springer Nature, 2019)

which helps decrease the toxicity of EPI on normal tissue. As a result, the fabricated NPs have shown potential for cancer therapy [212].

On the other hand, a study reported using a rapid and straightforward in vivo biosynthesis method to prepare AuNPs using heavy metal-binding proteins (HMBPs) expressed in recombinant *E. coli*. The HMBPs were determined to serve as reducing, stabilizing, and capping agents in the formation of 5–20-nm-diameter spherical NPs. In HeLa cancer cells, the cytotoxic effects of AuNPs synthesized utilizing recombinant proteins (AuNPs@HMBPs) were compared to chemically formed AuNPs using the MTT assay. A citrate reduction of $HAuCl_4$ was used to create chemically generated AuNPs. The cell death rate for AuNPs@HMBPs was found to be 57.99% at 1 μM concentration (Fig. 5a), and the IC_{50} value was confirmed to be more than 1 μM. Moreover, at a 1 μM concentration of chemically produced AuNPs, high cytotoxicity (82.42% cell death) was found (Fig. 5a), with the IC_{50} value determined to be 0.05 μM. Additionally, DOX was conjugated to biogenic AuNPs (AuNPs@HMBPs@DOX) to provide significant toxicity for cancer treatment, which may be a more effective anticancer agent than free DOX. Figure 5b depicted the procedure used to fabricate and evaluate the cytotoxicity of DOX-loaded biogenic AuNPs. HeLa cells were treated with DOX-loaded biogenic AuNPs or free DOX at concentrations corresponding to 1 mg/mL of DOX

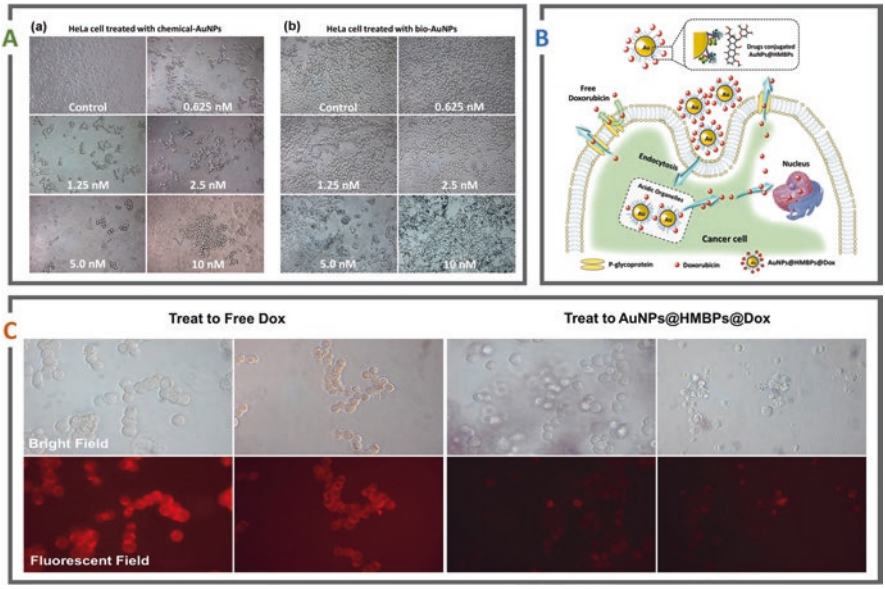

Fig. 5 (**A**) Dose-dependent cytotoxic effect of the (**a**) chemically synthesized AuNPs and (**b**) biologically synthesized AuNPs against HeLa cells; (**B**) schematic illustration of the preparation process used to synthesize biogenic AuNPs and subsequent conjugation of DOX. DOX-loaded biogenic AuNP complexes that are biodegradable may be easily fragmented to release DOX from AuNPs; (**C**) fluorescence photos of HeLa cells treated for 24 h with free DOX and DOX-loaded AuNPs@HMBPs. (Reprinted (adapted) with permission from Seo et al. [218])

before being studied with a fluorescence microscope equipped with phase contrast and fluorescence modules. Figure 5c revealed that DOX-loaded biogenic AuNPs had a stronger cytotoxic impact on HeLa cancer cells than free DOX. Cancer cells treated with DOX-loaded biogenic AuNPs had a higher proliferation inhibition than cells treated with free DOX. The morphology of the cells altered completely after incubation with DOX-loaded biogenic AuNPs, with cells acquiring a spherical shape rather than their characteristic spindle configuration, indicating apoptosis. The red fluorescence detected in cells treated with DOX-loaded biogenic AuNPs suggested cellular damage caused by AuNP uptake inside the cells. Nevertheless, no apparent apoptotic cellular death was seen in cells treated with free DOX, showing that AuNPs biosynthesized via HMBPs and DOX have synergistic toxicity [218].

Furthermore, a study reported the conjugation of DOX to pectin-capped AuNPs (PEC-AuNPs) for delivering it to hepatocellular carcinoma cells (HepG2 cells) wherein the asialoglycoprotein receptor (ASGPR) was overexpressed. As a bioreducing, capping, and stabilizing agent, pectin, a natural linear polymer comprising α-(1,4)-linked d-polygalacturonic acid residues, was utilized. DOX-loaded PEC-AuNPs had a high drug loading efficiency of 78%, were spherical with a particulate size of 17 ± 3 nm, and had a zeta potential value of −21.64 ± 2.93 mV, without any aggregation. The results of the in vitro drug release study revealed that the rate and quantity of DOX released from the DOX-loaded PEC-AuNPs demonstrated pH-responsive drug release behavior, with preferred release at pH 5. DOX will be released preferentially in an acidic environment since it will be released in endosomes and lysosomes following uptake by receptor-mediated endocytosis in target cells. MTT assay was used to assess the cytotoxicity of DOX solution, developed PEC-AuNPs, and DOX-loaded AuNPs against HepG2 and HeLa cells. Because of the intrinsic biocompatibility of pectin, the stability of PEC-AuNPs in the intracellular environment, and the absence of contact between anionic AuNPs and negatively charged cell membrane, synthesized PEC-AuNPs are noncytotoxic. DOX-loaded PEC-AuNPs were shown to have concentration-dependent cytotoxicity. The viability of HepG2 and HeLa cells reduced as drug concentration increased. In HepG2 cells, the IC_{50} values for pure DOX and DOX-loaded PEC-AuNPs were 4.11 and 0.74 μg/mL, respectively, demonstrating a considerable difference in IC_{50} values, which can be attributable to the interaction between DOX-loaded PEC-AuNPs and ASGPR. As a result, the intracellular drug concentration rises. In ASGPR-negative HeLa cells, though, the IC_{50} values for DOX and DOX-loaded PEC-AuNPs were 3.88 and 3.27 μg/mL, respectively, with no significant difference in the IC_{50} values. Furthermore, to understand the mechanism of PEC-AuNPs by HepG2 cells, the AuNPs were incubated with the cells at either a low temperature (4 °C rather than 37 °C) or in ATP-depleted conditions (cells pretreated with NaN_3). Following cell lysis and determination of the quantity of gold accumulated in cells, their results revealed that the underlying mechanistic route for uptake of PEC-AuNPs by HepG2 cells was receptor-mediated endocytosis. To establish that DOX-loaded PEC-AuNPs were internalized by ASGPR-mediated endocytosis, HepG2 cells were co-incubated with DOX-loaded PEC-AuNPs and galactose. Their findings proved that the addition of free galactose resulted in a considerable decrease in

absorption of DOX-loaded PEC-AuNPs in comparison to when galactose was not present. The authors stated that the decrease in absorption was caused by an overabundance of free galactose, which has a high affinity for ASGPR. The binding sites are effectively competed with and saturated by free galactose. As a result, the capacity of ASGPR to bind and internalize DOX-loaded PEC-AuNPs is reduced, resulting in a lower intracellular concentration of DOX [219].

Interestingly, a study developed a method to synthesize biocompatible green chemistry-based AuNPs by combining the gold solution with *Punica granatum* fruit peel extract and functionalizing it with folic acid for targeted delivery of 5-Fu to MCF-7 cancer cells, which are known to overexpress folate receptors. The average particle size of 5-Fu-loaded AuNPs was 70 nm, and 78% of the 5-Fu bound to AuNPs was discovered. The in vitro release profile of 5-Fu-loaded AuNPs revealed an early burst release of the drug during the first hour, followed by a steady, gradual drug release of 22.92% until 48 h. The authors argue that the presence of epigallocatechin in the plant extract might be a factor in the reduction of Au^{3+} to create Au^{0}. Furthermore, the AuNPs generated using this approach were shown to be exceptionally stable, which might be attributed to the inclusion of ellagitannins such as punicalagin. In zebra fish embryos, the in vivo toxicity of AuNPs, 5-Fu, and 5-Fu-loaded AuNPs was studied over 4 days. Toxicity was determined by examining the hatching rate, percentage survival rate, and embryo morphology. As shown in Fig. 6a, AuNPs at a concentration of 1000 ng were determined to be less toxic than the control. Up to the maximal concentration of AuNPs, the hatching rate remains constant. The larvae survival rate remained stable up to 800 ng AuNPs and then declined. The larvae's overall body form, heart, and pericardial sac were normal at all AuNP concentrations; however, poor pigmentation was detected as the AuNP concentration increased from 800 to 1000 ng. Figure 6a showed that the free 5-Fu and 5-Fu-loaded AuNPs did not exhibit any hatching delay or morphological alterations. When the concentration of free 5-Fu and 5-Fu-loaded AuNPs increased compared to the control, the survival percentage fell. The cytotoxicity of free 5-Fu, and 5-Fu-loaded AuNPs, against MCF-7 cells (breast cancer) was assessed using MTT, and it was discovered that the quantity of 5-Fu required to achieve 50% growth inhibition (IC_{50}) was substantially lower when compared to free 5-Fu. The inhibitory effects of 5-Fu-loaded AuNPs on MCF-7 cell cycle progression were investigated further using flow cytometry. In a dose-dependent manner, 5-Fu-loaded AuNP therapy caused G0/G1 phase cell cycle arrest. After 24 h of treatment with various doses of drug-loaded NPs, the fraction of cells in the G0/G1 phase increased compared to the control. In addition, in the presence of drug-loaded AuNPs, the fraction of cells in the G2/M and S phase was reduced. The high dose of 500 ng/mL, on the other hand, revealed a significant increase in G0/G1 phase (71.81%) compared to the control (29.17%), demonstrating that 5-Fu-loaded AuNPs promoted apoptotic cell death via G0/G1 cell cycle arrest. The study was conducted to determine if receptor-mediated pathways induced cell death by looking at whether the 5-Fu-loaded AuNPs downregulated Bcl-2 (B cell lymphoma 2 gene) and upregulated Fas and FasL (Fas ligand) expression. As shown in Fig. 6b, the IC_{50}

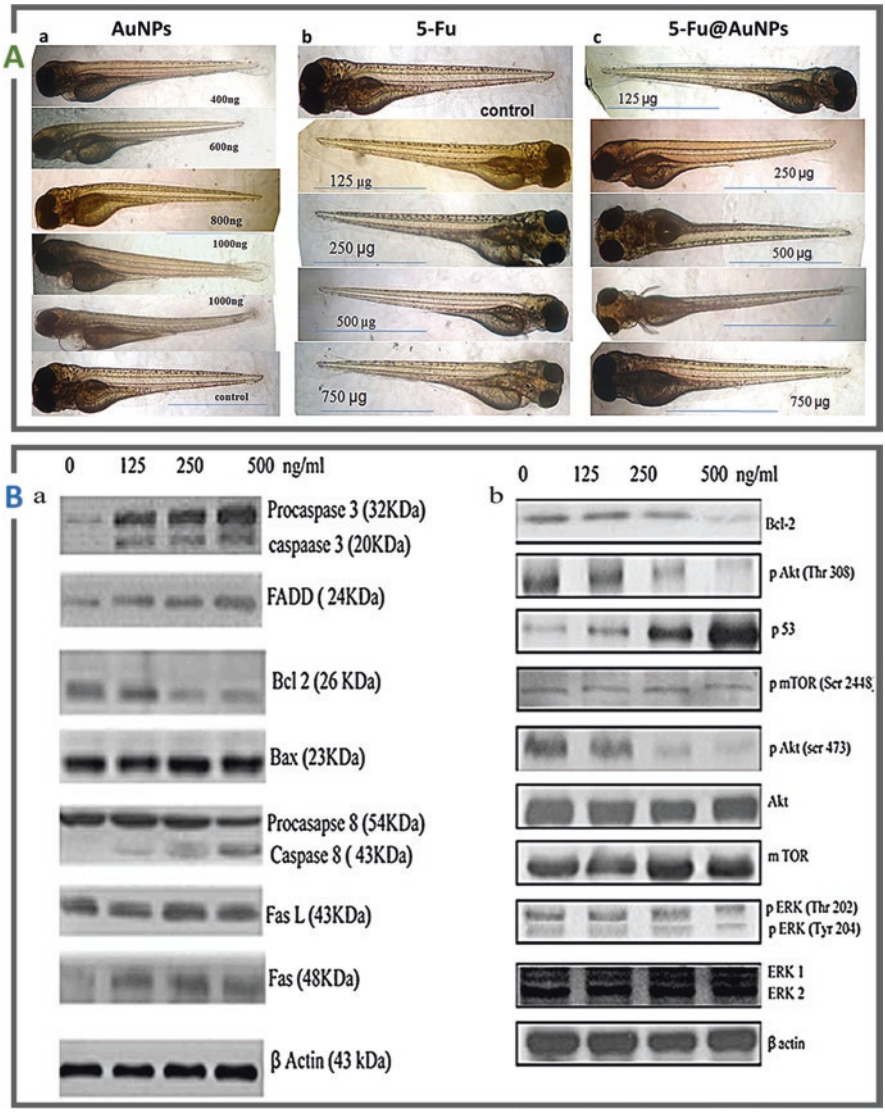

Fig. 6 (**A**) Morphologic analysis of zebra fish embryo toxicity of (**a**) AuNPs, (**b**) 5-Fu, (**c**) 5-Fu-loaded AuNPs; (**B**) (**a**) the effect of 5-Fu-loaded AuNPs-FA on Bcl-2 (B cell lymphoma 2 gene) downregulation and upregulation of Fas and FasL (Fas ligand) expression. (**b**) The effect of 5-Fu-loaded AuNPs-FA on the downregulated PI3K/AKT/mTOR activation pathway in MCF-7 cells. (Reprinted (adapted) with permission from Ganeshkumar et al. [220])

concentration of drug-loaded NPs elevated the expression of caspase 8 and pro-apoptotic proteins Bax and Fas/APO-1 receptor [220].

5 Conclusions and Future Outlook

Natural resources for developing efficient and safe formulations have recently grown at an exponential rate. To capitalize on the synergy between nanotechnology and natural resources, the green synthesis of nanomaterials has lately gained much attention. The "green" approach for synthesizing nanostructures, which is expeditiously displacing the current costly physical and traditional non-eco-friendly chemical techniques, seems to be of great interest due to its high sustainability, economic perspectives, convenience, nontoxicity (chemical-free), less energy-intensive solution, and variety of applications in several areas such as drug delivery, cancer treatment, gene therapy, antibacterial factors, as well as biosensors. In a related context, in the last few years, bioactive NPs have been synthesized using a variety of biological components that function as both reducing and stabilizing agents. The natural machinery used by all biocomplex systems to execute specific redox reactions in aqueous solution and under standard conditions of temperature and pressure offers a robust approach for generating a wide range of nanosized structures, often at a fraction of the expense of conventional chemical methods. However, the exact role of active metabolites and functional groups in partial or complete bioreduction of metal ions is still being debated. As a result, developing a logical strategy requires a detailed understanding of the bioreduction processes involved in reducing metallic ions to NPs.

To achieve effective and reproducible characteristics, biofabrication of MNPs is always accomplished under controlled reaction settings. It is worth noting that pH, reactants, temperature, and reducing agents all had an impact on the biosynthesis pathway of NPs as well as their pharmacological implications, which included antibacterial, antitumor, antioxidant, and drug delivery. More thorough research on the optimization of reaction conditions and the creation of hybrid biological resources is recommended to increase the production of proteins, enzymes, and other biomolecules engaged in the construction and stability of NPs. With that being said, biosynthesized NPs have been utilized in practically every sector where conventional NPs have been used. One of these applications is the role of biosynthesized MNPs as nanocarriers for active or passive drug delivery systems. As thoroughly explained earlier, although there are various advantages to depositing drugs on the surface of NPs, papers highlighting the potential of MNPs as nanocarriers are scarce. As a result, scientific investment and collaboration are required to develop the drug-metal conjugated complex. Considering various benefits, the toxicological factor is a major worry. As a result, substantial research in this field will be required to efficiently exploit noble MNPs coated with biopolymers in drug delivery.

Last but not least, given the amount and progress of nanoparticle biosynthesis research in recent years, the mentioned field looks to be on the verge of considerably more broad applied research. Meanwhile, no clear ideal condition has been identified for the proper synthesis and development of bioconjugated NPs with specified properties; consequently, greater study and enhancement of the underlying synthesis procedures are necessary for the described subject. Besides, extensive

pharmacodynamic and pharmacokinetic profiling is required to determine the precise pathway, distribution, and side effects. Long-term in vivo investigations of drug-conjugated nanocarriers are also required to assess the toxicity and effectiveness of biosynthesized MNPs.

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Nanobody-Based Delivery Systems for Diagnosis and Therapeutic Applications

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1 Introduction to Nanobodies

Nanobodies, also known as nanocarriers, nanoparticles, nanomaterials, etc., are being increasingly used for disease diagnosis, drug delivery, nanomedicine design, and bioimaging. Nanotechnology has brought a revolution into the field of drug delivery and thereby reshaped the approaches used by the pharmaceutical industries. Tremendous efforts are vested to improve the fate and function of nanobodies being employed for better health services. In modern medical science, nanotechnology is all over the areas of drug delivery, in vitro diagnostics, in vivo imaging, therapy techniques, biomaterials, tissue engineering, etc. [1]. Recent pharmaceutical techniques are increasingly inclining toward the utilization of multifunctional properties of nanomaterials and employ them as multipurpose smart nanobodies for diagnosis and therapeutic applications. Various engineering strategies are being continuously opted to improve their efficiency to achieve targeted delivery with precision [2]. These nanobodies can significantly contribute to overcome the barriers faced by the solo therapeutics and permeate through biological barriers like the

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blood-brain barrier (BBB), skin, small intestine, mucosa, etc. Nanobodies for drug delivery are increasingly contributing to overcome the challenges posed by patient heterogeneity. These nanobodies can protect drugs from premature degradation, improve their half-life inside the circulatory system, reduce immunogenicity, and enhance sustained delivery. Thus, both the toxic side effects and the administration frequency of drugs have got substantially reduced. Since its inception, this field of study is focusing on developing delivery platforms which can address numerous such problems to minimize any adverse impact but maximize therapeutic activity. After the first description of liposomes in the 1960s, a variety of nanobody-based drug delivery systems have been developed, and several of them are approved by the Food and Drug Administration (FDA). A summary of the progress of such nanobody-based drug delivery systems highlighting their important milestones is presented in Fig. 1 [3].

1.1 Basic Properties

Traditionally employed drug delivery systems often suffer from various drawbacks. They are sensitive to toxicity, demonstrate relatively poor targeted delivery, and result in reduced therapeutic efficiency. Moreover, due to their large size, it is often challenging for such materials to permeate through biological barriers, their uptake through endocytosis is inefficient, and the drug loading efficiency is poor. At this juncture, the necessity of nanobody-based drug delivery systems is huge. These nanobodies, having at least one of their dimensions below 200 nm, which is

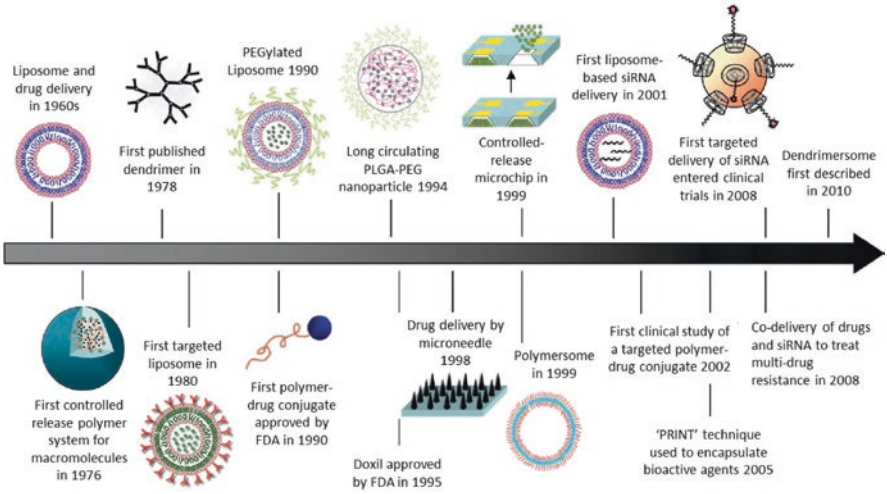

Fig. 1 A timeline to show the progress on the development and use of nanobody-based drug delivery systems highlighting some significant milestones achieved. (Adapted with permission from [3])

desirable for drug delivery carrier design, are often found to demonstrate improved cellular uptake and achieve sustained and targeted drug delivery. The properties of nanobodies are different than that of their bulk materials. These nanobodies can have a substantially high surface-area-to-volume ratio which thereby can exhibit high drug loading capacity. They can also be modified to load multiple drugs and be multi-stimuli responsive for drug release. Incorporation of a suitable fluorescent entity into these nanobodies is also possible and hence can simultaneously be used for disease diagnosis and drug delivery monitoring purposes through bioimaging. They can also be used for 3D live cell bioimaging purposes [4, 5]. These advantages have significantly contributed to explore the pharmacodynamics and pharmacokinetics of various therapeutics. In the field of biomedicine, these nanobodies contribute to improve water solubility/dispersibility, biocompatibility, bioavailability, biodegradability, antioxidant and antimicrobial activity, bioimaging, etc. In modern medical science, these nanobodies are also being used on medical devices, such as lenses, stents, etc., for localized drug delivery [6–9]. Being of nanoscale dimension, these nanobodies can easily pass through the tiny capillaries in physiological bodies. Thus, these nanobodies are often called "smart nanocarriers" and are advantageous over the conventional drug delivery systems [10].

1.2 Different Types of Nanobodies Used in Drug Delivery

The nanobodies used for drug delivery can be divided into three main categories, namely, (i) organic, (ii) inorganic, and (iii) hybrid nanobodies (Fig. 2a). Representative examples for each type of these nanobodies are presented in Fig. 2b [11]. Based on the materials used for their formation and application fields, these nanobodies can have multiple subcategories.

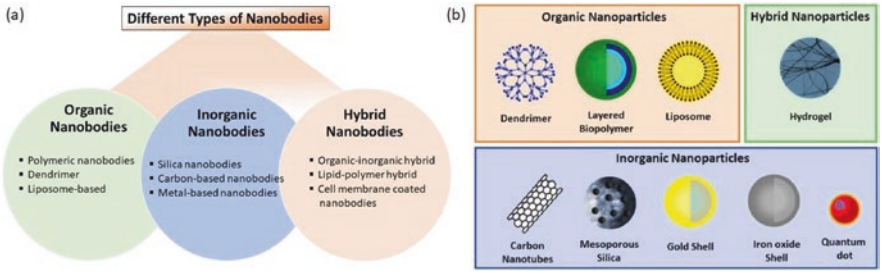

Fig. 2 (**a**) The three types of nanobodies popularly used for drug delivery, organic, inorganic, and hybrid nanobodies. (**b**) Schematic presentation of different types of nanobodies. (i) Organic, (ii) inorganic, and (iii) hybrid. (Adapted with permission from [11])

1.2.1 Organic Nanobodies

Organic nanobodies for drug delivery are mainly composed of dendrimers, polymers, and lipid-based (liposomes) materials. These nanobodies are being explored and developed for decades. The first nanobody-based drug approved by FDA in 1995 was liposome-based which had the outer layer made of lipid and the core as a hydrophilic milieu. Thus they can encapsulate both the hydrophilic drugs at the core and hydrophobic drugs at the outer layer [12]. *These nanobodies have gone through several breakthroughs and improved their circulation half-life* by modifying their size, charge, and composition. This has resulted in improvement in their stability and contributed to overcome the challenge of rapid clearance that often occurred after injection. It has been found that by modifying the surface of liposome shells using hydrophilic polymers, such as polyethylene glycol (PEG), the circulation lifetime can significantly be increased. This can reduce the adsorption of blood protein during circulation (opsonization) and thereby their clearance from the body. However, it is mentioned that a longer half-life may not be required for all the cases, especially when the drugs are to be released quickly after their intake by the body. Hence, various liposome-based nanobodies have been developed with controllable half-life. Due to the availability of PEG having different molecular weights (i.e., variable chain length), the circulation time, solubility in aqueous and/or organic media, improved biocompatibility, etc. can easily be achieved via the appropriate surface modification of liposomes. Improved elasticity, skin penetration, delivery of insoluble drugs, sensitivity to stimuli, and targeted delivery have contributed to the widespread applications of liposome-based nanobodies in clinical practices.

Polymer-based nanobodies also come under this category. Unprecedented progress has been made in developing various types of polymeric nanobodies for drug delivery. It is relatively easy to synthesize polymers of variable properties, such as different chain lengths, biocompatibility, biodegradability, conformational flexibility, ability to host ionic charges, and numerous functional groups. Hence, these materials are highly promising to introduce a variety of properties into the polymeric nanobodies [13–17]. Various types of organic nanobodies can be prepared for their use as drug delivery nanocarriers, such as nanomicelle, dendrimer, nanosphere, nanocapsule, nanogel, nanocomposite, polymersome, etc., and can be stimuli-responsive for drug loading and release (Fig. 3) [18]. The possibility of the use of the physical, chemical, and biological stimuli, both independently and together, has presented these nanobodies as promising candidates for various clinical trials. Moreover, the use of biodegradable polymers for fabricating nanobodies has shown the way to overcome the concern of chronic toxicity and high immunological response. Such nanobodies are already in use for designing surgical materials, bone replacement, vaccine delivery, plasma expander, etc. [18]. However, it is to note that some popularly considered biodegradable polymers have also been recently found to demonstrate some toxicity. This is because the properties of a polymer can get altered when they are in the form of nanobodies [19]. It is also to note that variation in sizes of nanobodies can also alter cytotoxicity, as demonstrated by Xiong et al. using poly(lactic-co-glycolic acid) (PLGA) nanobodies of

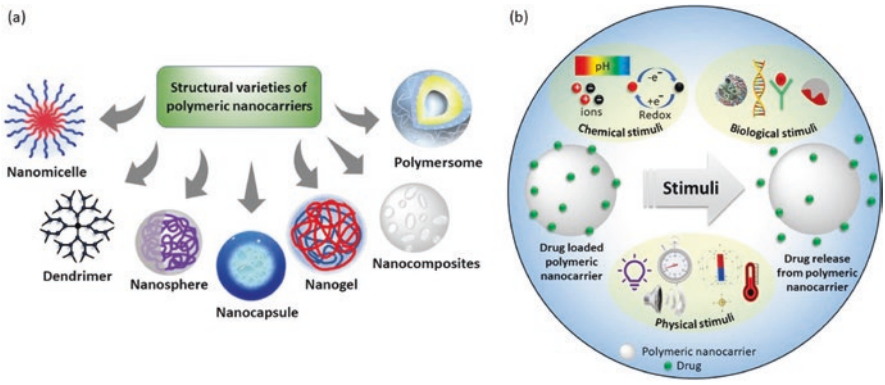

Fig. 3 (**a**) Different types of polymeric nanobodies based on their structural varieties and (**b**) various types of stimuli which can regulate drug release from these nanobodies. (Adapted with permission from [18])

different sizes [20]. Polymer-based nanomicelles can be prepared of different sizes ranging from 20 to 200 nm and have low *critical micelle concentration (cmc)* values which are beneficial in their biomedical applications as they would be comparatively more stable. The *cmc* values of micelles prepared using poly(poly-(ethylene glycol) methyl ether monomethacrylate were very low (1.4 to 2.6 mg L^{-1}), and the average diameter was in the range of 140 to 250 nm. These nanobodies exhibited 96% drug release when tested for a hydrophobic drug nifedipine at pH 7.4 within 24 h of administration [21]. Polymeric nanovesicles, also known as polymersomes, of size 80 to 150 nm have also shown good uptake by the biological cells [22]. These types of nanobodies can co-load both the hydrophilic and hydrophobic drugs using their aqueous interior and the exterior membrane, respectively [23]. Recently, nanogels that are the swellable nanostructures of polymeric materials are being popularly used due to their enhanced drug loading capacity and improved stability [24–26]. Organic nanobodies can also be of different structures, such as nanosphere, hollow, core-shell, multilayered, surface-functionalized, etc. They can be responsive to pH, magnetic field, temperature, light, biomolecules, etc. They can also be multi-stimuli responsive and at the same time capable of delivering multiple loads. For example, multi-stimuli-responsive polymeric nanogel has been investigated for the co-delivery of paclitaxel, Nile red, and doxorubicin [27]. Nanocapsules are another type of organic nanobody which hosts a cavity in which drugs can be loaded. The outer membrane of such nanobodies plays a role of protecting the loaded drug and thereby contributes to diminish premature degradation or delivery [28]. Another widely used organic nanobody is made of star-like oligomeric or polymeric dendrimers. These nanobodies can be of size 5 to 10 nm and hence highly preferable for intravenous as well as pulmonary systems. The size of these nanobodies can be altered depending on the number of generations, and the size can increase as the number of generations increases [29]. Their architectures can also be varied by altering molecular chirality, branching, and molecular composition of the dendrimer at G0 generation.

These organic nanobodies can also be prepared to be fluorescent and multifunctional materials that can respond to stimuli to deliver drugs at the targeted sites at the same time. Jana et al. have designed perylene-3-ylmethanol fluorescent nanobodies which have demonstrated four roles, (i) served as a nanocarrier for drug loading and delivery, (ii) responded to photons for drug release, (iii) functioned as a chromophore for imaging of biological cells, and (iv) real-time monitoring for drug release [30]. Thus, organic nanobodies are highly potential for drug delivery as they can be designed as multifunctional carrier systems. Despite various advantages of these organic nanobodies, their susceptibility to destabilization or disassembly influenced by an environmental milieu or harsh condition parameters often triggers them to cause premature delivery, which is a great challenge that needs to be addressed.

1.2.2 Inorganic Nanobodies

Inorganic nanobodies mainly include carbon-based nanomaterials (e.g., carbon nanotubes, quantum dots), metal and metal oxide-based nanobodies (e.g., gold, silver, copper, iron oxides), lanthanide-doped upconversion nanoparticles, silica nanoparticles, etc. [31]. Different types of inorganic nanobodies often used for drug delivery are presented in Fig. 4a [32]. These nanobodies are advantageous due to their high surface-area-to-volume ratio. They are comparatively more stable, and

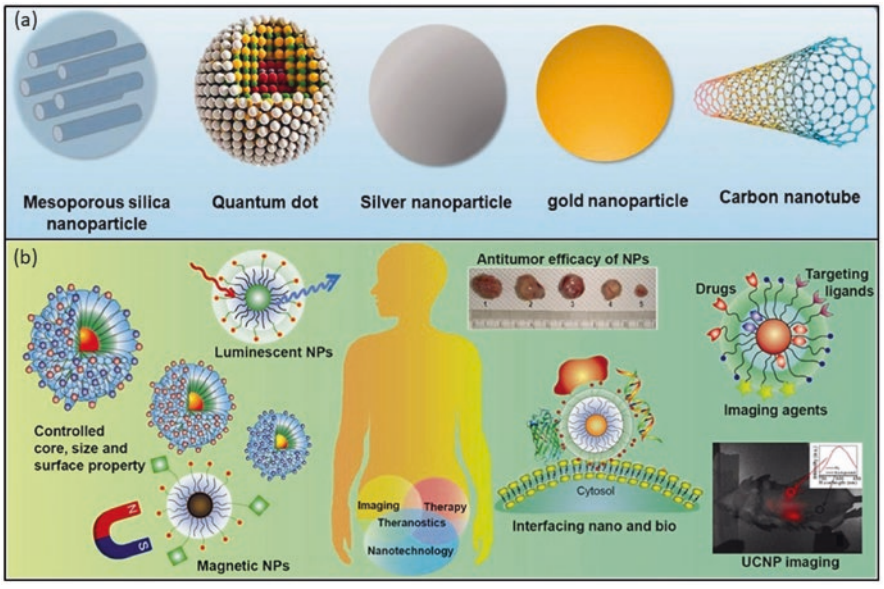

Fig. 4 (**a**) Different types of inorganic nanobodies often used for drug delivery. (Adapted with permission from [32]). (**b**) Use of inorganic nanoparticles in various bioapplications. (Adapted with permission from [36])

modification of their surfaces is relatively easier. However, they often exhibit poor biocompatibility and biodegradability resulting in non-negligible toxicity concerns and limited use in clinical practices [33]. Among various inorganic nanocarriers, gold nanobodies are most investigated for drug delivery [32]. Inorganic nanobodies have been prepared as nanospheres, nanorods, nanoshells, nanocages, etc. [34]. These nanobodies can also serve as nano-templates as their surfaces can be modified by various molecules suitable for loading, protection, and delivery of drugs. It has been found that such inorganic nanocores can also serve as nanoprobes so that bioimaging can be carried out [5, 35]. The surface modifications contribute to improve the biocompatibility and pharmacological properties of inorganic nanobodies and thereby have got various bioapplications (Fig. 4b) [36]. At present, various inorganic nanobody-based nanomedicines are being evaluated for their suitability through clinical trials [37]. Thus, the inorganic nanobodies can be used for therapy, theranostic, and imaging purposes.

Though these inorganic nanobodies have various advantages, their biological compatibility issues are prominent. Injected nanobodies are often engulfed by the reticuloendothelial systems within a few minutes to hours of administration which hinders their targeted delivery and can lead to nonspecific delivery of payloads resulting in damages to healthy cells or tissues. Of course, to maximize biocompatibility their surfaces are modified by biocompatible polymers, such as polyethylene glycol, etc.; nevertheless, their cytotoxicity, immunogenicity, and poor cellular uptake are still the challenges. Hence, more alternative ways are to be explored, such as the use of proteins, peptides, etc. for the modification of inorganic nanobodies [38]. The challenges of permeating through the biological barriers are also to be taken care of. Even though the permeation through biological barriers by inorganic nanobodies is often more efficient in comparison to other nanomaterials, modifying them to target antibodies is found to be advantageous as observed through various in vivo studies. Thus, the efficient permeation favors effective biodistribution of these nanobodies to the targeted pathological tissues and limits nonspecific delivery of drugs. These inorganic nanobodies are also designed to achieve better renal clearance. Nevertheless, renal clearance is one of the major challenges faced during their clinical translation because most of these inorganic nanobodies are made of heavy metals which are toxic to physiological bodies [39]. It is to note that there are not yet sufficient studies evaluating the toxicity of inorganic nanobodies, especially in vivo; hence there are various facts that remained unknown in this field. At present more than ten different compositions of inorganic nanobodies are being used in clinical practices. They are being used to improve pathological targeting, drug loading, and escaping the immune system. In addition, they are also being used as contrast agents for magnetic resonance imaging (MRI), X-ray, and computed tomography (CT) scan. These nanobodies are also in use for generating localized heat or reactive oxygen species (ROS) as they can be stimulated by external inducements, such as magnetic fields, near-infrared (NIR) radiation, radioactive exposure, etc. However, incautious use of these inorganic nanobodies always has the risk of adverse effects, such as diarrhea, skin rash, electrolyte disbalance, chest pain, decreased oxygen saturation, dyspnea, hypotension, and many more. Thus, more

studies are to be carried out to design novel inorganic nanobodies for future nanomedicine so that they can become suitable to transfer from bench to bed.

1.2.3 Hybrid Nanobodies

As mentioned earlier that both the organic and inorganic nanobodies have their benefits as well as drawbacks, hence it is always desired to design nanobodies that can incorporate benefits from both of these nanobodies while their drawbacks are minimized. To achieve this target, the most common strategy is to form nanobodies which are a hybrid of these two [40]. Thus, these hybrid nanobodies used for drug delivery are mainly composed of complexes formed using both organic and inorganic materials. Some of the examples could be organic-inorganic, lipid-polymer, cell membrane-coated nanobodies, etc. (Fig. 5) [41–43]. They can be in the form of nanospheres, nanocomposites, nanocapsules, nano-hydrogels, core-shell-type nanobodies, etc.

One of the very common practices is to combine the organic and inorganic nanobodies to prepare the organic-inorganic-type nanobodies for drug delivery. These nanobodies have gained the great interest for controlled delivery. For example, the surface of iron oxide nanobodies modified by chitosan-grafted polylactic acid is an example of such organic-inorganic nanobodies which have demonstrated good stability and high anticancer drug loading efficiency of doxorubicin at about 500 μg/mg of the carrier [44]. For targeted drug delivery, Liang et al. designed organic-inorganic nanobodies through self-assembly having multi-functionalized surfaces [45]. Here, they have designed KALA/heparin-biotin/heparin/chitosan/calcium

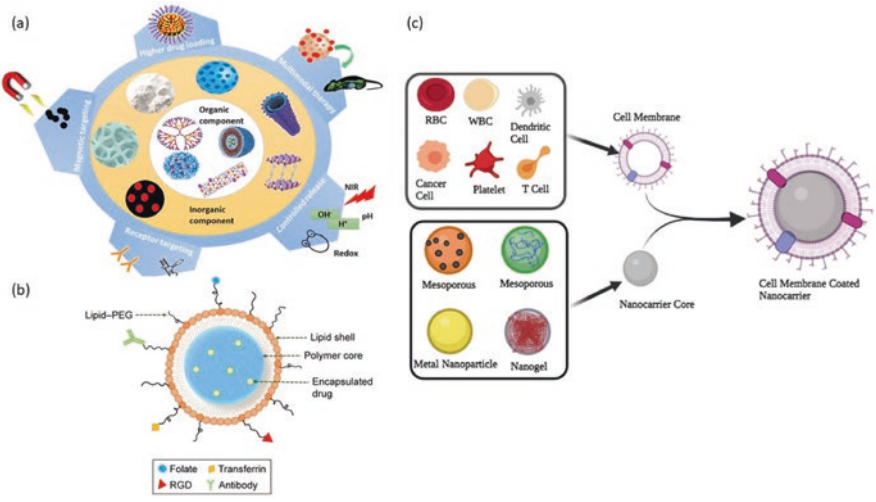

Fig. 5 Different types of hybrid nanobodies used for drug delivery. (**a**) Organic-inorganic [41], (**b**) lipid-polymer [42], and (**c**) cell membrane coated [43]. (Adapted with permission from [41–43])

carbonate (KALA is a cell-penetrating peptide) pH-responsive hybrid nanobodies which have been used to deliver anticancer drugs and showed active targeting phenomenon with penetrating capability. Kawamura et al. have designed bisphenol A-responsive organic-inorganic hybrid nanobodies that had cyclodextrin as ligands attached to a polymer that was on the silica nanobodies [46]. Similarly, liposome-silica hybrid nanobodies have been designed and used for treating breast and prostate cancers, where the silica nanobody served as the core and the lipid bilayer as a shell [47].

Similarly, lipid-polymer hybrid nanobodies have also shown promising results in delivering drugs to treat pancreatic, breast, as well as the prostate cancers. In most of these cases, polymer has served to form the core nanostructure, while the lipid formed the shell of such nanobodies. Dehaini et al. have developed ultrasmall lipid-polymer hybrid nanobodies having polymeric core coated with a layer of lipids, of size sub-25 nm to achieve the target delivery of chemotherapeutic docetaxel to treat mouse tumors [48]. Lipid-polymer-based hybrid nanobodies have also been found to use for the delivery of both the hydrophilic and lipophilic drugs for cancer therapy. Tahir et al. have designed such hybrid nanobodies using lecithin, poly(D,L-lactide-*co*-glycolide), and 1,2-distearoyl-Sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)]-2000 which delivered both the hydrophilic and lipophilic drugs [49]. Similarly, Gao et al. prepared iRGD (arginyl-glycyl-aspartic) peptide-modified lipid-polymer nanobodies to deliver isoliquiritigenin to improve anti-breast cancer effect and tumor-targeting capability [50]. Thus, these types of nanobodies often demonstrate high biocompatibility and biodegradability and can be prepared to load and deliver both the hydrophilic and hydrophobic drugs to provide better therapeutic effects. They can also avoid fast clearance from the body and get well internalized by cells [51].

Recently, cell membrane-coated hybrid nanobodies are getting increasing attention as this technique imparts biological properties directly onto the nanobodies [52, 53]. Naturally derived cell membrane can facilitate immune escape, long circulation time, drug delivery potency, and biocompatibility. The membranes used for nanobody coating can be derived from cancer cells, immune cells, red blood cells, macrophages, etc. [54]. Fang et al. have reported polymer nanoparticles coated with a layer of membrane derived from cancer cells for anticancer vaccination and therapeutic delivery [55]. Similarly, nanoparticles coated with the cell membrane and functionalized with neurotoxin-derived peptide have been used for the targeted delivery of drugs to the brain [56]. Stem cell membrane-coated nanogels have also been used for tumor-targeted delivery of drugs [57]. Though nanobodies coated with cell membranes are rising as next-generation therapeutics, it is found that achieving perfect coating of nanobodies is often challenging and hence requires further attention to improve the coating process. It is to note that nanobodies perfectly coated with membrane can enter the cells individually whereas the partially coated nanobodies get aggregated before their internalization by cells [58]. By controlling this phenomenon, the mechanism and efficacy of nanobody internalization by cells could also be controlled.

1.3 Nanobodies Achieving Various Targets in Drug Delivery

Targeted delivery of therapeutics at a desired site is an important characteristic of nanobodies which thereby reduces undesired damage to the normal cells or tissues. These nanobodies contribute to overcome the limitations of traditional drug carriers. There are several studies where the emphasis is given to design nanobodies to achieve targeted delivery.

Progress in targeted delivery is achieved by designing suitable nanobodies that can cross through various biological barriers, such as blood-brain barrier, skin, mucus membrane, etc. Suitable modification of nanobodies can facilitate targeted delivery. These nanobodies can improve the pharmacokinetics and pharmacodynamics of drugs and result in effective intravenous administration which thereby can maximize drug stability while minimizing its degradation. To achieve targeted delivery by supplying drugs at the diseased sites, these nanobodies are to be biocompatible, permeable through biological barriers, able to protect drugs from premature release and degradation, an optimized lifetime in the circulatory system, etc. Here, the shape and size of nanobodies along with their surface properties play crucial roles both in cellular uptake and targeted delivery. They can also enhance the solubility of otherwise poorly soluble drugs and thereby decrease their dosage which brings safety to untargeted healthy cells or tissues from undesired toxicity.

There are mainly two mechanisms for targeted delivery. One is known as active targeting and the other is passive targeting. Both of these mechanisms are employed to achieve targeted delivery. The active delivery pathway often results in a significant increase in drug delivery to the target cells in comparison to the passive delivery pathway or the delivery of solo drugs (without carrier). An example of an active targeting strategy could be the use of ligand on the surface of nanobodies, which is specific to an acceptor located at the target site. This is often practiced for drug delivery to the cancer cells or endothelial cells of tumors which overexpress specific receptors. Thus, a drug-loaded nanobody having ligands specific to these receptors will specifically interact with them and cause specific binding resulting in the arrival of drugs at the targeted cancer/tumor site. For example, hyaluronic acid (HA) is a suitable targeting moiety for the nanobodies made of poly(lactic-*co*-glycolic acid) (PLGA) as it can bind to CD44 receptors which are overexpressed in some tumor types. Cerqueira et al. designed PLGA nanobodies whose surfaces were modified with HA to achieve targeted delivery of paclitaxel to treat triple-negative breast cancer [59]. On the other hand, it has been observed that the chitosan nanoparticles can achieve targeted delivery to the tumors on specific organs through the enhanced permeability and retention (EPR); this process is known as the passive delivery pathway [60]. In some cases, to achieve even higher targeted delivery, both active and passive pathways can be adopted simultaneously. For example, 5-fluorouracil-loaded chitosan nanobodies incorporated with HA were used to target colon tumors by exploiting the elevated level of HA receptors expressed by the tumor cells as well as the EPR effect of the nanobodies [61]. There are a growing number of works employing these techniques to achieve targeted delivery of drugs with adequate success.

2 Necessity of Nanobodies in Drug Delivery

Traditional drug carriers have various drawbacks in comparison to the nanobodies used in modern health science. For example, traditional drug delivery carriers often show very poor loading capacity; as a result, the drug dosage must be high and frequent to heal a disease. This increases the possibility of side effects on the healthy cells and tissues. Additionally, due to their bulkiness, they often cannot pass through the tiny pores or passages in a physiological body to reach the targeted site. This also makes them available for a longer period in the circulatory system where they are susceptible to degradation by various enzymatic actions. Reaching the sufficient local concentration of a drug at the targeted site is highly challenging in traditional drug delivery methods. Thus, though the conventional delivery methods have been successful to some extent, the main disadvantages are their requirement of high dosage, inadequate bioavailability, harmful side effects, a rise in drug resistance, lack of targeted delivery, low therapeutic effects, etc. [62]. To overcome these drawbacks, various nanobodies for drug delivery are being developed.

2.1 Selected Challenges of Drug Delivery

Rapid developments in nanotechnology have a huge contribution to designing different types of nanobodies, and their applications in drug delivery science have resulted in unprecedented progress. With the help of nanotechnology, one can engineer various functional materials at the molecular level. A well-known advantage of this is the progress achieved in developing various nanomedicines. In drug delivery science, nanobodies are engineered in various forms, such as nanoparticles, nanomicelles, liposomes, nanoemulsions, polymer-therapeutic conjugates, nanocrystals, nanovesicles, nanogels, nanotubes, etc. Though these nanobodies have extraordinary contributions to nanomedicine, one of the most needed steps is to carry out proper physicochemical characterization before their in vivo applications. However, the diversity and complexity of different nanobodies often pose hurdles to prepare a general guideline, so the characterization of nanobodies could not be harmonized yet [63]. Moreover, since different nanobodies interact with biological components differently, a validated method to test their safety and toxicity concerns is yet to be established. This is one of the major challenges in evaluating the toxicity of these nanobody-based drug delivery systems. This is also a vital impediment to translating the discoveries from bench to bed. Though one of the most popular uses of nanobodies is their delivery to the cancer cell, the greatest challenge here is to deliver drugs efficiently and safely. In a review by Wilhelm et al., it is pointed out that the full benefit of nanobodies in drug delivery is not yet achieved [64]. Analyzing the data available for the last 10 years in nanobody-based drug delivery to tumor cells, they found that only about 0.7% of the administered nanoparticles have reached the targeted site. The reasons are attributed to low delivery efficacy,

competition between organs to receive the nanobodies, extravasation of nanobodies, inter-interactions between nanobodies at a targeted site, etc. In addition to these, large-scale production following the standards of good manufacturing practice (GMP), quality control, need for more toxicological studies, inadequate understanding of the interactions between the nanobodies and the cells/tissue, and low stability of nanobodies after in vivo administration are some of the challenges often faced during the development of suitable nanobody-based drug delivery carrier [65]. Hence, solving these issues can only expedite the translation of the findings from bench to bed.

2.2 Advantages and Disadvantages of Nanobodies in Drug Delivery

Nanobodies can prolong the duration of actions of the loaded drugs which thereby reduces the frequency of dosage. A suitable choice of materials for designing these nanobodies can also protect against premature drug degradation and increases their bioavailability. These nanobodies can minimize the negative side effects of the drugs and contribute to improve patient compliance. They can also stabilize the concentration fluctuation in plasma and thereby improve drug utilization. Along with these advantages, the nanobodies do pose some challenges such as the materials used for designing the nanobodies can be toxic and, in some cases even if their constituent materials are not toxic, the side products generated after their degradation can show toxicity. Sometimes surgery may be necessary to remove such materials from the body and the process may become tedious and expensive. The low stability of some of the nanobodies is also a big concern. Additionally, many of the nanobodies are unable to protect sensitive therapeutics, such as therapeutic proteins, from being degraded by harsh condition parameters, such as heat, high or low pH, the enzymatic actions of protease, etc.

2.2.1 Nanobodies in Cancer Therapy

The traditional cancer therapies, such as radiation therapy, chemotherapy, immunotherapy, and targeted therapy, often suffer from a lack of specificity, toxicity, and high drug resistance. Hence, the search for alternative therapies became an immediate necessity, which triggered the investigations of nanobody-based drug delivery systems. The use of nanobody-based therapeutics for cancer therapy has an immense contribution; in fact, the most investigated nanobody-based therapeutics are for cancer therapy (Fig. 6) [66]. This is mainly because the efficacy of a specifically designed nanobody to deliver the therapeutics to the target cancer cells is relatively high. They can deliver the cargo only after they reach the targeted site. As a result, the side effects of damaging the untargeted healthy cells or tissues become

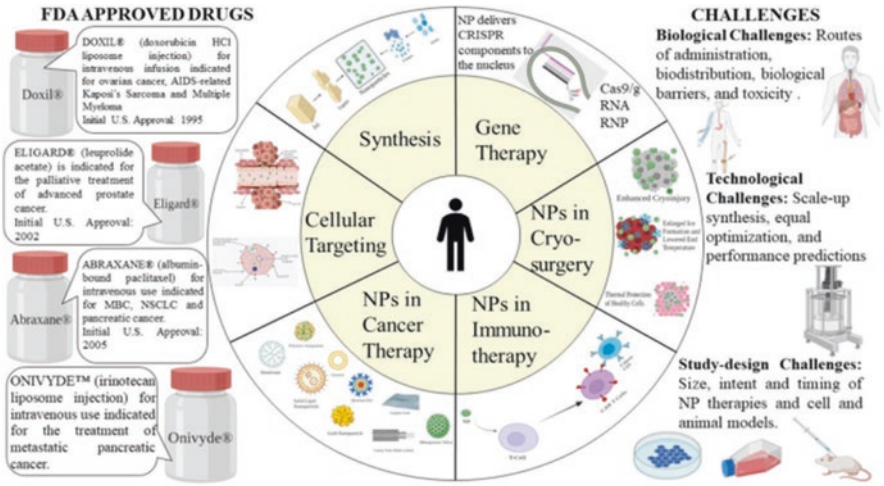

Fig. 6 Application of nanobodies in cancer therapy. (Adapted with permission from [66])

relatively low. These nanobodies can also show enhanced permeability and retention (EPR) effect as they can be designed employing the tumor and its environmental characteristics. With the progress in understanding the mechanisms of multidrug resistance phenomena, nanobodies are getting increasing attention for their employment in cancer therapy. All the three types of nanobodies, namely, organic, inorganic, and hybrid, are in use for cancer therapy. Some of the examples of nanobodies used for this therapy are made of polymers, dendrimers, extracellular vesicles, liposomes, solid-lipid particles, nanoemulsions, carbon-based materials, quantum dots, metal elements, magnetic materials, calcium phosphate, silica, etc. The stability of nanobodies in the blood, their biodistribution, endocytic pathway, and intracellular distribution as well as the bioavailability can be controlled by modifying the shape, size, and surface properties of nanobodies. Palanikumar et al. have designed a hybrid nanobody comprised of an anticancer drug-loaded PLGA core covalently wrapped with a bovine serum albumin shell which could escape interaction with serum proteins and macrophages and thereby facilitated target recognition [67]. Dendrimer nanobodies have also shown potential theranostic properties for the treatment of prostate cancer [68]. Exosome loaded with doxorubicin has been used for the treatment of breast cancer which exhibited enhanced cytotoxicity and minimized cardiotoxicity [69]. Liposomes have also shown successful delivery of various anticancer drugs such as paclitaxel, doxorubicin, and nucleic acid with improved bioavailability of the drugs and efficient anti-tumor effect [70]. However, due to relatively poor loading efficacy, liposome-based nanobodies could not achieve widespread applications. One of the advantages of liposome-based nanobodies is that they can transport both the hydrophilic and hydrophobic drugs simultaneously. Spirulina polysaccharide-loaded nanoemulsion showed improved anti-tumor effects of paclitaxel [71]. Carbon-based materials (fullerene modified with poly(ethylene glycol) (PEG)) have shown promising photodynamic effects on tumor cells [72]. It

has been seen that graphene quantum dots-aptamer-doxorubicin conjugate can target prostate cancer cells; however, due to the lack of an optimized development process, these carbon-based nanobodies have got limited applications [73]. Recently, quantum dots have also been used to achieve targeted delivery of doxorubicin in the human lung cancer cell [74]. Iron oxide nanobodies loaded with ferumoxytol have been approved by the Food and Drug Administration (FDA) to treat nodal metastases in both testicular and prostate cancer [75]. Thus, there are various potential candidate nanobodies to be considered in clinical trials.

2.2.2 Conquering Drug Resistance

Drug resistance is an action by the diseased cells or tissues to inhibit the function of a drug and thereby reduce the success of medication. In addition, tumor cells can also develop resistance to chemotherapeutic drugs. A drug, which acted well on tumor cells, may not be able to respond with the same efficacy as the cells may also develop an ability to resist actions of drugs resulting reduced therapeutic impact. Overexpression of efflux transporters, such as P-glycoprotein, which is also known as multidrug resistance protein, can reduce the intracellular drug accumulation and thereby suppress the drug efficacy. Similarly, hypoxic environment, low pH, defective apoptotic pathways, etc. can also favor drug resistance properties of cancer cells. There are various nanobody-based approaches opted so far to overcome these challenges. As more mechanisms of multidrug resistance are getting revealed, nanobodies with better capabilities are being designed to target them. Since drug resistance is one of the most significant reasons of failure in treating cancer by chemotherapy, hence various new nanobody-based strategies are emerging to address this. The most common primary drug resistance factors in chemotherapy are tumor microenvironment (TME) and heterogeneity, drug transporter and multidrug resistance, epithelial-mesenchymal transition (EMT), cancer stem cells, and tumor metastasis (Fig. 7a) [76]. Furthermore, secondary resistance can also develop during the therapeutic periods, such as overexpression of therapeutic targets and the activation of the alternative signaling pathways, which are initially responsive to cytotoxic agents. Other reasons for resistance could be genomic instability and gene mutations, methylation in DNA, protein acetylation, etc. All these resistances ultimately impair the traditional therapeutic delivery [77]. Nanobodies are found to be promising materials to overcome drug resistance as their physicochemical properties can be exploited to achieve safe and effective cancer treatment. To overcome tumor heterogeneity, such nanobodies can be used to develop precision nanomedicine which not only can have better performance in treating the disease but also can contribute to improve the prognosis of patients. The exploitable multifunctional properties of nanobodies can remarkably accelerate revolutionary cancer treatment. Based on tumor acidity, a variety of pH-responsive nanobodies can be designed which can release drugs triggered by the acidic environment of tumor. For example, PEG-functionalized polymeric micelle prepared through the self-assembly of amphiphilic copolymer and loaded with doxorubicin can release drug once it

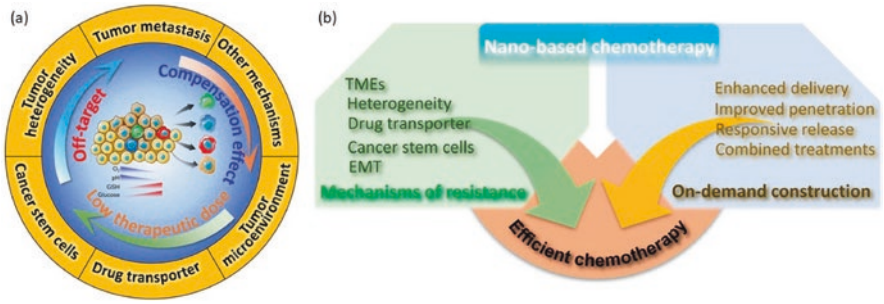

Fig. 7 (**a**) Schematic presentation of various drug resistances in chemotherapy and the crosstalk between them. (**b**) A schematic presentation of various chemotherapy resistance pathways and the advantages of nanobody-based chemotherapy. (Adapted with permission from [76]). Abbreviations: TME tumor microenvironment, EMT epithelial-mesenchymal transition

reaches the acidic environment of tumor [78]. Such nanobodies can also be designed to be dual- or multi-stimuli-responsive (e.g., pH and redox) and dual pH (e.g., two different pH values) and even can be co-loaded with dual or multiple drugs [79, 80]. Huo et al. have demonstrated pH-responsive co-delivery of paclitaxel/disulfiram from surface charge-switchable polymeric nanomicelle and their ability to overcome multidrug resistance in cancer [81]. Nanobody-based drug delivery carriers have also shown success in targeting the hypoxic tumor microenvironment. Tian et al. have designed nanobody-based drug delivery carrier by mimicking cancer cell membrane to carry oxygen and used it for breaking hypoxia-induced chemoresistance [82]. PEG-modified gold nanobodies have been employed to overcome drug pump efflux. Song et al. have used a hybrid nanobody composed of DNA origami and gold nanorods having doxorubicin and a tumor-specific aptamer MUC-1 to circumvent drug resistance [83]. Nanobody-based carriers have also been used for the co-delivery of chemotherapeutic doxorubicin and siRNA as excellent nanotherapeutic strategy for the reversal of resistance mechanism shown by human lung cancer cells [84]. Thus, there are various works demonstrating significant potentials of nanobodies to combat drug resistance at various circumstances.

3 Optically Active Nanobodies for Disease Diagnosis

Optically active nanobodies are nanomaterials that can respond to light of various wavelengths and are popularly being employed for bioimaging and targeted therapy. They have huge success rates of being used for noninvasive tracking and monitoring of therapeutics to explore their function both in vitro and in vivo. So far different nanobodies have been developed for the bioimaging of tissues and treatment of diseases including cancer, inflammation, cardiovascular diseases, etc. Optically active nanobodies are being employed in the fields of fluorescence imaging, persistent luminescence imaging, photodynamic therapy, NIR surface-enhanced Raman

scattering (SERS)-based imaging, photothermal therapy, photoacoustic imaging, optical-responsive therapeutic delivery, etc., to name some [85]. One of the prominent advantages of nanobodies to be used for optical imaging is that modifying their structures and composition makes it easy to modulate not only optical properties but also their physical properties. There are also some nanobodies that can be used for simultaneous imaging and therapeutic purposes. Though there are various challenges often faced during the production and use of these nanobodies, which include loading efficacy, image quality, in vivo stability, toxicity, long-duration storage, reproducibility, etc., they have found increasing use in imaging applications as these nanobodies are being continuously improved. By altering their structural elements and surface properties, these nanobodies are also being developed as multifunctional.

3.1 Optically Active Nanobodies

All the three types of nanobodies, namely, organic, inorganic, and hybrid, are in use as optically active nanomaterials. Inorganic nanobodies made of gold, silver, iron, carbon, silica, lanthanides, etc. have been studied for designing various optically active nanomaterials. Among various inorganic nanobodies, the most studied gold nanomaterials have been designed in various sizes (1 to 120 nm) and shapes (nanosphere, nanorod, nanocage), and by modulating their aspect ratio, various optical properties have also been achieved. These nanobodies have exhibited excellent stability and biocompatibility, and their surfaces can be modified to serve purposes of drug loading and targeted delivery along with bioimaging [86]. However, their long-term toxicity is a concern for their full-fledged clinical translation. Similarly, there are a variety of carbon-based nanobodies which can be optically active, such as carbon nanotubes, graphene, and its oxide forms, nanodiamonds, carbon quantum dots, etc. [87]. Modifying their surface using amine, carboxylic, epoxy, and hydroxyl groups widened the applicability of these nanobodies. Their inherent optical properties, such as photoluminescence, Raman scattering, etc., are exploited to track the status of various therapeutics in vivo. Such optical properties are also used to study pharmacodynamics, drug delivery efficacy, and disease detection [88]. Along with various carbon-based nanomaterials, graphene and graphene oxide-based nanobodies have recently attracted significant attention in bioimaging and biosensing [89]. Porous silica nanobodies have also gained huge attention in biomedical applications mainly due to their low toxicity to avoid many conventional side effects during noninvasive therapies. Porous silicon nanobodies can be engineered to form tunable nanocarriers, and they can degrade inside the physiological body and finally achieve renal clearance [90, 91]. They are also popularly known for their high enough (up to 1000 m^2/g) surface area which is favorable for high drug loading and bioimaging [92]. Nevertheless, these nanobodies need to be further studied to achieve sufficient data for their safe use in vivo. Lanthanide-doped upconversion nanoparticles can be excited using multiple photons of low energy, say NIR, and these nanobodies can

emit high-energy photons in the region of UV to visible [93]. Due to the high tissue penetration capability of NIR and minimal photodamage to cells, they are often used for bleeding-free noninvasive imaging purposes. These nanobodies are highly resistant to photobleaching and photoblinking; hence they can be used as nanoprobes having a negligible autofluorescent background [94]. However, sufficient surface modification and toxicological studies are required before they could be considered for clinical applications. Quantum dots are nanobodies displaying significant optical properties in terms of absorption and luminescence. They can be excited with a single light source to emit diverse colors with a minimal spectral overlap which is in demand for multiple imaging purposes [39]. Quantum dots are used as versatile and bright biosensors for both in vitro and in vivo imaging [95]. However, the possible hazardous properties of many quantum dots have often been a matter of concern which needs further improvements.

Though the inorganic nanobodies are widely employed, their safety concerns are keeping them limited. At this juncture, organic nanobodies are often favored. Aggregation-induced emission (AIE) fluorogens are often used as fluorescent nanoparticles for bioimaging [96]. These materials emit very week emission when in molecular state, while they can emit strong fluorescence while aggregated. They can also be designed to emit wavelength of broad range, such as from UV to NIR. The possibility of conjugating these to various biomolecules has allowed them to be used for bioimaging purposes. However, they often present complex molecular structure and require multistep synthesis processes. Organic semiconducting materials including polymer nanobodies are also in use as optically active materials [97]. These nanobodies have high absorption coefficient and their optical properties are tunable. They can be designed in various sizes. So far, various organic semiconducting nanobodies have been used for deep tissue imaging purposes [98]. They are also used for tumor and cardiovascular imaging. These nanobodies are promising candidates for real-time imaging as investigated by Shuhendler et al. for testing oxidative and nitrosative stress in live animal liver [99]. Polymer nanoparticles have also received huge attention for diagnosis and therapy of various diseases as they can load different types of drugs with high efficacy and exhibit extraordinary fluorescence intensity with good photostability, biocompatibility, and biodegradability [18]. However, more in vivo studies are required to improve their stability in the circulatory systems and to realize their further applications in clinical practices.

3.2 Optically Active Nanobodies for Bioimaging

Thus, optically active nanobodies have found their applications in bioimaging through various techniques, such as fluorescence imaging, persistent luminescence imaging, NIR-SERS imaging, photoacoustic imaging, etc. Fluorescence imaging techniques are popularly used in biological studies as well as fluorescence-guided surgical procedures. This technique can provide information even at the molecular level during their use to investigate the tumor microenvironment [100]. In the

persistent luminescence technique, the luminescence from nanobodies can continue for a few seconds to several days after the excitation is ceased which makes them valuable for bio-tracing. Nanobodies having this characteristic can demonstrate high sensitivity and signal-to-noise ratio. These nanobodies are emerging materials showing extraordinary advantages in nanomedicine. Using these nanobodies bioimaging can be carried out by UV, visible light, photostimulation, NIR, etc. that can emit from these nanoprobes [101]. These nanobodies are also being used for image-guided chemotherapy, photothermal therapy, and photodynamic therapy. NIR-SERS nanobody is typically consisting of nanostructures made of noble metals (e.g., Au, Ag, Cu), Raman-active reporter molecules, and the biocompatible surface coating. For example, gold nanorods wrapped with graphene oxide have been developed as robust nanoplatforms for ultrafast NIR-SERS bioimaging [102]. Even though the strong cytotoxicity of these nanobodies is often a challenge, their surface coating possibilities using various biomolecules can present them as biocompatible nanoplatforms. One of the advantages of such nanoplatforms is that they produce distinct signals (sharp band) which help to identify the target molecules very distinctively. Photoacoustic imaging is an imaging sensory system where heat-induced pressure transients are created by pulsed laser illumination which is detectable using traditional ultrasound. Here, a contrast agent converts the excitation light energy to thermal energy which thereby produces a wideband ultrasound. Both the organic and inorganic nanobodies are in use for this imaging technique. Recently plasmonic nanobodies have also been used for enhanced photoacoustic imaging [103, 104].

3.3 Optically Active Nanobodies for Targeted Therapy and Drug Delivery

As mentioned above, to achieve targeted therapy and drug delivery, optically active nanobodies have found their applications in the fields of photodynamic therapy, photothermal therapy, and light-responsive drug delivery. In photodynamic therapy, an organism having the diseased cells is allowed to intake a drug, which has a photosensitizer, and tissue having the diseased cells is illuminated in combination with molecular oxygen. On illumination, the molecular oxygen produces reactive oxygen species (ROS) which can kill cancerous cells by oxidizing various biomolecules and organelles. However, as the organic molecules are often susceptible to degradation by enzymes, recently the use of optically active nanobodies is being explored where it is proposed that in some cases plasmonic nanobodies can be suitable substitutes [105]. In addition, the use of nanobodies can also favor targeted delivery of hydrophobic photosensitizers to the hydrophilic bloodstream, enhance permeability and retention into the tumor microenvironment and surface modification of nanobodies to facilitate efficient cellular uptake [106]. Combining the optical properties of nanobodies and photosensitizers, multimodal imaging can be achieved. These nanobodies can also be chosen to transduce energy. Nanobodies made of polymers,

silica, noble metals, lanthanides, quantum dots, etc. are already in use for photodynamic therapies.

In photothermal therapy, a photothermal transduction agent capable of increasing the local temperature can trigger the death of cancer cells by converting the energy of light into heat during the illumination. An ideal transduction agent should be efficient in converting photons to heat, should have strong absorption in the NIR region, and should be able to accumulate in the tumor cells. So far both the organic and inorganic nanobodies are being used as photothermal transduction agents as they can be designed to achieve targeted delivery with enhanced permeability and retention. Nanobodies made of noble metals, carbon-based materials, chalcogenides, polymers, porphysomes, dyes, etc. are being used for photothermal therapy [107]. Especially, the recent development of nanoparticles which can generate heat on laser illumination is gaining quick attention. It is to mention that plasmonic photothermal therapy based on Au nanobodies has advanced toward clinical translation [108].

Light-responsive drug delivery systems can release drugs once they are directly or indirectly sensitized by light. There are different drug delivery nanobodies designed using various materials, such as carbon dots, plasmonic materials, photochromic moieties, photosensitizers, polymers, liposomes, dendrimers, etc. [109]. Carbon dots can be designed as drug carriers as well as nanoprobes for bioimaging. The triple-doped zinc gallogermanate photoluminescent nanobodies ($ZGGO:Cr^{3+},Yb^{3+},Er^{3+}$) showed superlong NIR persistent emission, and red light renewability has been successfully used for long-term oral-administered bioimaging and drug release in vivo [110]. Thus, such nanobodies can serve as drug carriers and can be triggered externally using light simultaneously.

4 Impact of Size, Shape, and Architecture of Nanobodies in Nanocarrier Design

The advantages of highly tunable physical properties of nanobodies along with the availability of an ever-expanding library of molecules for the surface modifications of such nanobodies have presented them as the most advantageous materials to design nanomedicines. Nanobodies of various sizes, shapes, and architectures are in use for drug delivery and bioimaging. The size of nanobodies is a crucial factor as it influences both the drug loading efficacy and delivery mechanisms. These nanobodies can be designed as spherical or nonspherical. For nonspherical nanobodies aspect ratio often determines their cellular uptake. It has been reported that the nanobodies having a higher aspect ratio demonstrated higher cellular uptake [111]. Such high uptake efficiency is due to the large surface area which thereby facilitates interaction between the nanobody with a high aspect ratio and the biological cell. Permeabilities of polymeric nanomicelles of different sizes in tumors have also demonstrated that as the size increases, the permeability decreases. Once the size

exceeds 100 nm, the biodistribution, as well as their pharmacokinetics, changes greatly as they can easily be detected by the liver, spleen, kidney, lung, etc. The size and shape of nanobodies can be controlled by varying the condition parameters adopted during their fabrication process. It is also to note that smaller nanobodies have high surface-to-volume ratio; however they can get aggregated easily. Thus, to design nanobodies for nanocarrier development, one must optimize their size for a particular use. Beside the size and surface properties, the shape or morphology of nanobodies also plays crucial role in drug delivery science. It has been observed that the nanobodies having spherical shapes can move smoothly whereas the nonspherical ones can tumble in the circulatory system which is found to be more prominent in filtering organs, such as the kidney, spleen, and liver. Eliezar et al. have shown that spherical micelles got more accumulated in the filtering organs while the worm-like micelles pass through smoothly [112]. Nanobodies can also be designed in various architectures, and accordingly their loading capacity or ability to load different types of drugs (multidrug loading) varies. For example, they can be designed as nanomicelle, dendrimer, nanosphere, nanocapsule, nanogel, nanocomposite, polymersome, core-shell type, nanostar, etc., and their drug loading efficacy will be different even when their size is similar. Furthermore, a simple nanomicelle can load say either a hydrophilic or a hydrophobic drug at a time, whereas a polymersome can load both the hydrophilic and hydrophobic drugs simultaneously [18]. Thus, based on the purpose, a nanobody with required architecture can be selected.

5 Stimuli-Responsive Nanobodies for Drug Delivery

Nanobodies can deliver loaded drugs as a response to stimuli, which can be chemical (e.g., pH, redox, ions, etc.), physical (e.g., light, sound, time, magnetic field, heat, etc.), and biological (e.g., enzyme, protein, DNA, etc.) in nature. pH-responsive nanobodies can donate or accept protons at pathological pH and thereby influence the drug-nanobody conjugation [113]. For example, a drug that remained conjugated to the nanobody at normal physiological pH 7.4 can get detached from the nanobody at low pH as it reaches the tumor microenvironment or in the early endosomal compartment. It is mentioned that the pH-responsive drug delivery may follow various mechanistic pathways, such as through physical dissociation between the drug and host nanobody or through the cleavage of chemical bonds that may result in the dissociation of a nanobody. It may happen through the cleavage of chemical bonds to release the drug, and there are also reports showing the release of payloads through the swelling of nanobodies [114]. Light-responsive nanobodies contain conjugated photochromes on to the nanobodies. In presence of light, they may undergo structural changes which thereby can release the drug to be free [115]. The sound may cause drug release via thermal effect. In this pathway, the acoustic energy may cause an increase in the local temperature resulting in the perturbation of the cell membrane which can facilitate the permeability of drug-loaded carriers into the intracellular milieu. The ultrasound-assisted drug release from nanobodies can also occur

via shear and shock waves from the collapsed nanobodies [116]. Amin et al. reported ultrasound-responsive lipid-coated mesoporous silica nanobodies which released drugs triggered by rupturing of the lipid layer [117]. This rupture was caused by the mechanical and thermal effects induced by the ultrasound. Enzyme-responsive drug-loaded nanobodies can selectively interact with specific enzymes expressed in the tumor which thereby trigger anti-tumor drugs [118]. This can result in targeted delivery and reduce toxicity to the healthy cells. Redox-responsive nanobodies can show high stability in the circulatory system. These systems respond to intracellular milieu having a high concentration of glutathione and can release the drug rapidly, and this happens only when they reach the tumor sites [119]. Furthermore, to overcome the challenges of achieving highly targeted and efficient delivery of a drug, the development of multi-stimuli-responsive nanobodies is also being increasingly investigated. An et al. have recently reported a multifunctional nanobody which exhibited NIR light/pH/reduction-responsive drug release property and intracellular drug translocation to treat cancer. This further demonstrated photoinduced hyperthermia to cause synergistic anticancer efficiency that led to tumor ablation [120]. Thus, stimuli-responsive nanobodies play a crucial role to achieve targeted drug delivery and reduced cytotoxicity with efficient drug release.

6 Cellular Compatibility of Nanobodies

It is understood that there are various advantages of using nanobodies for drug delivery; however, their biocompatibility and biodegradability are of great concern [121]. Many of such nanobodies need special attention to reduce their direct or indirect toxicity. In many cases, the surfaces of the nanobodies are modified by various biodegradable or biocompatible materials. The sizes of these nanobodies are also receiving serious attention so that they can be designed to get easily cleared from the body. There are also efforts being made to prepare these nanobodies completely made of biodegradable materials. Nevertheless, each system has its advantages and disadvantages. Hence, it is almost impossible to design a universal nanobody with no cytotoxicity. In this aspect, more studies are required, and further information is to be analyzed to decide on how to reduce the toxic effect of nanobodies on healthy cells and tissues and how they can get easily cleared from the body. In some cases, the nanobodies are modified using biodegradable molecules which finally get degraded to small molecules, and these small fragments can sometimes be toxic. Hence, this aspect has also to be considered. The success of nanobodies in biomedical applications and their clinical translations greatly depends on their biocompatibility and biodegradability. Thus, more attention must be paid to exploring the cellular response to nanobodies before they are considered for in vivo tests.

Though there are various advantages of nanobodies in the biomedical field, their toxicological impacts are to be investigated seriously. For example, inhalation of nanobodies may cause lung inflammation and heart problems. Nanoparticle dissolution can also induce significant toxicity to cells. They can also interact with various

biomaterials in the cellular milieu and generate ROS, increase inflammatory cytokine generation, and finally cause cell death. It is to note that improper choice of nanobodies may cause physical impairment to cell membranes, structural alterations in cytoskeleton components, disruption of transcription and oxidative damage to DNA, damage to mitochondria, lysosomal dysfunction, ROS generation, membrane protein dysfunction, generation of inflammatory factors, etc. [122]. Thus, histopathological studies of cells, tissues, or organs, once they are exposed to nanobodies, should be carried out to determine the level of toxicity.

7 Conclusion and Future Perspective

One of the biggest challenges of drug development is to deliver therapeutics at the targeted site at an effective concentration without causing any toxicity to the healthy cells and tissues. Here, the use of nanobodies for drug delivery plays a significant role. Such nanobodies can protect drugs from degradation and premature release. They can contribute to extending the lifetime of the drug in the circulatory system and improve their pharmacokinetics and pharmacodynamics. These nanobodies assist drugs to withstand environmental changes, such as changes in pH, ionic concentrations, actions of enzymes and heat, etc. Developments in nanotechnology have contributed to the design of nanobodies of various shapes, sizes, and structures. The possibilities of using various materials for designing these nanobodies and the ease of modifying their physicochemical properties via surface modification have contributed to overcome many of the challenges faced in drug development. Thus, nanotechnology has tremendous potential for advancing drug delivery and bioimaging; however, it is to note that these nanobodies can be toxic if suitable modification is not carried out. Therefore, toxicity needs to be reduced and standardized across all nanotechnology platforms to translate its full benefit to clinical practice. It is also to note that increasing the introduction of multifunctional properties into the nanobodies for drug delivery is also necessary to meet clinical requirements. Thus, the designing of nanobody-based drug delivery systems for diagnosis and therapeutic applications still has a vast scope for further development.

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Phytosomes Used for Herbal Drug Delivery

Mansab Ali Saleemi and Vuanghao Lim

1 Introduction

The twenty-first century has brought about a greater awareness of the importance of maintaining a healthy lifestyle, so the use of herbs and active extracts has gained widespread acceptance [1]. Due to the various advantages of herbal drugs, their increasing popularity has raised concerns about the safety and efficacy of modern drugs. Due to the limitations of modern medicine, the use of herbal medicines has increased. Apart from this, there is a rising interest in the safety and efficacy of synthetic drugs [2, 3]. Since ancient times, the active constituents of plants have been used to treat diseases. A number of plant-based compounds known as phytochemicals have been studied extensively for their various health benefits [4]. These compounds belong to the family of bioactive polyphenols, which can be found naturally in plants. Their various properties have been studied extensively and are being used by scientists for their cosmetic and dietary supplement applications. Their low cytotoxicity and potent bioactivity have been acknowledged by the scientific community [5]. One example is the extract of milk thistle fruit, which has been used for thousands of years to treat liver damage. Curcumins derived from turmeric have been shown to have antioxidant properties and anticancer effects. Furthermore, polyphenols, such as flavonoids, are among the most thoroughly investigated active components [4, 6].

Terpenoids, alkaloids, and phenolic compounds are separated into three groups based on their various structural elements. Some of these include geraniol, caryophyllene, and linalool. Pyrrolidine, pyridine-piperidine, and isoquinoline are among

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the most widely used alkaloids for their phytochemical effects. They can be used to inhibit enzymes or act as substrate holders [6, 7]. By improving the chelating properties of compounds, phytochemicals can help remove harmful substances from the gastrointestinal tract. They can also enhance the stability of various nutrients. Another benefit of phytochemicals is their antioxidant activity, which can help remove free radicals from the body and improve the uptake of various nutrients [8, 9]. As a phytochemical, salicin can be used as a precursor of drugs that contain nonsteroidal anti-inflammatory drugs (NSAIDs). Due to their properties, some phytochemicals are referred to as nutraceuticals. They can be extracted from various plant species to develop effective treatments for different diseases [10]. However, due to poor oral bioavailability, the clinical applicability of many potent plant compounds is a topic of debate. The low absorption rate of such elements could be attributed to their low lipid solubility, the presence of multi-ring polyphenols in their structures, and their high molecular weight [3]. It has been estimated that up to 70% of the new compounds that have been discovered could have low bioavailability. Most of the bioactive compounds in phytomedicines, such as flavonoids, phenolics, and glycosides, are water-soluble. Their poor absorption due to their large size and their inability to pass through the membranes of the small intestine limits their ability to interact with the intestine's lipid-rich outer membranes. Polyphenols' multi-ring structures are too big to be absorbed through passive diffusion or nonactive absorption [1, 4].

The properties of certain constituents are known to cause drawbacks when used as drugs. Therefore, a system that can deliver these constituents into the systemic circulation is needed. Various solutions have been suggested to improve the bioavailability of pharmaceutical drugs, such as the formation of emulsions, liposomes, or nanos [4, 10]. Nevertheless, the use of phyto-phospholipid complexes (phytosomes) is considered a great method for improving the bioavailability of drugs.

2 Background

The concept of phytosomes was first introduced in 1989 by Indena, a company based in Italy. A study conducted by these researchers reveals that phenolic compounds have poor bioavailability and can be complexed with phospholipids. The study shows that these compounds have a high binding affinity to phospholipids [2, 3]. The term phytosome comes from the words "plant" and "some," implying that the substance is cell-like [3, 11]. Phytosomes are complexes of phospholipids and naturally active phytochemicals that are bound in a structure, and these complex molecules are formed by the reaction between plant extracts and phosphatidylcholine (PC) [12, 13]. The advantage of phytosomes over other drug delivery systems is that they minimize any harmful effects of a drug while delivering it to a certain site of action. Aside from improving the drug's distribution, phytosomes can also control how much of the drug is used [2, 14]. Another benefit of this process is that it allows the drug to be transported in a controlled manner. The use of phospholipids

for drug delivery has been acknowledged as a promising strategy for increasing the effectiveness and efficiency of medication. Various types of phospholipid-related formulations, such as Doxil, Cleviprex, and Valium, have been widely used in the treatment of numerous conditions. Various phospholipids, such as PC and artificial PC, are commonly utilized in different formulations. Due to their versatility, they can be applied in many ways [1, 4, 12].

Phytosome production protects the valuable compounds of herbs from damage by gut bacteria and digestive secretions. A transition can also be made from a cell-free environment to a lipid-friendly one. Once they reach the cell membrane, they can enter the bloodstream [2, 3]. Water-soluble bioactive constituents, such as flavonoids, can become lipid-compatible molecular complexes by conversion to the outer membrane of the cells. This process can then be accelerated for them to reach the blood. A phyto-phospholipid complex is formed when a stoichiometric amount of lipoid (mostly PC) is combined with extracts or polyphenolic constituents, such as simple flavonoids, in an aprotic solvent [15]. PC is also the main component in cell membranes, which can be miscible in both water and oil phases. Its molecular properties make it a good candidate for phytosomes. The molecular group that phytosomes are linked to is known to form a bond between two molecules [16]. When combined with another flavonoid or PC-containing substance, this hybrid can convert into a lipid-miscible membrane. This bond can then transform into a hybrid structure that merges into the outer membrane of a cell [11, 15, 16]. As a biocarrier, PC is not just a means to carry the bioactive flavonoids of the plant into the bloodstream. It is also a known bioactive nutrient that can provide a variety of health benefits. When compared to commonly used preparations, these formulations have better pharmacological and pharmacokinetic features. The hydrophilic phytoconstituent-choline complexes are entirely covered by the lipid-soluble phosphatidyl part [2, 5, 6]. High drug encapsulation, better stability, and improved bioavailability are just a few of the advantages of phytosomes [17]. A higher rate of absorption also results in a lower dosage of the active constituents required to elicit a biological impact, which is true for both polar and nonpolar phytoconstituents [11, 12].

3 Properties of Phytosomes

1. Physicochemical Properties

Phytosomes are made up of natural substances and phospholipids, such as soy phospholipids. A complex is obtained by the reaction of stoichiometric amounts of a substrate and phospholipids in an aprotic solvent. The creation of hydrogen bonds and polar functionalities of phospholipids is thought to be the main interaction between phospholipids and their substrates. When a phytosome is treated with water, it assumes that a micellar shape forms liposomal structures. In phytosomes, the active principle is embedded in the polar head of phospholipase, and in

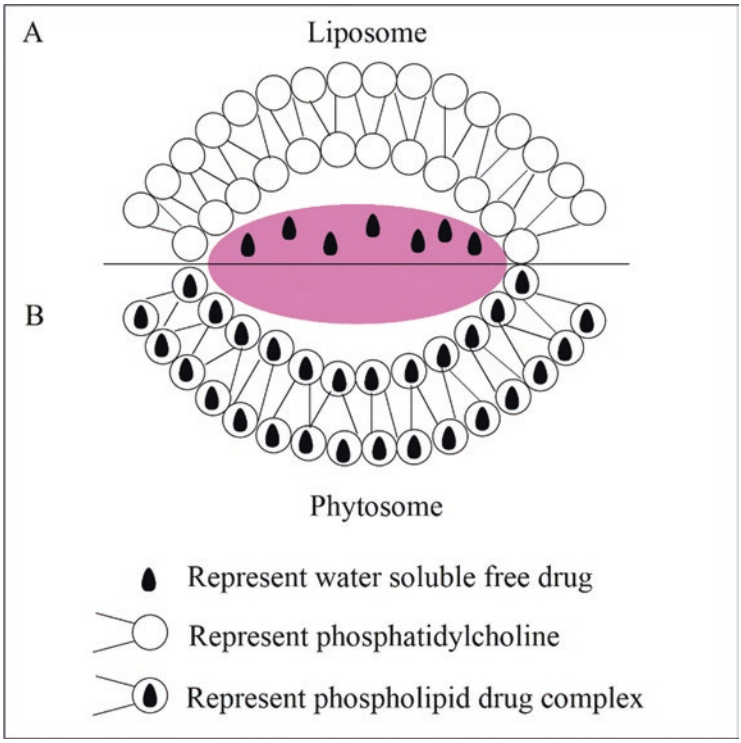

Fig. 1 Schematic representation of the differences between liposomes and phytosomes. (Created with ChemDraw Professional)

liposomes, it is dissolved in the inner pocket of the layered membrane. For instance, in the case of a catechindistearoylphosphatidylcholine complex, H-bonds between the phenolic compounds and the phosphate ions are formed. A comparison between liposomes and phytosomes is represented in Fig. 1. In addition, PC can be determined by comparing the complex's ^{1}H NMR and ^{13}C NMR spectra to those of the pure precursors. It has also been demonstrated that the presence of long aliphatic chains in the active principle allows the complex to produce a lipophilic envelope, which helps the complex to dissolve in low polarity solvents [18].

Phyto-complexes are formed as a result of the reactions among various substrate and polymer groups [19]. They are generally prepared in 1:2 or 1:1 ratios [19]. During contact with each other, the interaction between hydrogen bonds and substrate molecules can link the polar parts of phospholipids with their substrate molecules. This can be studied with a spectroscopic system [20]. Phytosomes are attached to the glacial tops of phospholipids. They can become an interior division of molecular film for the creation of bonding involving the phenol hydroxyls of flavone moiety [21]. It is possible to induce the similarity of the NMR of phytosomes by individualizing the untainted precursor, after which the signals of the fatty sequence are largely unaffected. This confirmation of the intention of the complex

was used to demonstrate the accessibility of phytosomes through the evaluation of the substance properties [11].

2. Biological Properties

Phytosomes are the sophisticated world of herbs used for making various drugs. The goal is to make these products more effective and efficient at reducing the side effects of drugs [22]. As a consequence of in vitro and in vivo investigations for the better invention of herbs in living things, it has been demonstrated that phytosomes are more useful for building bioavailability than noncomplex botanical herbs [23]. Phytosomes are advanced forms of herbs, making them more effective than traditional herbal extracts. Various pharmacokinetic investigations have demonstrated that phytosomes have higher bioavailability than non-complexed plant derivatives [24]. They are more advantageous in terms of reducing serum levels and improving the pharmacodynamic effects of various drugs [23].

4 Phytosomes' Significance in Herbal Drug Delivery

Phytosome is a novel, lipid-based delivery system that can improve the absorption of various phenolic compounds [25]. The company of Indena developed the first phytosome. Their goal was to improve the bioavailability of drugs by creating phospholipids. Phytosomes are made up of phospholipids, predominantly PC, that have been infused with standardized polyphenolic plant extracts [5]. The lipid vesicles in phytosomes are formed by an H-bonding interaction between bioactive compounds and non-polarizing phospholipids [26]. The hydrophilic moiety of phospholipids (such as choline) chemically binds the water-soluble polyphenolic rings of phytochemicals (such as flavonoids) to form the body of phytosomes, whereas the phosphatidyl lipophilic moiety of phospholipids forms a tail to incorporate the water-soluble phytoconstituents [27].

The absorption of phenolic compounds is significantly influenced by the encapsulation of poorly soluble compounds into phytosomal delivery vehicles. This effect helps in penetrating and enhancing the bioabsorption of phenolic compounds [28]. A phytosome is a bifunctional compound that can be used as a topically applied drug. Its capabilities have been demonstrated to improve the pharmacokinetic and pharmacodynamic properties of its formulations [29]. Phytosomes have been identified as a promising tool for developing new formulations due to their potential function in improving the characteristics of diverse polyphenolic substances. Phytosomes are often made by combining active biological phytoconstituents with phospholipids, such as phosphatidylethanolamine and phosphatidylserine (PS), in certain stoichiometric ratios under specific conditions [16]. To isolate the complex, aprotic solvents, such as methylene chloride, acetone, and ethyl acetate, are evaporated under a vacuum after mixing. The resulting phytoconstituents are then integrated into lipid vesicles [30]. The development of phytosome nanoscale technology for the delivery of bioactive compounds has the potential to improve the

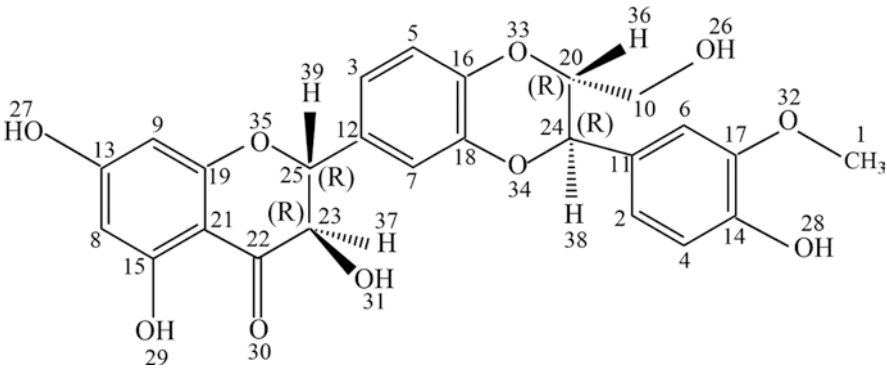

Fig. 2 The chemical structure of silybin, generated using the InChI code from http://pubchem.ncbi.nlm.nih.gov/

bioavailability of phytochemicals, such as polyphenolic compounds. Silybin is a water-soluble flavonoid that has antioxidant and hepatoprotective properties. The chemical structure of silybin is shown in Fig. 2. It is produced by the *Silybum marianum* plant [31]. Silybin has a low solubility and cannot be absorbed by a biological membrane that is rich in lipids. Its improved absorption is achieved through the use of milk thistle extracts [32]. It was also observed that the use of silybin-phytosome preparation exhibits excellent bioavailability in rats [12]. When broiler chicks are exposed to aflatoxin B1, the silymarin phytosome can protect them from the harmful consequences [33]. A study shows that silymarin phytosomes can improve fetoprotectant properties toward ethanol-induced behavioral deficits [34]. Studies reveal that the silymarin phytosome, which is derived from the seeds of the *S. marianum* plant, could protect pregnant women from ingesting ethanol [35]. One study discovers that silymarin, a standardized mixture of flavanolignans, forms a complex with phospholipids [36]. When compared to single constituents, phytosomes show a high level of activity, long-lasting action, and antioxidant properties [36]. Moreover, silybin from phytosomes enters the liver, which is the intended target organ. Nine patients who had their gallbladders removed were used to prove the effectiveness of this procedure. They were given a single oral dose of silybin, 120 mg. The total quantity of the drug recovered after 48 hours accounted for 11% of the total dose in the case of phytosomal silybin. Researchers discovered that about 3% of the silybin was retrieved in the case of silymarin. This data reveals that phytosomal silybin passes through the liver four times faster than silymarin [37]. The pharmaceutical scope of phytosomes includes the following:

(a) It appreciates the entrapment of drugs.
(b) It improves the oral and topical absorption of polar-insoluble phytoconstituents and their bioavailability.
(c) The dose required for active constituent(s) decreases as the rate of absorption increases.

(d) The chemical bonds between a PC molecule and its phytoconstituent help in maintaining a better stability profile for phytosomes.
(e) PC is a substance that can be used in the preparation of phytosomes and possesses a hepatoprotective effect.
(f) Phytoconstituents can be utilized as functional cosmetics after being applied to the skin to promote percutaneous absorption.

Another study analyzed silybin absorption after it was conjugated with phosphatidylcholine [38]. The silybin plasma level was determined after a single oral dose of the phytosome was administered. The effects of the silybin-phytosome on the liver function of patients with chronic hepatitis C were studied. Those taking the drug for up to 120 days showed a significant improvement in their liver function [39]. Using phytosome nanotechnology to deliver ginkgo extract has beneficial effects on the brain and vascular protection [40]. The study reveals that the addition of plant extracts to phytosome delivery systems boosts the absorption of flavonoid compounds [41]. Oleaselect is a polyphenols-based olive oil product. It possesses anti-inflammatory and antioxidant properties, in addition to other health benefits [42]. The preparation of *Centella asiatica* is shown to lower oxidative stress in diabetic patients and prevent strokes in rats [43]. Green select herbal extracts exhibit various beneficial effects, such as reducing free radicals, improving antioxidant properties, and reducing the generation of proinflammatory cytokine. Greenselect's formulation as phytosomes is shown to boost the extract's bioactivity and bioavailability [44]. Rutin phytosomal formulations have more skin absorption than conventional rutin formulations, resulting in increased anti-arthritis action [45]. The addition of poorly soluble curcumin to a phytosome delivery system improves liver injury protection by restoring the antioxidant glutathione [46]. The properties of flavonoids in curcumin have been observed to facilitate the absorption of bioactive curcumin in rats. This is due to the PC moiety of the 1,2-dimyristoyl-sn-glycero-3-phosphocholine in the bioactive curcumin [47]. The therapeutic activity of various phytoconstituents, such as *Echinacea angustifolia* [48], *Vitis vinifera* [5], and *Crataegus* species [2], is significantly improved when added to phytosomes.

Grape seed phytosomes are made up of several oligomeric polyphenols, which are found in grape seed extract. Moreover, procyanidin flavonoids are known to increase the antioxidant capacity of blood and stimulate the physiological antioxidant defense of plasma. They also offer protection against the effects of ischemia and reperfusion [49]. Researchers discovered that rabbits who were fed a high-cholesterol diet for 6 weeks had higher blood cholesterol levels and inflammatory lesions in their carotid arteries. One group of these rabbits was fed grape seed phytosomes during this study, and compared to the control group, they had a significantly less aortic plaque. Grape seed phytosomes were administered once daily to healthy young participants in a randomized human trial. The blood tartrate-resistant acid phosphatase (TRAP) was tested at various intervals during the first and fifth days. After administration on the first day of treatment, blood TRAP levels were elevated over the control group that was given conventional grape seed extract [50]. In one study, researchers used a simple and reproducible method to develop a

phytosomal complex containing quercetin-phospholipids. The complex exhibited better efficacy than a molecule used for liver injury [51]. The phytosomes of naringenin and curcumin were produced by a group of scientists in different experiments [51]. This study reveals that naringenin's phytosome possesses higher antioxidant properties. This could be because the prolonged duration of action limits the molecular elimination of the compound.

Citrus fruits are high in hesperetin, which is a phytomolecule. Despite numerous therapeutic benefits, the use of antioxidant drugs remains restricted primarily due to their shorter half-life and lower body clearance. Another group of scientists has successfully developed a novel hesperetin phytosome to overcome this limitation. The produced complex was examined for its antioxidant properties in CCl_4-instituted rats. The phytosome was discovered to have long-term release properties and increased antioxidant activity. The higher relative bioavailability of the phytosome is due to its higher concentration, according to a pharmacokinetic investigation [52].

5 Recent Phytosomes for Herbal Drugs

Phytosomes are known as effective nanocarrier delivery systems [9]. However, successful product commercialization is still far off. Despite the advantages of phytosomal drugs, few final products have been commercialized [9]. Safety proofing the product is a primary barrier to its entry into the market. There are numerous products available in the market that are based on phytosomes that work great in therapeutic applications and can be used to treat various conditions. Some of the products being formulated and commercialized are shown in Table 1.

Phytosomes are biological-neutral compounds that can be safely introduced into the human body [54]. Before they are commercialized, certain parameters should be considered regarding their nano-size structure, such as metabolism and excretion [55]. A curcumin phytosome prepared by scientists was successfully introduced into rats and was shown to produce high accumulation in bone marrow tissues [56]. Normal cells can also be passively targeted by phytosomes. Animal models and a clinical study should be conducted to investigate their biological effects [54]. However, various studies show the biological safety of phytosomes [57]. Further, designing a phytosome should involve the evaluation of its pharmacodynamic and pharmacokinetic properties. Finding the best dosage form is a step in the right direction for marketing a final product [58]. Phytosomes should also be reproducible and quality-monitored over time [55]. Popularity is another factor that influences a product's commercialization. Due to the safety and biocompatibility of natural products, their increasing popularity has become more prevalent in recent years. The process of introducing phytosomal technology to the industrial market is relatively simple due to the rapid commercialization of phytosomes [1]. In addition, polar phytoconstituents have been studied by several pharmaceutical industries for their biological activities and enhanced bioavailability.

Table 1 Bioactive phytochemicals based on phytosomes are available in the market

Source plant	Phytosome formulation	Application	References
Ruscus aculeatus	Roscugenin	Anti-inflammatory property	[53]
Crataegus species	Hawthorn	Antihypertensive property	[2]
Vitis vinifera	Leucoselect	Antioxidant activity	[5]
Ammi visnaga	Visnadine	Circulation improver	[14]
Curcuma longa	Curcumin	Hepatoprotective property	[47]
Crataegus mexicana	Crataegus	Antioxidant activity	[14]
Vaccinium myrtillus	Bilberry	Potent antioxidant activity	[14]
Echinacea angustifolia	*Echinacea*	Acts as immunomodulator	[48]
Ruta graveolens Sophora japonica	Rutin	Rheumatoid arthritis	[45]
Melilotus officinalis	Lymphaselect phytosomes	Indicated for insomnia	[14]
Horse chestnut bark	PA2 phytosomes	UV protectant and anti-wrinkles	[14]
Olea europaea	Oleaselect™ phytosome	Antioxidant and anti-inflammatory activities	[14]
Zanthoxylum bungeanum	Zanthalene phytosomes	Anti-itching and soothing	[14]
Serenoa repens	Sabalselect phytosome	Benign prostate hyperplasia	[14]
Camellia sinensis	Greenselect	Antioxidant activity	[44]
Polygonum cuspidatum	Rexatrol	Antiaging and antioxidant activity	[14]
Centella asiatica	*Centella*	Skin and vascular disorders	[43]
Terminalia sericea	Sericoside phytosome	Skin improver	[14]
Silymarin marium	Silybin	Liver protection and antioxidant activity	[12]
Ginkgo biloba	Ginkgo	Vascular and brain tissue protection	[40]
Europaea oil	Olive oil	Antihyperlipidemic, antioxidant, and anti-inflammatory activities along with cardiovascular protection	[42]

6 Targeted Delivery of Herbal/Phytochemical Phytosomes

Two key factors that determine the success of phytochemical drug carriers are targeted delivery and sustained release rates [59]. Various types of nano-systems are employed in disease imaging and therapy [60]. The use of nanocarriers for phytochemicals is widely based on their spherical shape [61, 62]. Some vesicular drug delivery systems have been developed, such as ethosomes, transfersomes, liposomes, and niosomes [63–67]. A schematic illustration of the numerous vesicle architectures involved in phytochemical delivery is shown in Fig. 3a.

7 Ethosomes

Ethosomes are carriers that deliver natural products and drugs to deep tissues and into circulation [68]. These are mainly composed of phospholipids. They include PC, PS, and ethanol [69]. The high concentration of ethanol in ethosomes helps to minimize the effects of impairing the skin's lipid bilayer. When ethanol is integrated into a cell membrane, it allows the ethanol to reach the targeted vesicles. The lipid membrane in ethosomes is less rigid than other vesicles due to the presence of ethanol, which increases drug trafficking capabilities in stratum corneum lipids [70]. Ethosomes are well-suited to various industries for different purposes, such as in pharmaceutical, veterinary, biotechnology, cosmetic, and nutraceutical applications. Also, these soft vesicles are used as carriers for the improvement of skin

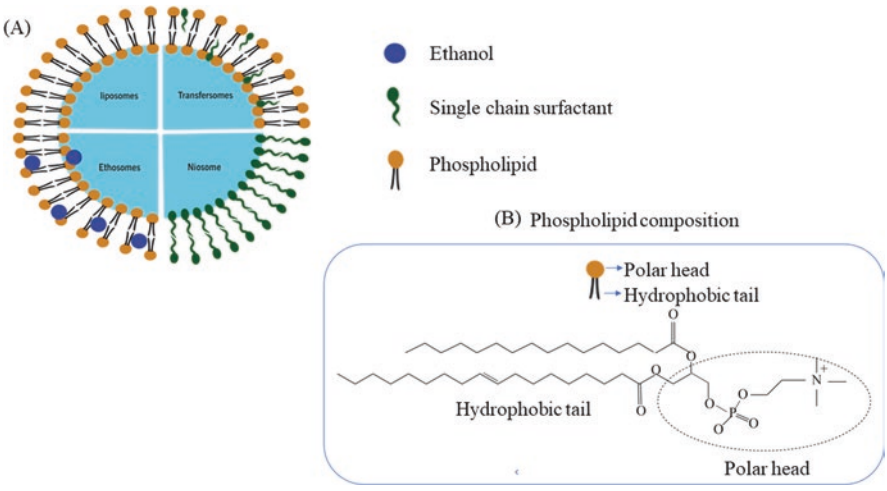

Fig. 3 Possible vesicles to produce phytosomes; (**a**) schematic illustration of various types of vesicles in phytochemical delivery, ethosomes, liposomes, transfersomes, and niosomes; (**b**) represents the composition of phospholipid moiety. (Created with ChemDraw Professional)

Table 2 A comparative evaluation of phytosomes, ethosomes, transfersomes, liposomes, and niosomes

Characteristics	Phytosomes	Ethosomes	Transfersomes	Liposomes	Niosomes
Composition	Phytoconstituents of phospholipids and polyphenolics	Water, phospholipid, alcohol, and polyglycol	Surfactant and phospholipid mixture	Cholesterol and phospholipids	Cholesterol and nonionic surfactants
Flexibility	Rigid	Elastic	Ultra-deformable	Rigid	Rigid
Significant application	Phyto-delivery	Skin delivery	Skin delivery	Gene and drug delivery	Drug delivery and cosmetics
Route of administration	Transdermal and oral	Transdermal and topical	Topical and transdermal	Topical, oral, transdermal, and parenteral	Topical, oral, transdermal, and parenteral
Key properties	High entrapment efficiency (EE) helps in releasing the contents slowly	Improves the efficiency of drug penetration across/through the skin	Deforming ability is high, allowing for deeper penetration into the epidermal layers	Biocompatibility, self-assembly capability, and the ability to transport large drug payloads	Improved dispersion of compounds with solubility difficulties and excellent stability
Commercialized product	Leucoselect, Sabalselect, etc.	MaccabiCARE, Trima, etc.	Daktarin	Doxil, Visudyne, etc.	L'Oréal and Lancôme
Limitations	The phytoconstituents' leaching, which lowers the targeted drug concentration, indicates that they are unstable	Poor yield, coalescence, disintegration when transferred to water, and product loss during transition from organic to water media	Cytotoxicity of surfactants	Low stability and low dermal penetration	Cytotoxicity of surfactants and low dermal penetration

delivery [71]. Ethosomes can range in size from nanometers to micrometers and are more effective at delivering drugs to the skin than liposomes. They have been found to have superior properties over other commercially available dermal delivery systems [72]. A comparative evaluation of the various components of nano-delivery systems, including phytosomes, ethosomes, transfersomes, liposomes, and niosomes, is presented in Table 2.

Many authors show that ethosomes are effective as topical vehicles of phytochemical compounds. A study conducted by scientists evaluated the cytotoxicity of different extracts loaded with ethosome for transdermal delivery. An evaluation of the cell-line data revealed that compounds containing the extract inhibited testosterone production and improved the viability of the cell, despite the fact that a histological analysis showed that the encapsulated vesicles did not affect the rat's epidermis layer [73]. The disadvantage of ethosomes is their poor consistency and evaporation. This contributes to the large size variation from nanometers to micrometers. Alcohol combined with trehalose and propylene glycol can manage this deficiency [74]. A brief description of surfactants and phospholipids that are used in the preparation of phytosomes, liposomes, niosomes, ethosomes, and transfersomes is presented in Fig. 3a. The phospholipid moiety of phytosomes, which consists of a polar head and a hydrophobic tail, is shown in Fig. 3b.

8 Transfersomes

Transfersomes are a type of flexible nanocarrier initially developed in the 1990s [71]. Regular liposomes do not penetrate the skin's outer layer and are restricted to the stratum corneum's outer portion [75]. Transfersomes are a new type of lipid vesicle developed to improve the efficiency of liposomes. They are flexible and elastic carriers that increase the delivery of compounds to deep dermal tissues [76]. They are composed of one amphipathic molecular, such as PC, and a bilayer softening agent. Transfersome components are designed to self-assemble into a lipid bilayer after being applied to an aqueous system [76]. Transfersomes have been proven in studies to penetrate the skin more deeply. They can also be used as drug carriers [77]. In a recent study, scientists developed resveratrol (RSV)-loaded transfersomes made up of PC and edge stimulators (EA) [78]. The results show that distilled water with 5% ethanol and 5% PC/EA might be utilized to generate an optimum formulation. The vesicles were found to be 40 nm in size and exhibited antioxidant activity similar to the free RSV group. Also, D1-20(W) demonstrated an improvement in its in vitro transdermal delivery performance. It was also noted that its cell viability was decreased by over 30% compared to the free RSV group [79]. Transfersomes are not chemically stable due to their susceptibility to damage caused by oxidation. Also, their purity is not ideal for use as a standard vehicle for drug delivery. However, large-scale production of transfersomes can be done without the use of additives [80].

9 Liposomes

The term liposomes come from the Greek words referring to fat and body parts [81]. Liposomes are spherical structures made up of phospholipids and cholesterol. The particles have a diameter of 0.05 to 5 micrometers. They are very promising carriers for various drug delivery systems because of their unique features, such as their ability to deliver drugs in different configurations [82–84]. The goal of this system is to deliver a drug to a specific site of action [85]. Liposomes are very stable and are equipped with a unique structure that traps various agents in their compartments [86, 87]. Liposomes are designed to be used in various pathological conditions, such as skin diseases and arthritis [88, 89]. Most liposome preparation methods involve lipid solvation by organic solvents, evaporation, condensation, and the hydration of lipid [90, 91]. Some synthesis methods can help improve the encapsulation of drugs [92, 93].

However, liposomes are designed to increase biological impact, solubility, and defense against degradation [94, 95]. Many reports suggest that the use of natural extracts in liposomes can improve their biological activity [96, 97]. For instance, one study reported that CD44 receptor-phyto-liposomes are loaded with stigmasterol (STS) for synergistic chemotherapy. In comparison to MCF-7 cells, the anticancer efficacy of HA-DOX-STS-lipo was significantly increased in MDA-MB-231, and CD44-overexpressing cells in vitro, showing an HA-mediated targeting effect. This suggests that the carrier system could be used against CD44-overexpressing tumors [98]. In another study, researchers prepared nanoliposomes by applying a simple hydration process with polyphenols. They studied the various aspects of these nanoliposomes, including their particle size, EE, and morphology. The study reported that the highest EE of nanoliposomes was 52.93%, which was composed of 1% lecithin and 1,000 ppm phenolic compounds. The Fourier transform infrared spectroscopy (FTIR) findings show that the phenolic compounds, hydroxy (OH) group, and phospholipid polar zone form hydrogen bonds. In addition, nanoliposomes have a long shelf life. The study also shows that liposomes can be used to enhance the maintenance and/or improvement of bio-functional active agents in food products [99]. A group of researchers studied the therapeutic efficacy of various drugs including rosmarinic acid, sinensetin, and eupatorin. They also evaluated the formulation of *Orthosiphon stamineus* extract OS-E nanoliposomes in rat plasma. All the compounds were poorly distributed and slowly removed from the body after intravenous administration of OS-E nanoliposomes. In contrast, the bioavailability of compounds was higher after oral administration of OS-E nanoliposomes. These findings show that the OS-E nanoliposome's higher bioavailability and solubility may be because of its use of liposome encapsulation [100].

Currently, it is observed that the antioxidant properties of *Capsicum annuum* pepper are extracted after being loaded with liposome. The effects of these extracts show no cytotoxicity, a reduced amount of reactive oxygen species (ROS), and an increased expression of endogenous antioxidants in the HepG2 cell line, as shown in Fig. 4 [101]. Aside from their improved ability to deliver phytochemicals,

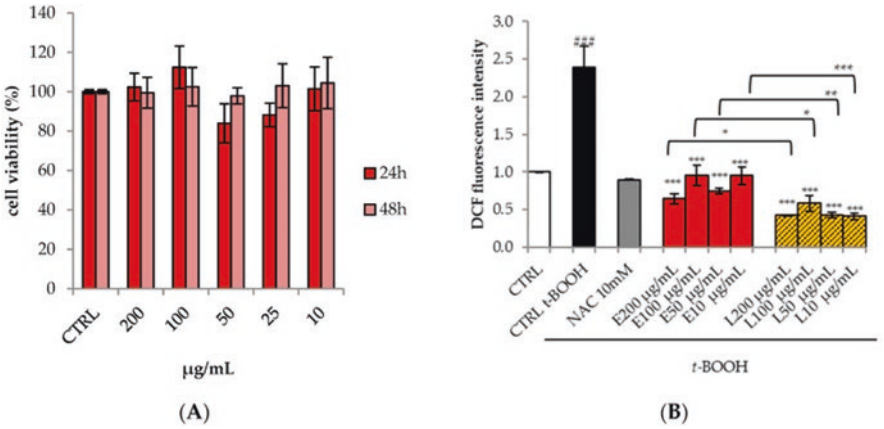

Fig. 4 (**a**) Viability of cells treated with different concentrations of *C. annuum* extract (CAE) for 24 and 48 hours; (**b**) effects of liposomes (L) and CAE (E) on intracellular ROS production in HepG2 cells induced by *tert*-butyl hydroperoxide (t-BuOOH); cells were pre-treated for 24 hours with extracts before being incubated with 5 mM *t*-BuOOH. The ROS production was evaluated by using 2',7'-dichlorodihydrofluorescein diacetate staining and flow cytometry. (Reproduced with permission from [101]; copyright [2020] MDPI; the article was printed under a CC-BY license)

liposomes also have disadvantages. For instance, the development of drugs for use in liposomes requires a high cost and can result in encapsulated drug fusion and leakage, and phospholipid liposomes may experience accelerated hydrolysis and/or oxidation, which can result in a shorter half-life.

10 Niosomes

Niosomes are nanometric lamellar vessels made up of nonionic surfactants [102, 103]. Nonionic surfactants can create a stable bilayer structure by effectively agitating and heating [104, 105]. The sections of the bilayer structure that are hydrophobic are guided away from the aqueous phase, while the sections with the hydrophilic tails remain in contact with the aqueous side. Biocompatible and non-immunogenic surfactants should be employed in the development of niosomes [106]. Both in vivo and in vitro studies demonstrate that niosomes mimic liposomes by prolonging the circulation of phytochemicals, altering organ distribution, and increasing bioactivity [107]. Niosome formulations are more prone to cause leaky patches than liposomes when mixed with the same cholesterol level [107]. The amount of cholesterol has a significant impact on vesicle leakage [108]. As a result, the efficiency of liposomal drug trapping is lower than niosomes [109]. The components of liposomes are unstable and require special handling and storage [110]. Niosomes are considered a new herbal drug delivery system that can improve the solubility of phytochemicals. In one study, it was determined that the niosome encapsulation of antioxidant phytochemicals includes lawsone, diosgenin, and *Carum* spp. [111].

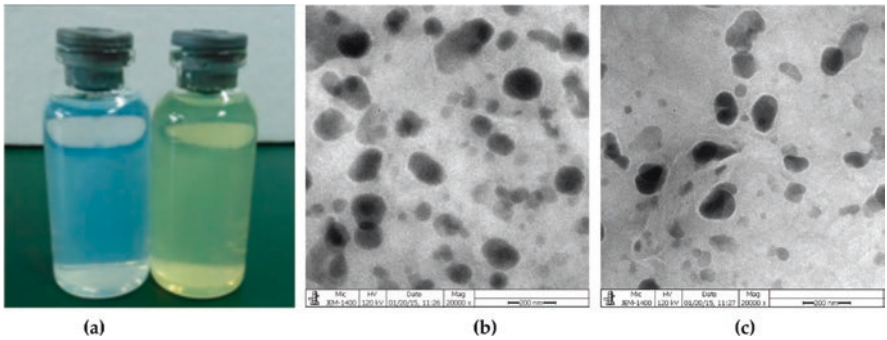

Fig. 5 (**a**) Appearance of blank niosome and quercetin-loaded niosome; (**b**) TEM image of empty niosome; (**c**) quercetin-loaded niosome. (Reproduced with permission from [8]; copyright [2019] MDPI; the article was printed under a CC-BY license)

Moreover, researchers also developed a natural anticancer niosome vesicle based on nonionic surfactants and *Carum carvi* extract. Various formulations were tested for in vitro cytotoxicity, DNA fragmentation, flow cytometry, and cell migration. *Carum*-encapsulated niosomes demonstrate good anticancer effects against cancer cell lines (MCF-7), based on the results of flow cytometry and MTT assay. The cell cycle analysis reveals that the formulations of *Carum*-encapsulated niosomes exhibit a G2/M arrest that prevents the migration of MCF7 cells [111].

However, niosome is formulated to increase the solubility and stability of antioxidant flavonoids. A group of researchers loaded phytochemicals into niosome and found that a spherical shape was generated with 87.3% drug trapping efficiency, and the quercetin demonstrated substantial whitening and antioxidant potential, as shown in Fig. 5 [8]. The transmission electron microscopy (TEM) image in Fig. 5b shows that quercetin-loaded niosome is oval or spherical. Another study reports an in vitro determination of nanovesicles incorporated with marigold extract and finds that marigolds can be entrapped in a bio-delivery vesicle containing surfactants. Their findings also indicate that noisome-based marigold extract could be used as a food additive. Niosomes significantly improve the photostability and bioavailability of quercetin. Quercetin-loaded niosomes exhibit prolonged release and increased skin absorption compared with quercetin solutions [112]. Niosomes have various benefits over liposomes, but they also have drawbacks. For instance, nonionic surfactants of niosomes are not generally considered safe. They are also more of an irritant than liposomes [113].

11 Phytosomes in Clinical Trials

Several phytosome-based drugs have progressed to the level of human trials after completing clinical studies. The effects of these drugs on the human body are being investigated. This is the first step toward receiving the Food and Drug Administration

(FDA) final approval. In 2007, the first clinical trial for a phytosome-based formulation was performed (ClinicalTrials.gov Identifier: NCT00487721). Silybin, which has anticancer properties, was integrated into a variety of phytosomes for use in prostate cancer patients. [114]. The researchers discovered that a high concentration of silybin-phytosome might result in a high blood concentration, suggesting that it could be used as an alternative treatment for prostate cancer patients [6, 115]. The silybin-phytosome formulation was used in the second clinical study (ClinicalTrials.gov Identifier: NCT02146118). Siliphos was developed using a combination of erlotinib (Tarceva) and phytosomal formulations. Although this research is still in its early phases, experts believe that this combination could have a synergistic effect on the treatment of epidermal growth factor receptor (EGFR) mutant lung cancer patients. In 2014, a study was conducted to evaluate the effects of green tea extract in reducing obesity (ClinicalTrials.gov Identifier: NCT02542449). A clinical trial was carried out to study this using an administrated formulation. The clinical trial for this study is currently in phase IV. The results reveal that green tea extract phytosomal preparation has a significant effect on maintaining weight in obese patients [7]. Moreover, a clinical trial was conducted to investigate the efficacy of grape seed extracts against lung cancer when synthesized as a phytosome-based formulation (ClinicalTrials.gov Identifier: NCT04515004). The study reveals that the formulation of phytosomes could delay the surgery by up to 14 days. When coupled with dry extracts from artichoke leaves, the anti-hypercholesterolemic effect of bergamot-phytosome formulations was investigated in patients with mild hypercholesterolemia (ClinicalTrials.gov Identifier: NCT04697121). The results demonstrated that administering the produced formulation has a beneficial effect on lipid and metabolic parameters, indicating a strong anti-hypercholesterolemic efficacy. The most recent clinical trial was conducted on the effects of quercetin phytosome on the treatment of COVID-19 patients (ClinicalTrials.gov Identifier: NCT04578158). It is hypothesized that quercetin phytosomes will boost the subjects' natural immunity and prevent the development of the COVID-19 disease. At present, this study is still being investigated. The numerous phytosome-based formulations presented in clinical trials are summarized in Table 3.

12 Patent Perspective

Numerous academic scientists and industrialists are working on phytosome technology in various regions of the world, resulting in multiple breakthroughs. A search on www.lens.org with the keyword "phytosome," a date range of January 1, 2020–January 1, 2022, and "granted patent" as the document type yielded the ten latest patents granted on phytosomes. This includes the phospholipid complexes of extracts of standardized extracts in medicinal and cosmetic formulations for skin aging prevention. This invention relates to a composition that includes a skin-healing extract of *Centella asiatica* and *Mori cortex* phytosomes. The composition of this extract has been shown to reduce the production of iNOS and COX-2 and

Table 3 Various phytosome-based formulations on clinical trials

Condition	Phytosome formulation	Clinical trial phase and number	Outcome	References
COVID-19	Quercetin	Phase III (NCT00487721)	Under investigation	–
Early stages of lung cancer	Grape seed extract	Phase II (NCT04515004)	Delayed planned surgery of >14 days	–
Hypercholesterolemia	Bergamot	Not applicable (NCT04697121)	Anti-hypercholesterolemic activity	–
EGFR mutant lung adenocarcinoma	Silybin	Phase II (NCT02146118)	Under investigation	–
Prostate cancer	Silybin	Phase II (NCT00487721)	High blood concentration of silybin	[6, 115]
Obesity	Green tea extract	Phase IV (NCT02542449)	Maintaining weight following weight loss	[7]

The data was retrieved from www.clinicaltrials.gov; accessed on November 21, 2021

inhibit the secretion of histamine in atopic-induced mast cells. This finding is made possible by the composition of the two plants' phytosomes, which are expected to be used as therapeutic agents for the treatment of inflammatory diseases. Other inventions include a formulation that provides a variety of nutrients and prevents age-related macular degeneration (AMD). It contains the optimal daily doses of various nutrients such as vitamin C, zinc, and copper. Phytosomes used in topical applications that contain a broad spectrum of active substances (sorbitylbenzal and sorbitylvanillinal) can help nourish and protect the skin from free radicals. It can also contribute to healing processes by reducing the spread of inflammatory factors. Table 4 summarizes the several patents on phytosomes and their related innovative technologies.

13 Future Prospects

The use of complex active ingredients with phospholipids has been identified as a prominent method for improving the pharmacokinetics and pharmacodynamic profile of phytoconstituents. A phyto-phospholipid complex that was initially developed for cosmetic use has been successfully used as a drug carrier [3, 5]. Although the field is still relatively new, studies are being conducted to improve the clinical capabilities of these systems. A deep dive into the literature shows that various studies are still being conducted on the use of herbal active ingredients for drug delivery. Researchers should also pay attention to the systems that can transport the various plant components needed for their effective delivery.

Although solvent evaporation is commonly used for the production of phyto-phospholipids, the process is often time-consuming and carries various steps that are not optimized in any studies. The supercritical fluid technique allows for the

Table 4 Patents on phytosomes and related technologies

No.	Title	Innovation	Patent no. (granted year)
1	Formulation and method for supporting retinal health and reducing the risk of AMD	The incorporation of about 10–30 mg of Bio-Quercetin phytosomes into an appropriate dosage form to reduce the risk of AMD	US 11090274 B2 (2021)
2	Composition for prevention or treatment of skin inflammation comprising *Centella asiatica* phytosome and *Mori radicis* Cortex extracts	Phytosomes of *Mori cortex* and *Centella asiatica* extracts for preventing, ameliorating, or treating skin inflammation	KR 102073009 B1 (2020)
3	Topical compositions comprising extract of *Coriolus versicolor* for autoimmunity enhancement	Composition of *Coriolus versicolor* for preventing and/or treating by vaginal or cervical administration	US 10874702 B2 (2020)
4	Insulin-lipid complex, preparation method therefor, and preparation thereof	Preparation of an insulin-lipid complex	US 10611852 B2 (2020)
5	Cosmetic formulation to reduce facial flushing	Compositions of novel cosmetic skin care products for the treatment of flushed or rosacea-affected skin	US 10596090 B2 (2020)
6	Compounds with antioxidant properties against free radicals, anti-inflammatory activity, and corresponding pharmaceutical compositions for skin care	Topical composition with anti-inflammatory and antioxidant properties	EP 3274347 B1 (2020)
7	Compositions containing silymarin and sulfoalkyl ether cyclodextrin and equal usage methods	Composition comprising silybin A, isosilybin A, and sulfoalkyl ether cyclodextrin	EP 3270941 B1 (2021)
8	Ferment extract of *Eupenicillium crustaceum* and cosmetic use thereof	A ferment extract used to treat and/or care for the skin, mucous membranes, hair, and/or nails on a cosmetic, nontherapeutic basis	EP 3265184 B1 (2020)
9	Administration of berberine metabolites	A technique for regulating an individual's glucose tolerance	US 11026929 B2 (2021)
10	Multi-supplement compositions	Compositions including multiple dietary supplement formulas	US 11207388 B2 (2021)

The data was retrieved from https://link.lens.org/96alkkDrO5h, accessed on January 23, 2022; keyword, phytosome; date range, January 1, 2020–January 1, 2022; document type, granted patent

precise control of the particle size and distribution, which can improve the systemic bioavailability of pharmaceutical products. The CO_2 supercritical fluid's properties make it an excellent choice for the development of sensitive drugs [23]. However, further studies are needed to analyze its effects on the in vivo properties of drugs. Although it has been commonly used to formulate drugs and phospholipids at a 1:1

molar ratio, much of the research has been focused on the use of compounds with a different ratio. Studies reveal that a drug with a higher phospholipid content can produce a better product in terms of its pharmacological properties [3, 5]. The exact steps involved in producing and drying phyto-phospholipid complexes vary depending on the method used. The statistical technique can also be used to design a plant extract with superior quality and trapping efficiency [23]. It can also be used to determine the proportions of phospholipids and plant extracts that are required to achieve this goal. Aside from targeting antigens, phyto-phospholipid complexes can also be used to target other cellular structures. This will allow users to treat other debilitating conditions, such as arthritis and osteoporosis [9, 23].

14 Conclusion

Due to the rise in phytochemicals, their medical benefits are expected to be studied more extensively in the future. However, their low solubility and sensitivity limit their use in food and pharmaceutical products. Understanding the characteristics of vesicular drug delivery systems can help improve their usefulness in the development of phytochemicals. Phytosomes are complex compounds that consist of phytochemicals and phospholipids. They contribute to the improved absorption and bioavailability of drugs. In terms of release efficiency, transfersomes, niosomes, and liposomes are the most commonly used nanocarriers for the preparation of phytochemicals. As for phytosomes, their development is also related to the increasing demand for plant-based nutraceuticals. This step involves carrying out a comprehensive analysis of the product's stability and release dynamics.

This chapter provides an overview of the characteristics of phytosomes, which are mainly used as delivery products. Studies show that their use can improve the bioavailability of formulated compounds. The presence of a clinical study does not always mean that a product is superior to its components. In most cases, the superiority of a product's formula is only assessed in comparison with its components. Most of the clinical evidence supporting the use of certain phytochemicals for various applications has been collected. However, the results of these studies are not yet clear enough to support the biological activities of these preparations. The development of standardized products that have superior efficacy will be the main focus of future studies.

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Porphysomes and Porphyrin-Based Nanomaterials for Drug Delivery System

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1 Introduction

1.1 Evolution of Phototherapy

Phototherapy or light-based therapy dates back to several thousand years used to treat various diseases by various ancient civilizations. In 1903, Finsen used phototherapy to treat skin cancer by applying eosin dye topically over the target area and irradiating it with white light, which earned him the Nobel Prize. By the end of the nineteenth century, research on the usage of PDT (photodynamic therapy) to treat various cancer began to flourish, leading to the development of new classes of photosensitizes. Light-absorbing compounds and nanostructures are widely used for biomedical applications, ranging from diagnosis to therapy. Owing to their enhanced

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destruction coefficients, gold inorganic nanoparticles are employed for therapeutic and imaging applications, such as fluorescence imaging, photoacoustic imaging, lateral flow immune sensing, photothermal therapy, and targeted delivery. Quantum dots are used for MRI (magnetic resonance imaging), fluorescence imaging, PDT, and targeted distribution due to their photostability and superior brightness to proteins and fluorescent dyes. Conversion of these inorganic nanoparticles from research to real-time applications has been stalled owing to their indefinite long-standing possessions on the body. Liposomes (organic nanoparticles) are known for their biocompatibility, biodegradability, tunability and as a carrier for drug loading. Nevertheless, these do not fundamentally absorb light and often act as a dye loading and delivery carrier. Porphyrins are naturally occurring organic, light-absorbing molecules found in many plant and animal systems. Most clinically approved photosensitizers (PS) used for PDT-based cancer therapy are porphyrin derivatives, except methylene blue dye.

1.2 Porphyrin

Porphyrins are a group of macrocycle organic compounds (heterocyclic). It comprises pyrrole rings (four conjugated) settled in a circle-like fashion via methylene bridges (=CH−) at their α-carbon atoms [1]. Porphyrin is made of 26 π-electrons, of which 18 are arranged continuously in a planar form. The name "porphyrin" was derived using the Greek word "Porphyra," which means the color purple. Porphyrins could absorb light in the region of visible light and exhibit photonic properties such as fluorescence or PDT, aided by the large conjugated structure. Various classes of porphyrin exist in nature, such as in the blood, and the cytochrome chain aiding aerobic mechanisms. The elementary porphyrin structure and its notable group associates are shown in Fig. 1.

Hematoporphyrin is the first porphyrin molecule discovered by Scherer in early 1841 while exploring the properties of blood [2]. The fluorescence properties and PDT abilities were not explained until 1871 and 1911, respectively, followed by the demonstration of hematoporphyrin's photosensitization by Meyer-Betz in 1913, self-injecting himself with porphyrin [3]. First, PDT was attempted to suppress the

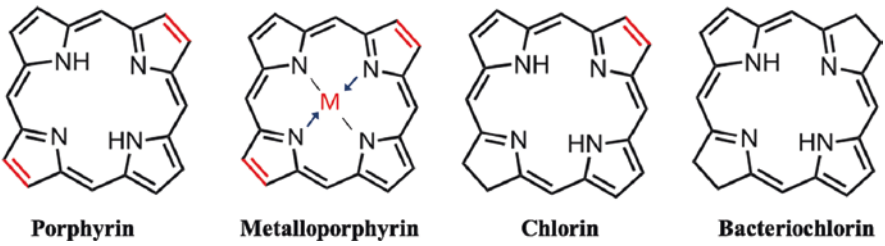

Fig. 1 Basic structures of porphyrin and its family

tumor growth in bladder cancer, followed by skin and lung cancer. Even though porphyrins were discovered in the eighteenth century, their unlimited potential was not explored until the late nineteenth century. In 1984, Dougherty demonstrated PDT-based tumor suppression in animal models by administering porphyrins and irradiation with red light [4]. In 1984, the first human trial of PDT involving a single patient with bladder cancer, who did not respond to all conventional treatments such as chemotherapy, radiotherapy, and surgery, was administered with porphyrin. On irradiation, the fluorescence at the tumor site, followed by tumor necrosis in selected malignant tissues, was observed. Only after reflecting on the affinity of porphyrin to tumor tissue, many studies targeted combining the fluorescent and phototoxic effects of porphyrin with tumor-selective localization properties in the cancer treatment, leading to the expansion of photoimaging and photodynamic therapy [5].

1.3 First- and Second-Generation Porphyrin-Based Drugs

Porphyrin-based compounds that are clinically approved and under clinical trial for detection and treatment of cancer and other diseases are listed in Table 1. Hematoporphyrin, isolated from the blood (RBCs), is the first isolated porphyrin compound. Further purification of hematoporphyrin resulted in Photofrin®, the first-generation porphyrin-based drug used as a photosensitizing agent in PDT. Photofrin® (porfimer sodium) was the first officially approved PS in over 120 countries for photodynamic therapy of several cancers, such as lung adenocarcinoma and esophageal, superficial bladder, gastric, cervical, and endobronchial cancers. The drug is administered intravenously near the tumor, allowing accumulation in the tumor surroundings. The tumor was irradiated with a laser at a wavelength of 630 nm, 24–48 h after the injection, resulting in necrosis of malignant tissues. Despite the advantages offered by Photofrin®, there are various limitations such as long photosensitivity over several weeks due to prolonged half-life time, inability to target deep-seated tumors due to short wavelength, erythema, constipation, and poor chemical purity [1].

Second-generation porphyrin-based drugs attempted to improve on the limitations of the previous generation PS. The chemical purity of the drugs was enhanced using various techniques. Furthermost, the other-generation PS were grounded on modified porphyrin and chlorin structures. Either the core was modified, or additional side chains or functional groups were introduced to improve their specificity to tumor cells. Also, the first-generation PS had a very short excitation wavelength. The structural modifications enabled excitement at a sophisticated wavelength, which allowed innermost tissue dispersion to treat deep-seated tumors. Some of the second-generation PS include Foscan (temporfin), Visudyne (verteporfin), talaporfin, redaporfin, Lutrin® (motexafin lutetium), motexafin gadolinium, Tookad (palladium-bacteriopheophorbide), etc. [6]. Their chemical structures are given in Fig. 2.

Table 1 List of selected first- and second-generation porphyrin-based PS

Generic name	Chemical name	Application	Excitation wavelength and drug-light interval	Dose (mg/kg)	Power (J/cm^2) and fluence (mW/cm^2)	Advantage	Disadvantages/ side effects	Clinical approval status and year
First generation								
Photofrin®	Hematoporphyrin derivative (HpD), porfimer sodium	Lung adenocarcinoma, esophageal, cervical, superficial bladder, gastric, and endobronchial cancers	630 nm; 24–48 h	2 mg/kg	120–180	First developed. Preparation is less heterogenous than the original hematoporphyrin derivative	Long photosensitivity, erythema, mild constipation, long drug-light interval	Yes, 1993
Second generation								
Foscan®/ temporfin	*meta*-etra(hydroxyphenyl) chlorin (m-THPC)	Head and neck cancer	652 nm; 90–110 h	0.15 mg/ kg	20 J/cm^2 100 (mW/cm^2)	High singlet oxygen yield, chemically pure	Headache, hemorrhage, dysphagia, edema, scarring, long photosensitivity, long drug-light interval, hydrophobic	Yes, 2001
Visudyne®/ verteporfin	Benzoporphyrin derivative monoacid ring A (BPD-MA)	Advanced pancreatic cancer, age-related macular degeneration, myopic choroidal neovascularization	690 nm; 15 min	6 mg/kg	50 J/cm^2; 600 (mW/cm^2)	Induce tumor necrosis, rapid clearance, short drug-light interval, better selectivity	Pain, edema, inflammation, hemorrhage, discoloration, visual impairment, visual field defects	Yes, 2000

Generic name	Chemical name	Application	Excitation wavelength and drug-light interval	Dose (mg/kg)	Power (J/cm^2) and fluence (mW/cm^2)	Advantage	Disadvantages/ side effects	Clinical approval status and year
Laserphyrin®/ talaporfin	Mono-L-aspartyl chlorin e6	Early lung cancer (central), head and neck cancer	664 nm; 90–110 h	1 mg/kg	200 J	Chemically pure, rapid clearance	Long drug-light interval, short excitation wavelength	Yes, 2004
Redaporfin®	5,10,15,20-tetrakis(2,6-difluoro-3-N-methylsulfamoylphenyl)-bacteriochlorin	Biliary tract cancer	749 nm 3–20 h	NA[a]	NA[a]	Long wavelength, significant ROS generation, and cellular uptake	Hydrophobic, skin photosensitivity	In trial
Fotolon®	Chlorin e6-PVP	Skin melanoma, nasopharyngeal sarcoma	665 nm 2–3 h	2–2.5 mg/kg	50–600 J/cm^2	Effective and selective	Shorter excitation wavelength	In trial
Lutrin® Xcytrin®	Motexafin lutetium Motexafin gadolinium	Prostate cancer Brain and lung cancer	730 nm 3 h	0.5–2 mg/kg	25–150 J/cm^2	Chemically pure, deeper tissue penetration, soluble	Photosensitivity, pain, multistep synthesis, cancer recurrence	No, approved for AMD
Purlytin®/ rostaporfin	Tin ethyl etiopurpurin	Kaposi sarcoma, metastatic breast cancer	660 nm; 24 h	1.2 mg/kg	200 J/cm^2 150 mW/cm^2	Effective for treatment against AMD	Not effective, shorter excitation wavelength	No, approved for AMD

(continued)

Table 1 (continued)

Generic name	Chemical name	Application	Excitation wavelength and drug-light interval	Dose (mg/kg)	Power (J/cm^2) and fluence (mW/cm^2)	Advantage	Disadvantages/ side effects	Clinical approval status and year
Radachlorin®	Sodium chlorin e6	Skin cancer, advanced non-small cell lung cancer	662 nm; 3 h	0.5–2.4 mg/kg	200–300 J/cm^2	Short drug-light interval, better selectivity	Shorter excitation wavelength	Yes, 2009; Russia
Photochlor®	2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a	Head and neck cancer	664 nm	NA[a]	NA[a]	Chemically pure	Shorter excitation wavelength	In trial
Tookad®	Palladium bacteriopheophorbide	Prostate cancer	762 nm; 15 min	NA[a]	200 J/cm 150 mW/cm	Soluble, short half-life, long wavelength, high singlet oxygen yield, pure, rapid clearance	NA[a]	In trial, 2005
Talaporfin®	Mono-L-aspartyl chlorin e6	Liver cancer, colorectal neoplasms, hepatocellular carcinoma	664 nm; 0.25–4 h	1 mg/kg	200 J/cm^2	Water soluble, rapid elimination	Shorter excitation wavelength	In trial

[a]NA information not available (table was prepared by one of the authors – Dr. Pon Janani Sugumaran)

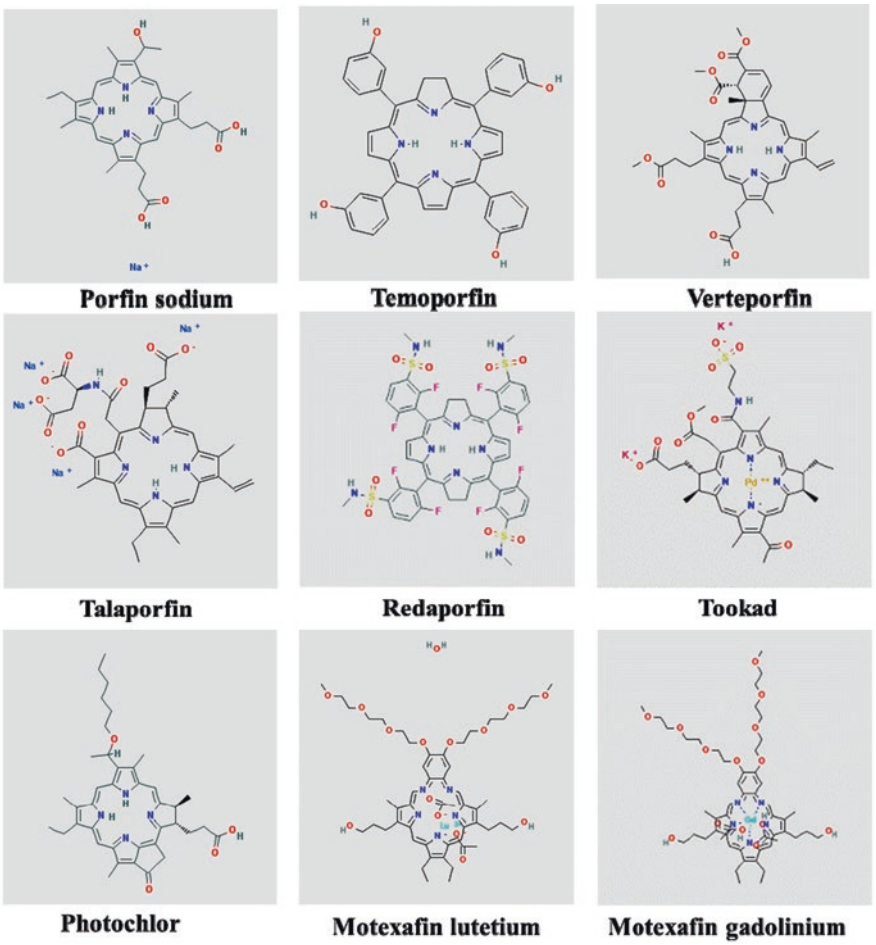

Fig. 2 Porphyrin-based photosensitizers (clinically approved/in trial)

Foscan/temporfin is a clinically approved second-generation photosensitizing agent used to treat head, neck, breast, and pancreatic cancers. After the injection of the drug, temporfin is allowed to accumulate at the tumor for not less than 90 h and is exposed to a wavelength of 652 nm, which triggers the generation of ROS. It had good specificity to cancer cells and a more than 50% survival rate. Metal-based porphyrin complexes, commonly known as metalloporphyrin compounds such as motexafin lutetium, motexafin gadolinium, and palladium bacteriopheophorbide, were also used as PS for PST [7, 8]. In general, most metal-associated porphyrin complexes tend to have longer excitation wavelength, making them suitable for deep-seated tumor PDT. For example, Tookad is activated at 762 nm, allowing deep tissue penetration. They are frequently used as PDT (vascular-targeted), in which after the excitation, it experiences universal movement leading to the intravascular

group of hydroxy and superoxides, which triggers PDT. Another main advantage of Tookad is the short drug-light interval and faster clearance. Light can be instantly administered within 10 min after administration, and it has a very short half-life time of 0.02–0.03 h. The most common disadvantage of porphyrin-based PS, prolonged skin sensitivity to light after treatment, is addressed by Tookad's faster elimination rate [8]. The chemical name, excitation wavelength, drug-light interval time, application, advantages, and disadvantages of first- and second-generation porphyrin-based PS are listed in Table 1 [1, 6, 9, 10].

Even though the chemical purity was improved, the second-generation PS still suffer from several limitations: poor water solubility, prolonged photosensitivity, photobleaching, low penetration depth, poor selectivity, and a slower clearance rate. To address this, third-generation PS are targeted to improve tumor selectivity through targeting and longer excitation wavelength activation. Poor water solubility can be addressed by encapsulating the existing PS in nanocarriers that render hydrophilicity to the complex. Sensitivity can be improved by conjugating the complex with ligands/antibodies for targeting specific tumor receptors. Porphyrin-based nanomaterial for drug delivery is further discussed in detail in the latter part, after discussing the mechanisms and requirements of photodynamic therapy (PDT) and photothermal therapy (PTT).

2 Drug Delivery Mechanism

Cancer is the subsequent foremost cause of passing worldwide, secretarial for roughly 10 million deaths in 2020. If the cancer is detected and treated early, the number would be considerably less. Despite constant improvements, conventional cancer treatment methods such as radiotherapy and chemotherapy still pose some serious disadvantages. Their hydrophobicity and poor stability limit the usage of several potential drugs. Development in nanotechnology has led to some breakthroughs in drug delivery [11, 12]. Several nano-drug delivery systems have been developed for cancer therapy that is more localized and selective and can surpass the protected organization and even cross the blood-brain barrier. By suitable surface ligands, the nanoparticle system could selectively target and bind to the cancer cells, offering excellent selectivity and not affecting normal cells [13]. An ideal drug carrier should be (a) biocompatible, (b) non-immunogenic, (c) biodegradable, (d) able to form a stable drug-ligand complex, (e) specific to the target cells, and (f) able to cross the blood-brain barrier and should (g) release the drug at a target site, making them active, and (h) provide hydrophilicity and good water solubility.

It is mandatory to understand the tumor environment to develop a better delivery method. In general, all cells undergo cell division to sustain life and undergo cell death after a certain number of cycles, or the immune system eliminates an error in the division. Tumor is formed when such a normal cell undergoes random mutagenesis, escapes from the immune system, and divides uncontrollably, becoming cancer. Such divided cells secrete certain chemical signals, like vascular endothelial

growth factor (VEGF), which results in the formation of newer blood vessels and supplies nutrients to the tumor leading to further growth. These newly formed blood cells are imperfect, irregular, and dilated. The tumor does not have a proper lymphatic drainage system. The combination of imperfect dilated blood vessels and poor lymphatic drainage contributes to the enhanced permeation and retention (EPR) consequence, mostly taken benefit of unreceptive pointing. The nano-drug carriers take advantage of this tumor microenvironment. When the drug is administered intra-tumor or near the blood vessel that caters to the tumor, the nano-drug gets accumulated more in the tumor tissue than normal cells due to the dilated blood vessels. It gets retained there due to poor lymphatic drainage. The tumor cells are also characterized by over expression of certain receptors, such as folate receptors, G protein-coupled receptors, etc. The conjugation of ligands specific to certain receptors/markers to the drug molecules can aid in the active targeting of the drugs to the tumor [14–16].

2.1 Photodynamic Therapeutics (PDT)

Photodynamic therapy is a localized and robust cancer treatment. PDT is a multi-step process concerning the direction of photosensitizer (PS) at the tumor site, which is activated on the absorption of light of suitable wavelength and triggers a series of biological and photochemical processes that result in irreparable photodamage to cells [17]. PDT relies on three important constituents: photosensitizer, suitable excitation wavelength, and oxygen. The photosensitizer is administered via intra-tumor injection or at nearby areas, either targeted or accumulated at the site through EPR. When excited at a suitable wavelength, the photosensitizer excites the ground state (PS_0) to a higher energy state ($^1PS^*$). This is followed by the short-lived excited singlet PS ($^1PS^*$) movement to the ground state. These drops to the ground state occur directly or via an intermediate step to the long-lived triplet state ($^3PS^*$) before reaching the ground state. The movement from triplet PS ($^3PS^*$) to the ground state (PS_0) initiates the necessary pathways for photodynamic therapy. Based on the mechanism of electron transfer/reaction with oxygen, it is classified as (i) electron transfer (Type I) and (ii) energy transfer (Type II) pathways [18, 19].

In the Type I pathway, an electron allocation mechanism occurs between the organic component and excited PS of the cell, which results in the generation of reactive free radical species (ROS), such as hydroxyl radical (•OH), hydrogen peroxide, and superoxide ($\bullet O_2^-$). These ROS molecules are extremely robust and attack the neighboring cells, resulting in irreversible cell membrane damage via lipid peroxidation, abetting in PDT. The Type II pathway occurs when the eager triplet PS reacts straight with the O_2 molecules and causes the generation of highly reactive singlet oxygen ($^1O_2$). The triplet PS ($^3PS^*$) interaction with biomolecules present in the cell wall/membrane can instigate oxidative damage and impair the cells. This can prompt functional and morphological changes in the cells, resulting in one or more damages leading to cell death, such as impairment in the membrane transport

system, cell membrane mutilation, cytoplasmic leakage, mitochondrial damage, lipid peroxidation, metabolic inhibition, DNA impairment, etc. The damage may be triggered by Type I/Type II pathways or by acting together. However, damage instigated by singlet oxygen generated via Type II pathway is the most common. The singlet oxygen and ROS generated to have an inadequate generation and effective action radius. Typical singlet oxygen generated has a short lifetime of 0.04 μs and a radius of 0.02 μm; this enables localized treatment and necessitates the need to activate the photosensitizer at the target site. Figure 3 shows a representation design of the mechanism of PDT and associated cell death pathways. PDT provides better tumor selectivity than conventional therapies and can be used for multiple rounds of treatment without damaging the immune and hematopoietic systems. Some studies have shown that the ROS-mediated PST process can successfully reverse the chemoresistant tumor. This could be attributed to the ROS-mediated conversion of nicotinamide adenine dinucleotide with hydrogen (NADH) to nicotinamide adenine dinucleotide (NAD), which in turn reduces the ATP available for the efflux pumps.

PDT's efficiency is dependent on the dosage of PS, excitation wavelength, and exposure time. The wavelength of light required depends on the type of PS, ranging from UV-visible light (300–700 nm) to NIR light (700–1000 nm). The penetration depth depends on the wavelength of light. Short-range wavelength is typically used for surface/skin cancer, whereas long NIR-based wavelength is used for deep-seated tumors [19–21].

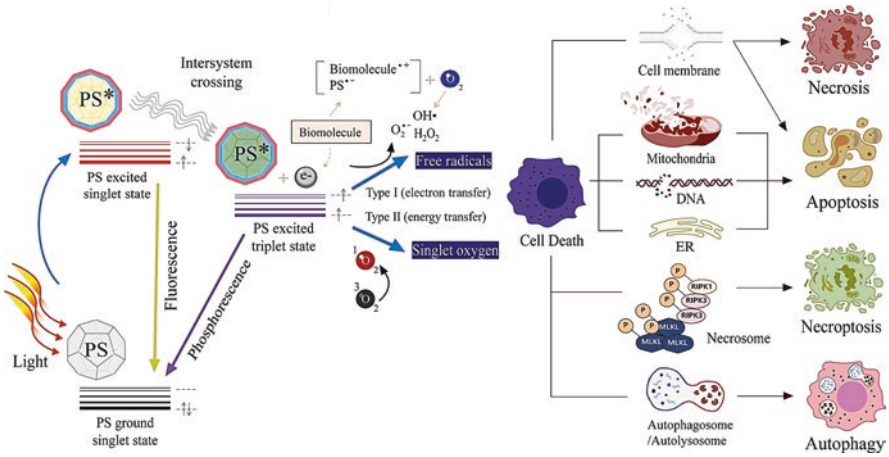

Fig. 3 Schematic illustration of the mechanism of PDT and associated cell death pathways. A PS took energy from light to killing tumor cells through ROS group. The induced modes (PDT) of cell passing include necroptosis, necrosis, autophagy, and apoptosis, depending on the cell category, concentration or PS type, oxygen partial pressure, light dose, and intracellular localization. PDT, photodynamic therapy; PS, photosensitizer; ER, endoplasmic reticulum [18]. (Reproduced from open-access journal under the term of Creative Commons Attribution License (Ref. [18]) Copyright © 2021)

2.2 Photothermal Therapy (PTT)

Photothermal or hyperthermia is a targeted, localized, and minimally invasive treatment modality with negligible toxicity. Thermal ablative therapies have shown promising results in preclinical and clinical studies. Localized heat generation could be achieved by several thermal therapies, including administration of inorganic gold nanoparticles or magnetic hyperthermia in which exposing magnetic nanoparticles such as an iron oxide to a magnetic field, radiofrequency ablation, and high-intensity focused ultrasound results in heating causing tumor destruction [16, 22–24]. Thermal therapy discussed in the chapter excludes those techniques and discusses only the photosensitizers-based PTT. PTT involves irradiating target tumor tissue with light, causing localized heat generation due to the light absorption and subsequent vibration of molecular chromophores [25]. PTT is mainly based on activating a localized photosensitizer by electromagnetic radiation, such as NIR, or visible light to generate heat, damaging the surrounding cancer tissue and leading to cytotoxicity. Cells are highly sensitive to their surrounding environments, such as temperature or pH. When the temperature goes beyond 42 °C, it causes denaturation/oxidation of protein resulting in several dangerous phenomena, such as membrane damage, altered cell signaling mechanisms, inhibition of cell proliferation, and nuclear protein damage inhibiting DNA repair mechanisms, thereby leading to apoptotic cell death. For even higher temperatures above 45 °C, the cell undergoes necrosis [5, 11, 25, 26]. Cancer cells are inherently sensitive to heat compared to normal cells. Treatment/exposure time depends on the heat generated at the tumor site. Lower temperatures need extended exposure time for the cell to undergo deterioration, whereas higher temperatures result in rapid necrosis/carbonization of the tumor, with heat radiating the entire tumor volume.

After administration of the photosensitizer and intra-tumoral accumulation, PS is activated with irradiation of light, typically near-infrared (NIR) light. It causes the PS conduction band electrons to undergo synchronized oscillations to convert NIR light into heat, eventually leading to cell death. The main advantages of PTT associated to predictable chemotherapy or radiotherapy are the localized approach and minimal non-targeted cell expiry in the neighboring well tissue. NIR has the ability for bottomless tissue diffusion and targeting deep-seated tumors. Unlike photodynamic therapy, PTT does not depend on the presence of oxygen for the generation of ROS. This facilitates the PTT in hypoxic regions in a tumor, where other treatments are ineffective. The supreme photosensitizing agent should possess huge absorption cross sections for optical wavelengths, affluence in functionalization, small deadliness, biocompatibility, and good solubility. PTT is often used in association with conventional treatment modalities like chemotherapy, as PTT could act even in oxygen-deprived hypoxic tumor regions and make the tumor sensitive to chemotherapeutic drugs [26, 11].

3 Porphyrin-Based Nanomaterials for Drug Delivery

The sympathetic of structures (porphyrin) has progressed significantly in current ages, and numerous journals have defined complete metalloporphyrin and porphyrin structures and their dissimilar parts in applications of biomedicals. Commonly, porphyrins are a class of macrocycles belonging to the tetrapyrrole type with a 2-D or 1-D or occasionally 3-D skeleton. By substituting the carbon α- and β-atoms of porphyrins with nitrogen atoms and the additional replacements, their belongings and applications biomedically can alter intensely. Additionally, characteristics of the porphyrin's chemistry are associated to act as a ligand in coordination complexes, corresponding to their properties structurally, which are measured by bond distances and bond angles, as well as the nature of the metal and also the occurrence of functional clusters. Commonly, transition metals (first row) are used in the structure of porphyrins; poisonousness is diminished to the lowest. Nevertheless, some second- and third-row transition metals such as Pt, Pd, and Ru also have little cellular poisonousness. By using amine-based ligands as the alternatives, the applications biomedically in drug delivery, photodynamic therapy, and gene delivery are expanded. Nevertheless, carboxy-based linkers are also effective for the delivery of drugs due to their potential attraction to numerous drugs through H-bonds.

3.1 Polymer-Based Nanomaterials for Drug Delivery System

Conjugated polymers have been shown for various biological applications like biosensors, drug delivery, cell imaging, in vivo imaging, and PDT. For PDT applications [27], conjugated polymers have been generating the singlet oxygen ($^1O_2$) by their efficient energy and absorption coefficient passing to the multichromophoric photosensitizers. The large length of the conjugated chain of polymers exhibits an absorption peak in the red wavelength area, further developing the tissue infiltration of excitation light. In one methodology, photosensitizer atoms can be physically captured in conjugated polymer nanoparticles by hydrophobic connections. The tightly packed multichromophoric nanoparticles contained conjugated polymer and photosensitizer in the closeness that works to enhance singlet oxygen age by energy transmission [28].

Notwithstanding, the encapsulation tactic has certain difficulties. For instance, the photosensitizer particles captured in the nanoparticles incline to form collections, limiting the loading capacity and reducing singlet oxygen generation [29]. Conjugated polymerized photosensitizers have limitations such as low water solubility, poor absorption coefficients, and leaching from delivery carriers. Also, the photosensitizer can filter from the nanoparticles, bringing about undesirable fears such as dark poisonousness. In elective methodologies, electrostatic compounds of anionic porphyrins and cationic conjugated polymer could enhance the oxygen (singlet) production when contrasted with the corresponding polymer or porphyrin species.

Moreover, porphyrin as a suspended group was joined covalently to cationic polythiophene, which increased the oxygen production (singlet) effectively by energy moving from the conjugated backbone to porphyrin pendants [30]. Covalent-based conjugated porphyrin polymer has made the effective polymer-dot photosensitizer. Spectroscopic analysis indicates that the light-harvesting polymer overwhelmingly moves the excitation energy to the porphyrin unit, yielding effective singlet oxygen production for photodynamic treatment. The polymer dots (Pdots) also have great security that defeats the photosensitizer leaching problem experienced in additional nanoparticle transporters. Photodynamic adequacy and in vitro cytotoxicity of the Pdots have been assessed in MCF-7 cells by in vitro test, showing that the Pdots can effectively destroy cancer cells. The high product singlet oxygen formation and outstanding stability of porphyrin-added Pdots are hopeful for the photodynamic action of cancer [30]. Porphyrin-containing conjugated polymer (PCP) and a fluorescent conjugated polymer of poly(9,9-dihexylfluorene-alt-2,1,3-benzothiadiazole (PFBT) were coloaded into nanoparticles, and the obtained PorCP-PFBT-Tat nanoparticles permit clear perception of nanoparticles which take up into cancer cells. The fruitful model along these lines offers another chance to create multi-useful nanoparticles for imaging and therapy applications [31]. This study has exhibited that the encapsulation of meso-tetra(hydroxyphenyl) porphyrin (p-THPP) into sterile and freeze-dried sub-130 nm nanoparticles should be measured as a viable technique for delivering p-THPP to cancer cells. The somewhat low drug concentrations and short incubation times expected to incite good photodynamic harms after cell treatment, particularly with 50:50 PLGA (poly(D,L-lactide-co-glycolide)) nanoparticles, show these plans offer predominant photoactivity. Considering the possible clinical uses of nanoparticulate definitions for photodynamic treatment, a more noteworthy comprehension of the biological interactions and the involved PDT mechanism is of most extreme significance [32]. Because of the hydrophobic properties of porphyrins and their partiality to aggregate by stacking the planar atoms, they are hard to work within aqueous media. Consequently, epitomizing them in nanoparticles (NPs) or connection to dissimilar transfer vehicles has been utilized to develop distribution features further. Porphyrins can be utilized in a composite planned material with belongings that permit exact directing, extended tissue lifetime, improved hydrophilicity, and immune tolerance [33].

3.2 Microporous Organic Polymers

Microporous organic polymers have enormous explicit surface regions and porous structures. Microporous natural polymers have entered the structure through physical interactions or hydrophobicity [34]. This new type of porous material stands out because of its huge pore sizes, high clear surface regions, specific take-up of small particles, and optical or magnetic responses to the consideration of guests [35]. Metal-organic frameworks (MOFs) and covalent organic frameworks (COFs) are

crystalline hybrid microporous organic polymers. In COFs, the covalently bonded organic ligands, while in MOFs, polydentate organic linkers are linked to metal cations of transition elements. Mutually COFs and MOFs have been widely exploited as drug delivery or nanocarriers for various applications biomedically. Specifically, COFs and MOFs consist porphyrins which are examined in the accompanying sector [33].

3.2.1 Metal-Organic Frameworks (MOFs)

Metal-organic frameworks (MOFs), additionally called coordination networks or coordination polymers, are a group of hybrid materials enclosed by the groups of metal particles and polydentate bridging ligands normally below slight circumstances. Because of boundless mixes of ligands and metals, the physicochemical properties of MOFs can be prudently adjusted for precise requests. Therefore, mass stage MOFs have guaranteed various assorted applications, including nonlinear optics, light-harvesting, catalysis, sensing, separations, and gas storage, since MOFs can display extraordinarily extreme external areas with huge pore sizes and additionally have been researched for applications in controlled release and loading of numerous molecules of the drug [36].

Nanoscale metal-organic framework (MOF)-based porphyrin system was developed from metal particle/particle groups and natural spanning ligands of porphyrins. Nanoscale metal-organic framework (MOF) has as of late arisen as a talented nanocarrier step for remedial and imagination specialists. Contrasted with other nano-transporters, nanoscale metal-organic framework (MOF) merges numerous gainful elements into a single distribution phase and involves tuneable crystalline structures and chemical compositions, biodegradability, and high porosity. Hf-based DBP-UiO nanoscale (DBP-UiO,1,5-di(p-benzoato)porphyrin-Universitetet i Oslo from ruixi.UiO series MOFs) has been utilized as a profoundly dynamic photosensitizer (PS) or drug delivery system for PDT of the resistant neck and head cancer. DBP-UiO capably produces $^1O_2$, attributable to site isolation of porphyrin ligands, upgraded intersystem crossing by metal Hf centers, and effortless $^1O_2$ dissemination through porous DBP-UiO nanoplates. Thus, DBP-UiO showed incredibly improved PDT efficiency both in vitro and in vivo, prompting total tumor destruction in the portion of the mice getting a single DBP-UiO dose and a single light exposure. Metal-organic framework (MOF) subsequently addresses another class of profoundly powerful PDT specialists and holds extraordinary guarantee in treating resistant cancers in the clinic [37].

The chlorin-based nanoscale metal-organic framework (NMOF), DBC-UiO, has been intended for the upgrade of PDT's action. While receiving the stability, porosity, nanoplate morphology, and crystallinity of DBP-UiO, DBC-UiO sensitizes a more creative $^1O_2$ group and displays upgraded PDT adequacy on two colon malignant growth mouse models because of their better photophysical possessions. Both immunogenic cell demise and apoptosis added to the slaughter of malicious growth cells in DBC-UiO-induced PDT. Figure 4 explains singlet oxygen generation by

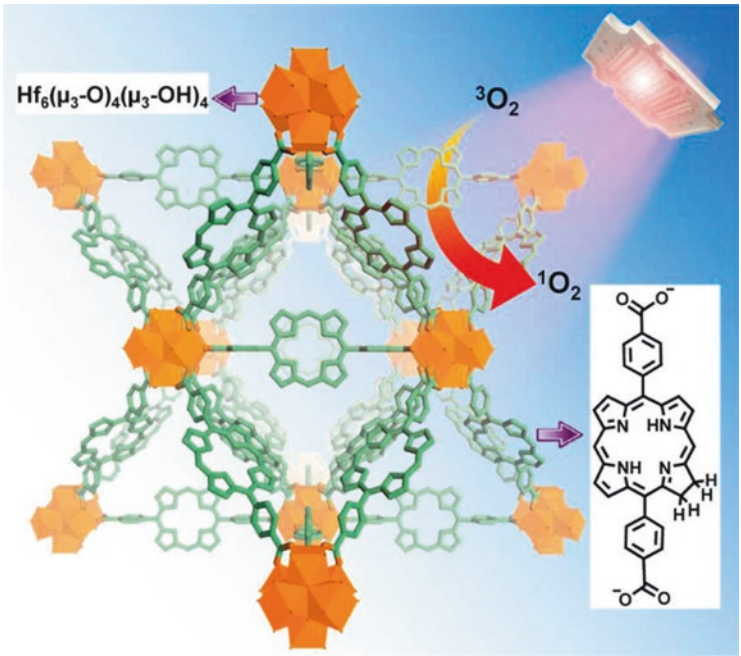

Fig. 4 Schematic explanation of singlet oxygen production by DBC-UiO photosensitization with LED light [38]. (Adapted with permission from (Ref. [38]) Copyright © 2015, Journal of the American Chemical Society)

DBC-UiO photosensitization with LED light [38]. Furthermore, a chlorine-based MOF planned with huge channels for extremely effective PDT while simultaneously loading an indoleamine 2,3-dioxygenase (IDO) inhibitor into its channels to accomplish mixed treatments of PDT checkpoint blockade immunotherapy. The outcome can raise the complete tumor-specific immune retort rates of checkpoint blockade cancer immunotherapy and lead to clinical advantages for the therapy of metastatic colorectal cancer and other problematic-to-treat cancers. Figure 5 shows the graphical representation of immunotherapy and combined PDT by IDOi@TBC-Hf [39]. Other studies show the focus on photodynamic treatment (PDT) with Zr(IV)-based metal-organic framework (MOF) nanoparticles. The size of subordinate cell take-up and resulting PDT were examined with different sizes of PCN-224 (PCN represents porous coordination network) nanoparticles with an imaging and a remedial methodology from the porphyrinic linker. Likewise, further functionalization with folic acid (FA) onto the Zr_6 group in the metal-organic framework showed upgraded PDT viability inferable from the active targeting of the altered metal-organic framework nanomaterial [40]. Nanoscale metal-organic frameworks (MOFs), Ti-TBP, made out of Ti-oxo chain second structure units (SBUs) and photosensitizing 5,10,15,20-tetra(p-benzoate) porphyrin (TBP) ligands, for hypoxia-open minded sort I PDT. Upon light illumination, Ti-TBP sharpens singlet oxygen creation. In addition, it moves electrons from excited TBP* species to Ti^{4+}-based

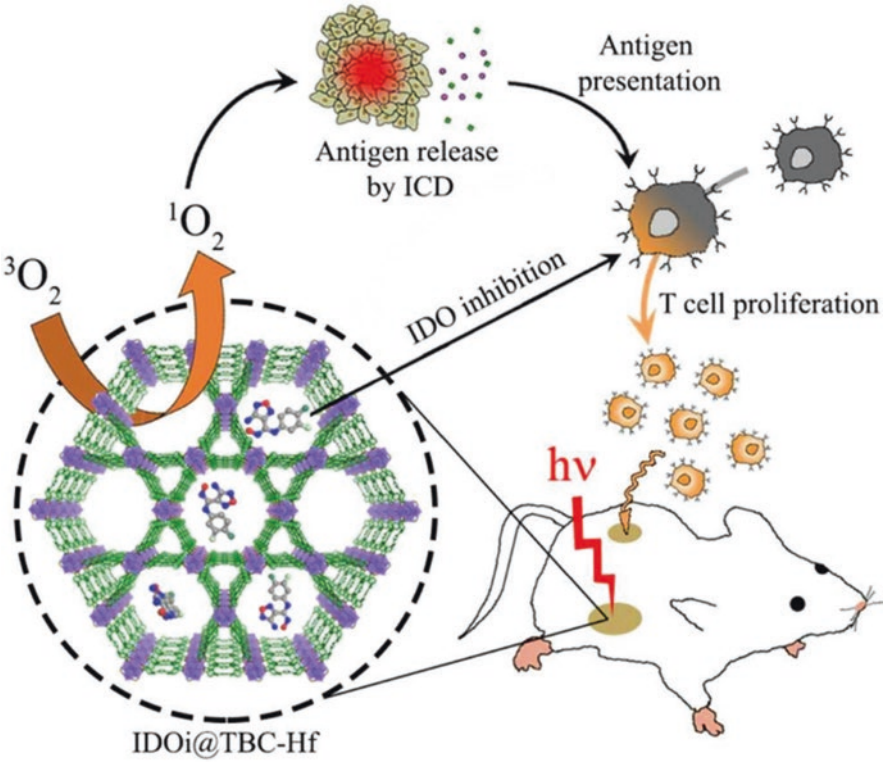

Fig. 5 Schematic presentation of combined PDT and immunotherapy by IDOi@TBC-Hf. Local injection of IDOi@TBC-Hf and irradiation of light to generate sensitive oxygen species, causing immunogenic cell death (ICD) and discharging tumor-associated antigens, which are presented to T cells. Meanwhile, the IDO inhibitor released from IDOi@TBC-Hf modulates tryptophan/kynurenine catabolism to activate the immunosuppressive tumor microenvironment. The combination of antigen presentation from PDT and checkpoint blockade by IDO inhibition causes T-cell proliferation and infiltration, leading to the eradication of local, treated tumors and the rejection of distant, untreated tumors [39]. (Adapted with permission from (Ref. [39]) Copyright © 2016, Journal of the American Chemical Society)

SBUs to afford $TBP^{\bullet+}$ ligands, and Ti^{3+} focuses consequently spreading the age of superoxide, hydrogen peroxide, and hydroxyl extremists. By producing four unmistakable ROS, Ti-TBP-intervened PDT inspires wonderful anticancer capability with >98% cancer relapse and 60% cure rate [41].

A short cytotoxic porphyrin-based metal-organic framework (MOF) PCN-221 acted as an oral drug carrier. Methotrexate (MTX) was picked as the classical drug element, assimilated into channels and inward pores of MOFs by diffusion. PCN-221 showed high drug loading and sustained release behavior under physiological climate without "burst impact." The controlled pH-responsive arrival of medications by PCN-221 uncovered its promising application in oral drug delivery [42]. A multifunctional nanoparticle-based porphyrinic Zr-metal-organic framework is utilized

simultaneously as the photosensitizer and the conveyance vehicle of vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor lapatinib [43]. Porphyrin-based MOF nanomaterials have been used as drug delivery for biomedical applications in tumor therapy and biosensing [44]. Porphyrin-based, paddle-wheel system (PPF) structures are joined with slow-dissolving dual-kinetic sorafenib (SOR) nanocarriers which are powerful components for releasing the drug from the nanocarrier against hepatoma. Obtained drug delivery systems with the slow and fast release of SOR affected the malignant growth cell expansion, albeit in an alternate way (reflected with ERK and EC50 1/2 phosphorylation level). The in vivo examinations demonstrate that quick delivered SOR@PPF lessens the growth extent extensively, while the sluggish delivered SOR@PPF much better keeps from lymph hub contribution and far-off metastases [45].

3.2.2 Covalent-Organic Frameworks (COFs)

COPs (covalent organic polymers), POP (porous organic polymers), or COFs (covalent organic frameworks) are recently developing functional and permeable compounds, which are made from multipurpose organic monomers and connected by covalent bonds. Inferable from high surface area and good stability, these materials display incredible gas storage and separation capacities, catalysis, optoelectronic devices, and energy-related applications [46]. Porphyrin-having materials could create cytotoxic singlet oxygen ($^1O_2$) productively, which will oxidize biomacromolecules and in this way act as drug delivery to the cancer cell apoptosis and necrosis because porphyrin-based conjugated polymers are predictable to show photothermal effect and good stability owing to the stretched conjugated structures and strong π-π stacking communications. 2-D polymers (conjugated) have been generally considered for PTT and PDT [47]. Nanoscale porphyrin-containing covalent organic polymers (PCOP) have been used for drug delivery. The subsequent nanoparticles have high photothermal transformation effectiveness (21.7%) and brilliant photodynamic consequence.

Interestingly, the in vivo and in vitro tests showed an improved antitumor efficiency for PCOP with consolidated PTT and PDT. This learning successfully deals with creating nano COP and shows the extraordinary capability of porphyrin-containing COP for applications in biomedicals [48]. The nano-based fluorinated covalent organic polymers (COPs) are synthesized by cross-linking the photosensitizer meso-5, 10, 15, 20-tetra(4-hydroxyphenyl) porphyrin (THPP) with PFSEA (perfluorosebacic acid) and PEG (polyethylene glycol) through one-pot esterification to empower synchronous cancer photodynamic and oxygenation therapy. Because of the occurrence of PFSEA, the THPPpf-PEG shows effective loading of perfluoro-15-crown-5-ether (PFCE), a type of perfluorocarbon, and consequently molecular oxygen, the two of which would essentially improve the photodynamic impact of THPP. After chelating THPP with a radioisotope, 99mTc (technetium), both THPPpf-PEG and PFCE-loaded THPPpf-PEG (PFCE@THPPpf-PEG) can be distinctively pictured under the single-photon emission computed tomography

(SPECT) imaging, which uncovers effective cancer aggregation of those COPs' post-intravenous injection. Attributable to the oxygen delivery capacity of PFCE, effective tumor oxygenation is noticed for mice postinjection of PFCE@THPPpf-PEG, which further prompts incredibly improved photodynamic handling of cancers. This learning offers the creation of an exceptional kind of numerous functional fluorinated COPs with a distinct structure, long blood flow time, and sustained tumor oxygenation capacity, showing incredible potential for likely clinical interpretation in the photodynamic treatment of cancer [49]. A porphyrin-containing nanoscale nCOP-PX-12 is noticeable in vitro and in vivo because of meso-tetrakis(4-aminophenyl) porphyrin (TAPP). With the acid-cleavable imine group, it exhibits pH-responsive drug discharge. Also, the delivered TAPP accomplishes mitochondria targeting, which can prompt mitochondrial-subordinate growth cell apoptosis with laser illumination. The combination treatment can make immunogenic cell passing and APC initiation, while PX-12 is found to redesign the growth hypoxic microenvironment, impeding the downstream flagging pathways of thioredoxin-1. The nanosystem additionally displays tumor accumulation and biosafety. After a single intra-tumoral dose of nCOP-PX-12 with moderately low density of laser, 98.5% of primary cancer, 65.3% untreated distant tumors, and 83.2% lung metastasis are restrained. Generally speaking, this study works on the combination of multifunctional nCOP and proposes a strong photodynamic treatment with high explicitness to conquer the opposition of hypoxic tumors [50]. The porphyrin nanocage installed in single subatomic nanoparticles (porSMNPs) can be used as a theranostic stage. Positron emission tomography imaging gives dynamic bio-dissemination of porSMNPs, confirming their excellent circulation time and particular aggregation at the tumor site, crediting the improved retention and permeability consequence. Besides, the enclosure structure altogether advances the photosensitizing impact of porSMNs by restraining the π-π stacking interactions of the photosensitizers, removing the cancer without decline by exploiting photodynamic treatment [51].

3.3 Porphyrin Based Self-Assembly Nanoparticles

Atomic length scales, colloidal or molecular, are self-assembled to form a new complex structure through chemical forces through metal coordination, hydrophobic, hydrogen bonding, π-π aromatic stacking, van der Waals forces, electrostatic, etc. [52]. Self-assembling of molecules helps obtain stable assemblies of materials, viz., liquid crystals, phase-separated polymers, semicrystalline, and molecular crystals. It also changes the development of huge molecules [53]. Self-assembly of nanostructures from amphiphiles (vesicles, hydrogels, and micelles) occurs because of different physical interactions. Current developments in drug delivery have undone up fresher roads to foster novel drug delivery systems (DDSs), and self-assembly nanostructures have demonstrated their great potential to be utilized as easy and productive materials for this purpose [54]. Nanomaterials of porphyrin with the

donor-acceptor-donor compound have formed by self-assembly. This nanomaterial has improved fluorescence quenching and near-infrared absorption and showed enhanced drug delivery for photodynamic/photothermal combination cancer therapy due to its good biocompatibility, $^1O_2$ generation volume, and high photothermal conversion efficiency [55]. Self-assembled meso-A_2B_2 porphyrin nanomaterials have enhanced the near-infrared absorption and increased the drug delivery activities with desired fluorescence imaging presentation for photodynamic cancer therapy [56]. Self-assembled zinc porphyrin nanoparticle (ZnTPyP) has enhanced the light absorption for increased drug delivery properties of photodynamic therapy. The electron spin resonance (ESR) spectroscopy results indicate that porphyrin nanoparticles generate a high production of singlet oxygen and increased antibacterial photodynamic therapy activity [57]. In addition, water-soluble porphyrin nanomaterials have connected with various lengths of glycol chains. These nanomaterials have enhanced drug delivery due to their hydrophobic and hydrophilic units. Minor molecular self-assembly is a capable method to understand a water-soluble photosensitizer with a clear structure used in the clinic [58].

3.4 Porphysomes

Porphysomes are self-assembly of pyropheophorbide-conjugated phospholipid (pyro-lipid) forming liposome-like structures [59, 60]. Each porphysome nanoparticle comprises about 80,000 porphyrin molecules and is approximately 100 nm in size. Several studies have explored the use of porphysomes in both the structurally intact (nanoparticle) state and their disrupted states (pyro-lipid subunits). The porphyrin molecules are tightly packed in the porphyrin-lipid bilayer in the structurally intact nanoparticle state. This results in the dissipation of absorbed light energy, leading to poor fluorescence emission, resulting in almost 99% of emission quenching. In the disrupted state, the pyro-lipid subunits are loosely packed, allowing fluorescence and photochemical reactions. The conversion of porphysomes from their entire state to a disrupted state is dependent on time. The use of porphysomes is explored in therapy and imaging, such as for targeted photothermal therapy, drug delivery, fluorescence imaging, magnetic resonance imaging, positron emission imaging, and photodynamic therapy [25, 61–64]. Due to their smaller size, they are taken up due to EPR (enhanced permeation and retention) effect and cleave into several pyro-lipid subunits at the tumor sites. The spreading and movement of dissociated pyro-lipid and porphysome nanoparticles in the tumor are shown in Fig. 6.

Porphysomes show high extinction coefficients in the near-infrared and shifting of absorption spectrum contingents on the type of metal present in the porphyrins-lipid chelating bilayer. Porphysomes show high self-quenched fluorescence due to the high-density packing of porphyrins in the bilayer, forming a supramolecular arrangement of porphyrin-lipid molecules that permits close intermolecular interaction [65]. Figure 7a shows the mechanism of hydrophobic self-assembly porphysomes. Porphysomes with a monodisperse diameter of 100 nm might improve their

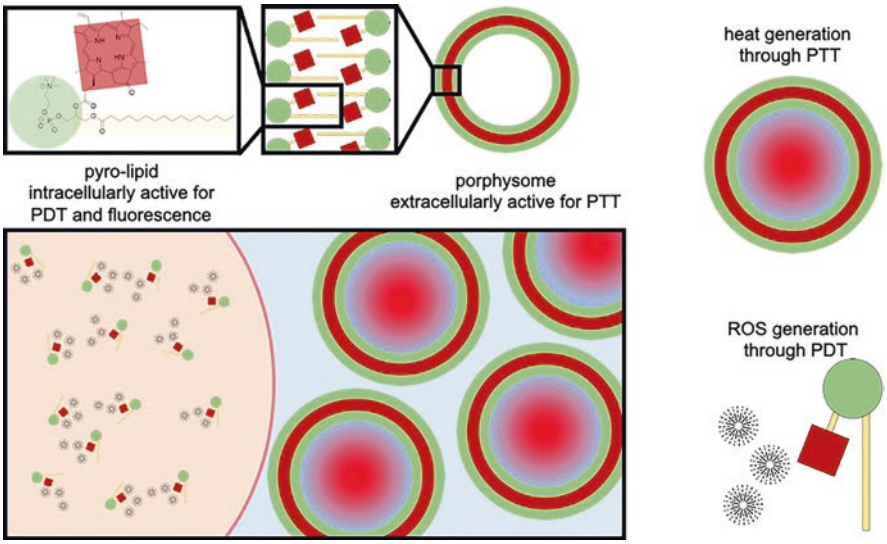

Fig. 6 Schematic representation of the activity and distribution of dissociated pyro-lipid and porphysome nanoparticles in the tumor, irradiated with 671 nm light; the complete porphysomes in the extracellular space reply photothermally, while the interrupted intracellular porphysomes (pyro-lipid) are unquenched and generate reactive oxygen species (ROS; e.g., $^1O_2$) for photodynamic therapy [61]. (Reproduced from open-access journal under the term of Creative Commons Attribution License (Ref. [61]) Copyright © 2021)

passive accumulation in tumor tissues through the osmotic cycle effect (Fig. 7b). Moreover, a diameter of 100 nm of porphysomes can be loaded with approximately 8×10^4 porphyrin molecules. Also, porphysomes can be degraded in living cells (Fig. 7c) [11]. Pyropheophorbide porphysome is a multifunctional structure square to gather nanomedicines from the bottom-up. Pyropheophorbide porphysome created in the definition, multi-modular targeting, and imaging of a praiseworthy "one-for-all" nanomaterial, and it is preserved from a hitherto unfamiliar clinical proposal and expansion viewpoint [61, 66].

Vesicular drug delivery system assumes a significant part in particular targeting of different kinds of drugs. Among them, porphysomes are an arising and a more liked drug delivery system. It tends to be utilized as a substitute drug delivery system for liposomes as it presents the same structure as that of liposomes. Porphysomal drug delivery systems have a wide scope of utilization in photothermal treatment, photoacoustic imaging, radiolabelling, and so on. It has extremely high bioavailability when contrasted with different drug delivery systems [63]. In 2014, Huynh and Zheng have reviewed that porphysome nanovesicles in supramolecular structures have been removed from simple provision to a therapeutic agent and intrinsic imaging and submissions in imaging and therapy, as well as multimodality imaging, photothermal therapy photoacoustic imaging, and activatable fluorescence imaging and photodynamic therapy [67]. In addition, applications and future works of multifunctional properties of porphysome derivative nanoparticles were discussed [60].

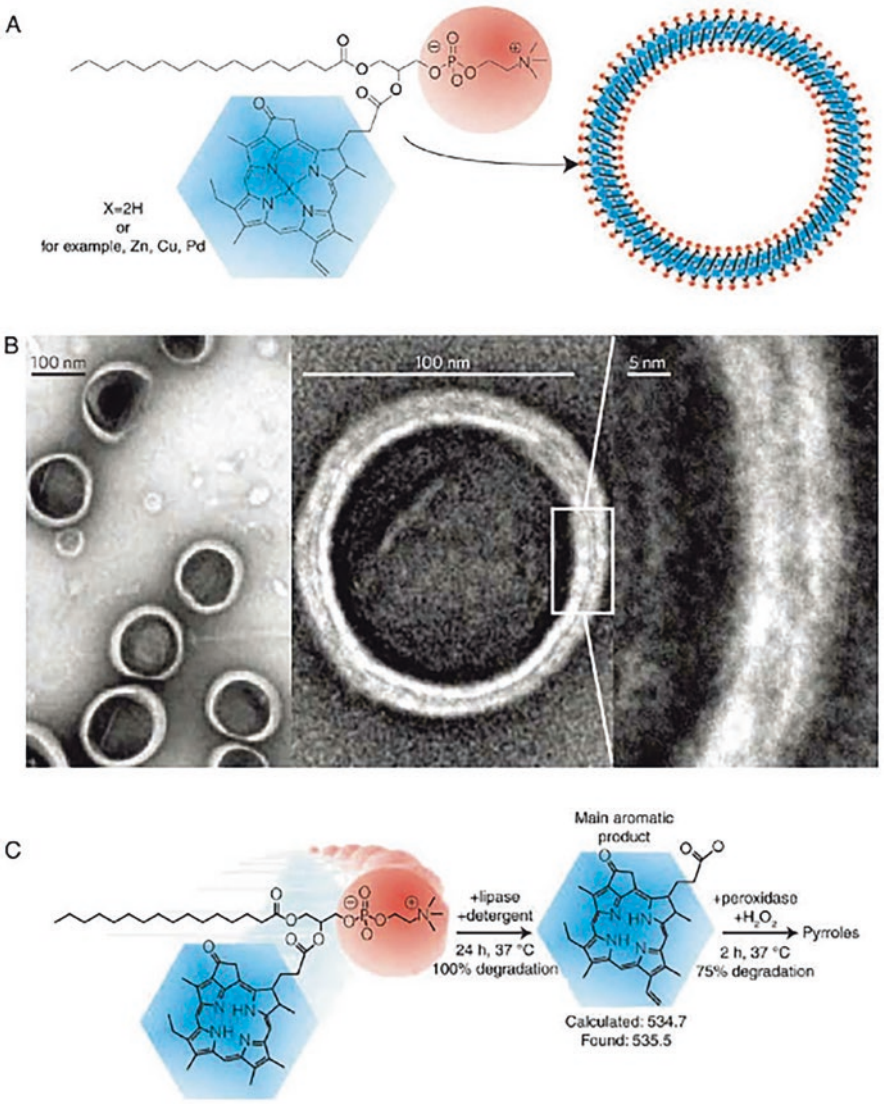

Fig. 7 (**a**) The self-assembling mechanism of porphysomes; (**b**) scanning electron microscopy (SEM) images of porphysomes; (**c**) the intracellular degradation mechanisms of the porphysome [11] (Reproduced from open-access journal under the term of Creative Commons Attribution License (Ref. [11]) Copyright © 2019)

Phospholipase A2 from honey bee venom (PLA2HBV) and lipase from *Thermomyces lanuginosus* (LTL) have been used for sn-1 and sy-2 porphyrin-lipid conjugates isometrically. The sn-2 porphyrin-lipid may be used to ablate tumors using porphysome-mediated photothermal therapy. The results indicated physically

similar biodegradation of nanoparticle-based regioisomeric phospholipid conjugates [68]. Photoacoustic and photothermal belongings of porphysome nanovesicles have permitted the delicate imaging of lymphatic systems employing photoacoustic tomography. The near-IR fluorescence shows that porphysomes have reinstated dissociation, generating chances for low-background fluorescence imaging and biodegradability and made slight acute toxicity. The huge aqueous core of porphysomes was loaded. Resulting in complete management, porphysomes gathered in tumors of xenograft-bearing mice, and laser irradiation-induced photothermal tumor ablation. Porphysomes prove the multimodal potential of porphyrin-lipid nanoparticles for biophotonic imaging and therapy [62]. High-density porphyrin nanoparticle loading imparts improved photonic properties and empowers high-payload tumor delivery. A patient-determined orthotopic pancreas xenograft model was utilized to assess the attainability of porphysome-enhanced PTT for pancreatic cancer. Biodistribution and tumor collection were assessed by utilizing fluorescence intensity measurements from homogenized tissues and imaging of extracted organs. Tumor surface temperature was recorded by utilizing IR optical imaging during light illumination to screen treatment progress. Histological examinations were directed to decide the degree of PTT thermal damage. These investigations might understand the impact of the heat sink effect on thermal treatment dosimetry for all-around perfused pancreatic cancers [25]. The metal particles can be straightforwardly joined into the porphyrin building blocks of the preformed porphysomes, opening their potential for PET and MRI. By changing the way porphyrin-lipid gathers, HDL-like porphyrin nanoparticles (~2 μm) gain porphyrin vesicle (~100 μm), and a mixture of porphyrin-gold nanoparticles are prepared, growing the domain of porphyrin nanophotonics. High-ordered porphyrin aggregates into supramolecular assemblies were introduced by mimicking light harvest systems in photosynthetic bacteria with extraordinary photonic properties (e.g., reversible photoacoustic nanosensors). Such optical properties are likewise liable to the ultrasound-initiated microbubbles-to-nanoparticle transformation peculiarity, which might make way for sidestepping the improved penetrability and maintenance impact while delivering drugs to cancer cells, moreover, by "growing" the four-coordination condition of porphyrins to the five-coordination condition of texaphyrin-lipid building blocks with stable chelation of 18 metal particles, hence releasing the metal chelating force of texaphyrins for a wide cluster of nanomedicine applications. Together, the straightforward yet characteristic multimodal nature of porphyrin self-gathering addresses another outskirt in cancer imaging and treatment [69]. The single-molecule of ^{65}Cu porphyrin molecule in a porphysome nanoparticles has shown the properties of bot fluorescent and PET active (Fig. 8) [70]. Liu et al. (2013) confirmed the first in vivo application of ^{64}Cu-porphysomes in clinically important orthotopic prostate and hard metastatic malignant growth models. They show a clear multimodal depiction of orthotopic growths on both the large scale and the tiny scales (utilizing both PET and fluorescence) and sensitively detected small bony metastases (<2 mm). The novel and diverse properties of porphysomes offer a promising across the board, prostate malignant growth imaging specialist for cancer

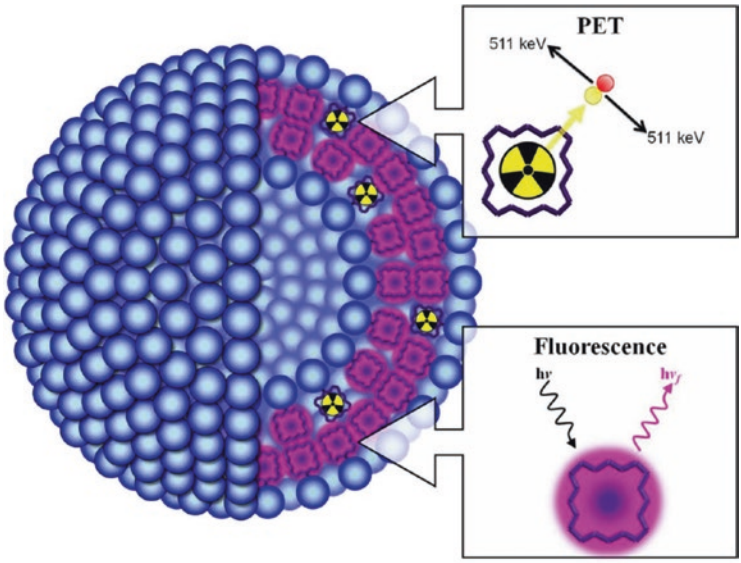

Fig. 8 Schematic diagram of the multimodal properties of ^{64}Cu-porphysomes as a result of directly radiolabeling a fraction of the porphyrin-lipid bilayer of preformed photonic porphysomes creating intrinsic multimodal nanoparticles [70]. (Adapted with permission from (Ref. [70]) Copyright © 2013, Journal of the American Chemical Society)

identification and therapy reaction/repeat observing utilizing both radionuclide-and photonic-based procedures [70].

Microbubble containing bacteriochlorophyll-lipid or porphysome has changed over nanoparticles of different sizes. Their nano-sized particle empowers better diffusion into tissue; however, it negatively affects acoustic reflectivity. The delivery of drugs can be traced by image direction in light of ultrasound imaging and estimating the drug concentration in the tissue by fluorescence imaging [71].

The supramolecular porphysome nanovesicles have been prepared from amphiphilic porphyrin glutathione with high doxorubicin (DOX) loading capacity. The drug loading and in vitro drug discharge examinations show that these nanovesicles can embody doxorubicin (DOX) to accomplish DOX-loaded nanovesicles and could especially deliver the loaded drug triggered by high centralization of glutathione (GSH). Hence, the porphysome nanovesicles showed between smart delivery systems and imaging-directed medication discharge [72]. These initial research discoveries are inspiring, but there are restrictions to address and more capacities to investigate (Fig. 9) [70].

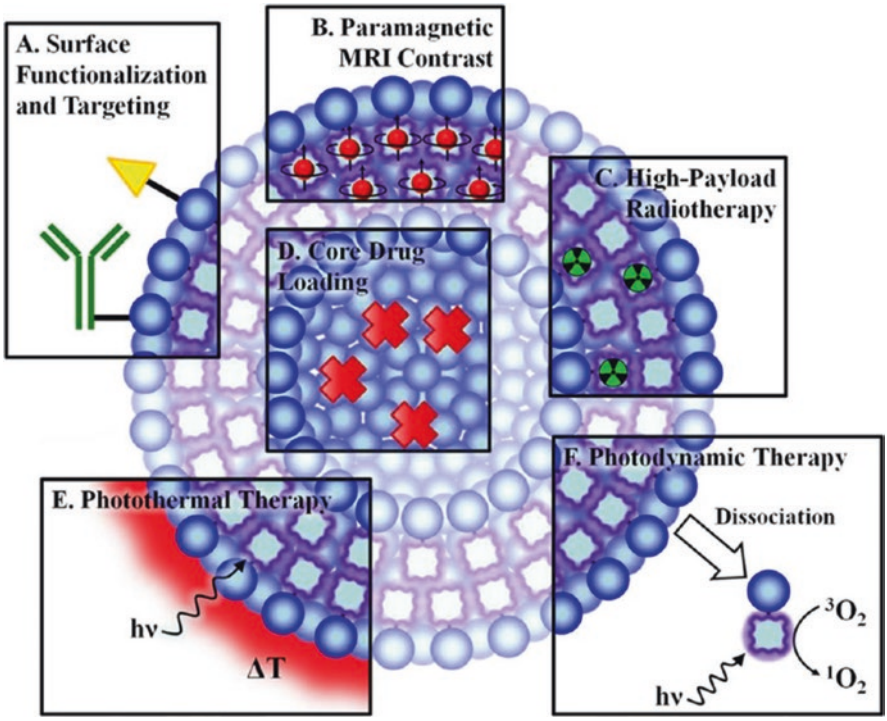

Fig. 9 The forthcoming porphysomes' potential: (**a**) pointing the nanoparticles by functionalizing with antibodies, receptor ligands, etc., (**b**) chelating paramagnetic metal ions with the porphyrin building blocks for MRI contrast, (**c**) exploiting porphysomes' high-payload chelating abilities to deliver radiotherapeutics, and (**d**) entrapping soluble drugs or contrast agents within the aqueous core and exploiting the strong absorbance of porphyrins for (**e**) photothermal therapy with highly quenched, intact porphysomes and (**f**) photodynamic therapy with porphyrin-lipid monomers following dissociation of the nanostructure [70]. (Adapted with permission from (Ref. [70]) Copyright © 2013, Journal of the American Chemical Society)

3.5 Inorganic-Based Porphyrin Nanoparticles

Porphyrins can be formed inorganic nanoparticles to give organic-inorganic hybrid systems for different applications. An enormous assortment of work in this class is centered around shipping PDT agents through the bloodstream to the target site [65]. Homogeneously multifunctional GdTPP/ZnTPP nanocomposites (GZNs) from gadolinium porphyrin (GdTPP) contrast agent and zinc porphyrin (ZnTPP) photosensitizer have been constructed. The GZNs acquire surprising fluorescence imaging, high relaxation rate, and singlet oxygen creation from GdTPP and ZnTPP metalloporphyrins. In the wake of disguising with homotypic cancer cell layer for immunologic escape, the HeLa membrane-covered GZNs (mGZNs) show upgraded in vivo MR/FL imaging-directed antitumor targeting efficiency of 80.6% for HeLa cells. Homogeneously multifunctional GdTPP/ZnTPP nanocomposite

photosensitizer to improve drug delivery for photodynamic therapy [13]. The gold nanoparticle was formed by coupling between 5,10,15,20-tetrakis(pentafluorophenyl)-21H,23H-porphine (PF6) dye and nanoparticle of gold (Au). Highly stable Au nanoparticles were accomplished utilizing PF6 with poly(N-vinyl caprolactam-co-N-vinyl imidazole)-g-poly(D,L-lactide) graft copolymer hybridization. These polymers were used to enhance drug delivery for photothermal and photodynamic treatments. The core-shell PF6-Au nanoparticles were ardently taken up by cells and exhibited cell phototoxicity upon illumination with 300 W halogen lamps. The structural arrangement of PF6 dyes in the core-shell particles guarantees singlet oxygen creation efficacy [23]. The nanoparticles of iron porphyrin have been manufactured with a crystalline structure of size 1.4 nm. Its electrochemical sensor activity has good performance for H_2O_2 detection. The iron-porphyrin-based covalent organic framework could be used as a mimic peroxidase to apply in biological fields [73]. Doxorubicin-gold porphyrin nanoparticles (DOX@TPPS-AuNPs) go about as nanocarriers (Figs. 10 and 11). DOX is stacked on the porphyrin-altered gold nanosurface noncovalently with high epitome viability (~90%) and firmly related under typical physiological circumstances. However, it is equipped for delivering ~81% of medication in a low-pH climate. In this manner, DOX-stacked TPPS-AuNPs displayed higher restraint of cell metastasis, attack, and angiogenesis, proposing that TPPS-altered AuNPs could work on the therapeutic viability of the drug particle.

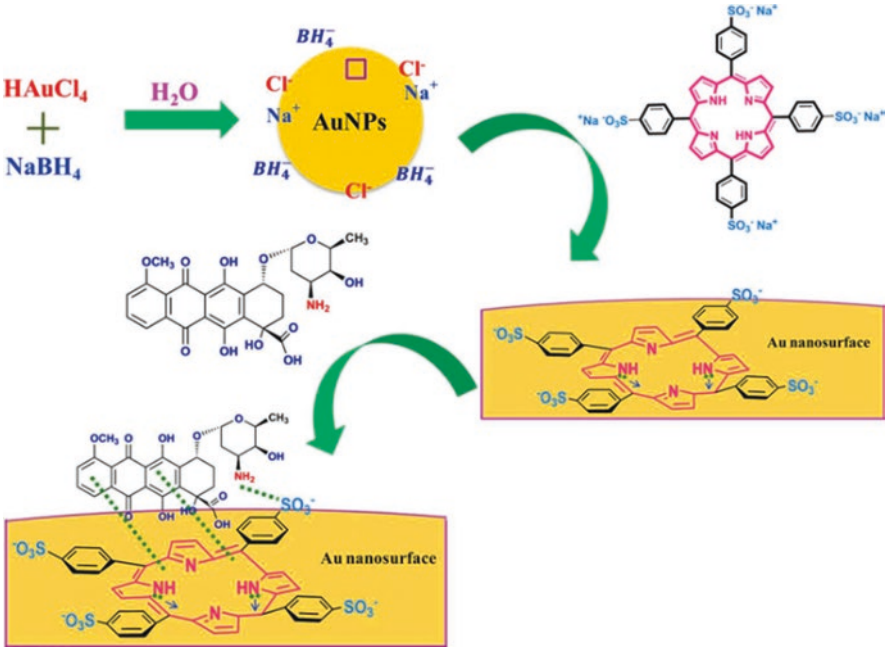

Fig. 10 Graphical depiction of the conceivable mechanism of establishment of TPPS-conjugated AuNPs and succeeding loading of DOX on their surface [16]. (Adapted with permission from (Ref. [16]) Copyright © 2018, Journal of the American Chemical Society)

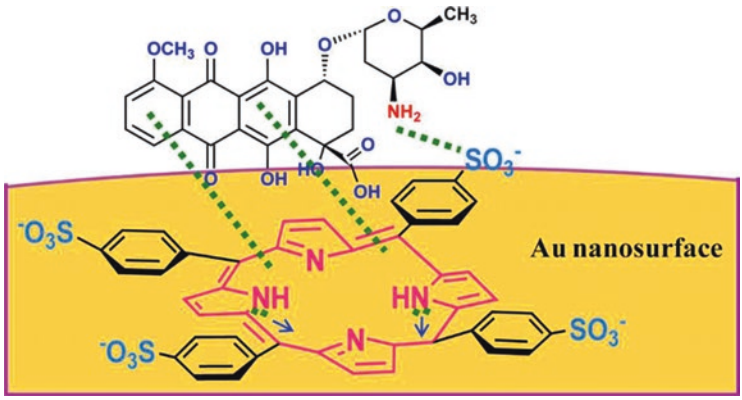

Fig. 11 Schematic representation of electrostatic interaction and hydrogen bonding between adsorbed TPPS and adsorbed DOX molecules on AuNP surface [16]. (Adapted with permission from (Ref. [16]) Copyright © 2018, Journal of the American Chemical Society)

Doxorubicin-gold porphyrin nanomaterials enhanced the drug delivery for cancer therapy due to porphyrin interacting with the gold nanosurface through the coordination between gold and pyrrolic nitrogen atoms of the porphyrin and forming a strong association complex [16].

Mn (III)-porphysome inorganic nanoparticles that can match gadolinium diethylenetriamine pentaacetate (Gd-DTPA) have increased drug delivery. Their MRI contrast production, photothermal effectiveness, and photostability are remarkable for an all-organic nanoparticle made up of a single functional component [74]. Recently, porphyrins have been attached with different nanomaterials to enhance drug delivery and biodistribution. These mixtures permit nanoparticles to improve photodynamic therapy (PDT) cancer treatment and improve photodynamic diagnosis (PDD) to the reaction [5].

4 Advantages and Disadvantages of Porphyrin and Porphysome-Based Drug Delivery

Most of the clinically approved photosensitizers used for PDT and PTT-based cancer therapies are porphyrin derivatives. Porphyrins are naturally occurring organic, light-absorbing molecules found in many plant and animal systems. Porphyrins could absorb light in the visible region and exhibit photonic properties such as fluorescence or PDT, aided by the large conjugated structure. The fluorescent and phototoxic effects of porphyrin with tumor-selective localization properties have led to many developments in photoimaging and photodynamic therapy, aiding cancer diagnosis and treatment. PTT is often associated with conventional treatment modalities like chemotherapy, as PTT could act even in oxygen-deprived hypoxic tumor regions and make the tumor sensitive to chemotherapeutic drugs [10].

Despite the advantages of porphyrin derivatives, there are various limitations such as long photosensitivity in patients over several weeks due to their prolonged half-life time, and short excitation wavelength that cannot target deep-seated tumors, poor chemical purity, erythema, etc. Recently developed second-generation porphyrin compounds address these issues by developing metal-associated porphyrin derivatives that tend to have longer excitation wavelength, which makes them suitable for deep-seated tumor PDT. Even though the chemical purity has been improved, the second-generation PS still suffer from several limitations like poor water solubility, prolonged photosensitivity, photobleaching, low penetration depth, poor selectivity, and a slower clearance rate [33, 67].

5 Future Work

To address the challenges imposed by first- and second-generation PS, third-generation PS are targeted to improve the tumor selectivity through targeting and longer excitation wavelength activation. Poor water solubility can be addressed by encapsulating the existing PS in nanocarriers that render hydrophilicity to the complex, and sensitivity can be improved by conjugating the complex with ligands/antibodies for targeting specific tumor receptor. By incorporating metal compounds or by improving the structure, the excitation wavelength of the PS should be longer, rendering them useful in the treatment of deep-seated tumors. The half-life time of the PS should be reduced to prevent prolonged photosensitivity in patients.

6 Conclusion

Multifunctional properties of porphyrin nanomaterials have shown the possibility of enhanced drug delivery of phototherapy agents to designated tissues utilizing porphyrin-based active targeting because porphyrins display great properties of phytochemicals, particularly singlet oxygen production, which makes them a reasonable contender for PDT. Along these lines, various nanoparticles have been planned to ship the porphyrin of hydrophobic type of atoms in the biological environment. Porphyrins' multifunctional nature and their derivatives are relied upon to assume a crucial part in upcoming experimental treatment. The profound abuse of original porphyrins and their by-products has newly acquired expanding researcher interest. Growing new porphyrins with various belongings is of significance for some biological applications. Future examinations into broadening the kinds of porphyrins utilized could additionally extend the capacities of these particles. Investigating very much evolved porphyrin nanoparticles that are utilized in non-medical fields regarding photomedicine may likewise offer extra chances.

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Ethosomes for Dermal and Transdermal Drug Delivery Systems

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1 Introduction

The process by which a therapeutic agent or drug is administered to the body for achieving desired effects is known as drug delivery. The most important criteria for choosing the drug delivery route are enhanced drug efficacy and site-specific release without any unwanted side effects in patients [1]. The drug delivery route is selected depending upon the disease condition, the desired effect on the body, and the type of drug intended for administration. Depending upon the disease and the target organ, the drug administration routes can be:

(a) *Oral route*: It is one of the most commonly preferred drug delivery routes which has excellent efficacy and patient-friendly. One of the drawbacks of this route is the slow release of drug with chances of drug degradation due to stomach acid and enzymes [2].

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(b) *Parenteral route*: This route comprises the administration of the drug through intravenous, intra-arterial, and subcutaneous routes. For quick delivery and efficacy of the intended drug, the parenteral route is preferred as the administered drug directly reaches the systemic circulation. Some of the drawbacks of this route are patient discomfort for needle fearing patients, local infections, and expensive IV injections [3].
(c) *Transdermal route*: In this route, the drug is delivered topically through the skin. It is one of the most patient-friendly drug delivery routes favoring instant local effect. The intended drug is released at regular intervals and gradually reaches the systemic circulation. The stratum corneum of the epidermis becomes the main hindrance to effective delivery of the drug which becomes one of the drawbacks in this drug delivery route [4, 5].
(d) *Nasal route*: This route is mostly preferred when dealing with respiratory diseases, whereupon administration the drug directly reaches the lungs and increases its bioavailability [6].

The *stratum corneum* is the outermost layer of the epidermis which is composed of 15–30 layers of the dead keratinocytes making it the toughest layer for penetration of any outside component. The permeability barrier of the stratum corneum is provided by the presence of an equimolar ratio of cholesterol, ceramides, and fatty acids [7, 8]. To counter the problems arising during the transdermal delivery route, various drug delivery carriers are specifically designed to overcome the barrier function of skin guarded by the tough stratum corneum. The *ethosomes* were therefore conceptualized for providing an appropriate mechanism for the delivery of intended drugs directly to the targeted area through the transdermal route. The concept and design of ethosomes were first reported by Elka Touitou in 1996 during her research on lipid vesicles. Ethosomes are soft, malleable vesicles which are primarily composed of phospholipids (phosphatidylcholine/phosphatidylserine/phosphatidic acid), ethanol, and water. The percentage of ethanol in the ethosomes governs their morphological aspects with their surface charge. The barrier function of the stratum corneum is disrupted by the collective action of ethanol and phospholipid. Being a penetration enhancer, ethanol disturbs the arrangement of the lipid bilayer in the stratum corneum, thereby facilitating the soft and malleable ethosomal vesicles to squeeze and fuse with the inner lipid bilayer. The drug is subsequently released to the deeper layer of the skin for intended therapeutic application. Consequently, the drug is released into the systemic circulation after providing its effect on the local and periphery targeted area [9].

2 Structural Organization of the Skin

The skin comprises the largest organ of our body which comprises around 16% of the total body weight and is commonly referred to as the integumentary system. Primarily its main function is to provide a protective shield against any physical, thermal, and mechanical injury as well as protect the harmful UV radiation of the

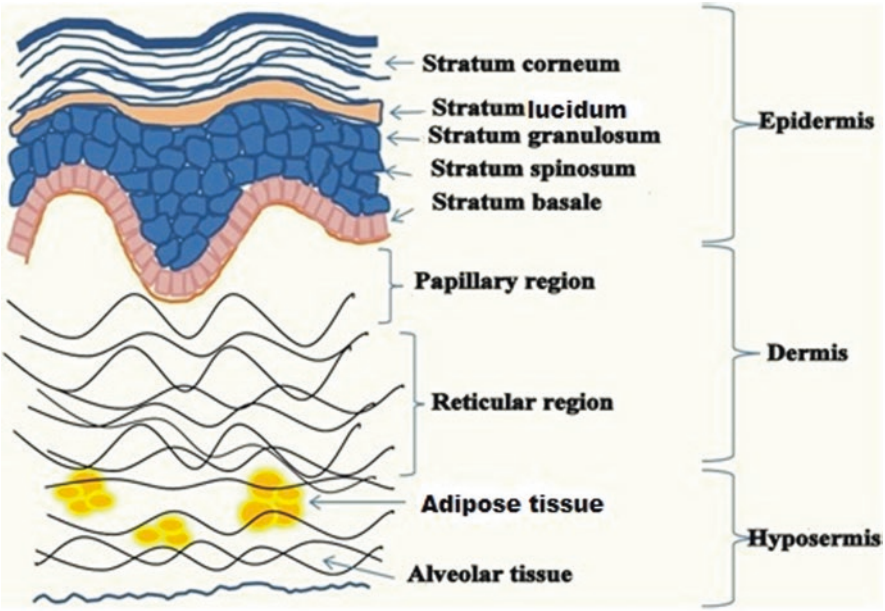

Fig. 1 Structural anatomy of the human skin showing a different layer of the epidermis

sun and prevent excessive loss of water from the body. Apart from this, the skin also helps in regulating the body temperature and synthesis of vitamin D, scrutinize the attack of any facultative opportunistic microbial invasion, and acts as a sensory organ [10]. This multi-dynamic property of the skin is assisted by the three structural layers, i.e., the epidermis (outer), dermis (middle), and hypodermis (inner) (Fig. 1).

The hypodermis is the innermost layer of the skin which is mainly composed of adipose tissue and connective tissues. This layer is important because it connects the dermis to the muscles and bones. Further, this layer also helps in regulating the body temperature and stores fats which help in insulating the body against any trauma and water loss. The dermis constitutes the main foundation pillar of the integumentary system which contains blood and lymph vessels, sweat glands, nerves, and hair follicles. It also helps in the production of collagen, elastin fibers, mast cells, and macrophages. This layer is further differentiated into the papillary layer which is composed of a loose mesh-like layer of collagen and elastin fibers. This papillary layer contains the Meissner corpuscles (touch layer), phagocytes, and nerve and lymphatic capillaries. Under the papillary layer lies the reticular layer which contains a strong mesh-like presence of connective tissues with a robust supply of sensory and sympathetic nerves.

The uppermost layer of the skin is the epidermis which does not contain any blood vessels. The epidermis is mainly composed of keratinocytes, melanocytes, Merkel cells, and Langerhans cells. Depending on the site, the epidermis is further subdivided into five layers, namely, stratum basale, stratum spinosum, stratum

granulosum, stratum lucidum (present only in palms of hands and soles of feet), and stratum corneum. Stratum basale is the deepest layer of the epidermis connecting to the dermis layer by the formation of the dermal papilla. From the basal cells, keratinocytes are continuously produced followed by periodic replacement of old cells by newly forming cells from the stratum basale. The other two important cells that are found in the stratum basale are Merkel cells (receptors for stimulating sensory nerves) and melanocyte cells (producing pigment melanin). Above the stratum basale lays the stratum spinosum which is primarily composed of many layers of keratinocyte interlinking through desmosomes. Among the layers of keratinocytes, the Langerhans cells are dispersed which act as macrophages. The waterproof nature of the skin lies in this section of the epidermis as during the synthesis of keratin water-repelling glycolipid is released at this layer. Following the stratum spinosum lays the stratum granulosum. Due to the continuous production of the basal layer, old keratinocytes are further pushed upward to the stratum granulosum where they appear more granular due to the large production of keratin and keratohyalin which are mainly deposited as lamellar granules in the cytoplasm.

Just above the stratum granulosum lies the translucent layer of stratum lucidum which is majorly composed of dead keratinocytes. From the keratohyalin, a protein called eleidin is produced that is rich in lipid, thereby providing water barrier property to the skin. Stratum lucidum is strictly found in the palms of hands and soles of feet and digits. The outermost layer of the epidermis is the stratum corneum which is mainly composed of 15–30 layers of dead keratinocytes making it the toughest layer for penetration of any outside component (Walters and Roberts, 2002). The permeability barrier of the stratum corneum is provided by the presence of an equimolar ratio of cholesterol, ceramides, and fatty acids. These lipids are produced through the catabolic modification of the lamellar bodies of the stratum corneum mediated by secreted co-enzymes [11]. The dynamic nature of the stratum corneum is maintained by the periodic replacement of dead layers with new layers of cells. The brick-and-mortar model of the stratum corneum is associated with the corneocytes being the bricks and extracellular lipids being the mortar.

2.1 Problems Associated with Drug Delivery

The skin is one of the most vital organs of our body. Being the largest organ, the skin covers every part of our body. Hence, its detailed biochemical organization and composition should be taken into consideration before designing any drug delivery vehicle [12, 13]. The most important concern in transdermal drug delivery is skin irritability reactions which may otherwise prove to be fatal with numerous underlying side effects [14, 15]. Further, in most the drugs, the biochemical composition of the transporting vehicle may initiate a hypersensitization reaction that may subsequently lead to microbial growth if used for a prolonged period [16].

2.2 Drug Absorption via the Skin

India being the land of Ayurveda has a vast treasury of traditional knowledge which is locally preserved community-wise. Most of the form of this knowledge is transported by word of mouth from generation to generation. Hence, in the premedieval ages, for treatment of bone fractures and chest congestions or other skin-related problems, various plant parts (leaf, bark, roots, flowers) were used [17–19]. However, it is clear in modern times that drug delivery through the transdermal route takes place by passive diffusion. The skin is the outermost covering and an important barrier; thus, most the encounters occur with the skin such as bacterial, viral, fungal, and parasitic attacks, mechanical injury, and sometimes skin carcinomas [20–22]. Topical ointments or gels are preferentially used for their treatment; hence, barely the active drug molecule reaches the systemic circulation. Therefore, the chances of any systemic side effects are rarely seen. Whenever higher doses are required, the drugs are administered by intradermal injection. The delivery of hydrophilic drugs through the transdermal route is a challenge, as most of the delivery vehicles used are hydrophobic in nature. Hence, the nature of the delivery vehicle, the incorporated drug, and their concentration play a significant role in determining the success of the transdermal drug delivery [23, 24].

3 History of Transdermal Drug Delivery System

The transdermal delivery route uses the skin for the delivery of specific drugs to the systemic circulation. The advantages of the transdermal delivery system are as follows: it eliminates the chances of first-pass mechanism in the oral route, evades the harsh acidic and alkaline conditions of the gut, directly reaches the systemic blood circulation, and facilitates controlled release of drugs at the specific site of action and patient-friendly approach for delivery of drugs [25]. Transdermal delivery also has an added advantage that apart from being inexpensive, it does not increase the biohazard burden on the environment with extra biomedical waste which is dangerous and a potential source of infection (reuse of the needle) [5].

The knowledge of Ayurveda and its importance are now getting recognition in recent years as they provide a holistic approach to treatment. Although the delivery of drugs through the dermal route is gaining popularity in the present century, it was practiced more than 2500 years ago in India. Despite being advantageous, till now only eight drugs (scopolamine, clonidine, estradiol, oxybutynin, nitroglycerine, fentanyl, testosterone, and nicotine) are approved for delivery across the skin since its inception in 1979 [26]. The greatest challenge faced by the drugs for penetration into the skin is the tough stratum corneum. The presence of intracellular lipids as mortar and corneocytes as bricks provides the barrier function of the stratum corneum [27]. Targeting the intracellular lipids that are present between the adjacent corneocytes provides a successive platform for penetration or diffusion of drugs to

the deeper layers of the skin where they can reach their targeted site of action or the systemic circulation [9].

The three basic components of transdermal delivery are a reservoir, matrix system, rate-controlling system, and the adhesive which remains fixed with the skin. The transdermal delivery systems can be broadly divided into three categories based on their generation: first-generation transdermal delivery systems, second-generation transdermal delivery systems, and third-generation transdermal delivery systems [28, 29].

3.1 First-Generation Delivery System

The first-generation transdermal delivery systems consist of the early developed transdermal patches that are currently available in the market after passing the clinical trials. Patient compliance with easy delivery of selected drugs has made them very popular and widely accepted by the masses. In this delivery system, the drug is contained in a reservoir which is released through a semipermeable membrane that controls its flow rate and an adhesive that sticks to the skin surface. The main challenge of the first-generation delivery systems is the thick stratum corneum which hinders the drug to reach its proper target site [9].

3.2 Second-Generation Delivery System

The second-generation transdermal system consists of more advanced techniques to overcome the barrier function of the stratum corneum by employing skin permeability enhancers.

Surfactants: Many commercially available cosmetic and therapeutic formulations contain surfactants that have the potential to solubilize lipophilic active compounds. Thus, the surfactant works by solubilizing the lipid layers present in the stratum corneum, thereby providing a free passage for the drug to reach its destination point. Some of the examples of popularly used surfactants are sodium lauryl sulfate, cetyltrimethylammonium bromide, and dodecyl betaine. Some of the drawbacks associated with surfactants are that they cause epidermal water loss and skin irritation and some of them interact with intracellular keratin [30].

Liposomes, *dendrimers*, and *microemulsions* are also well-known chemical enhancers that not only act as carrier vehicles but also increase the permeability and drug solubility across the skin surface [5]. Through these carrier vehicles, very low molecular weight drugs and small molecules are transported across the skin which has found its utility in topical creams and dermatological products. *Iontophoresis* is the use of continuous low voltage current to increase skin permeability across the stratum corneum. This methodology is mostly favored for small molecules that carry a charge and thus they can be either moved through electrophoresis or the

electroosmotic flow of water generated by mobile cations (Na^+). The advantage of iontophoresis is that the patient can control the flow rate of drug molecules by modulating the electrical charge, whereas the disadvantage is that upon an increase in time duration, skin irritation and pain increase [31]. *Non-cavitational ultrasound* was the first extensively accepted penetration enhancer used commonly by a physiotherapist. The non-cavitational ultrasound acts by disrupting the lipid structure of the stratum corneum and thereby forcing the drug molecules to the inner layers owing to the enhanced permeability of the membrane. The disadvantage of this methodology is that it can damage deeper layers of the skin with permanent damage to the stratum corneum [32].

3.3 Third-Generation Delivery System

The third-generation transdermal delivery systems are the most advanced delivery systems using high-end techniques making the drug delivery options more accurate and medically profitable. The techniques employ more vigorous disruptions of the skin barrier function with more effective, deeper, and specific delivery of formulated drug molecules [5]. Some of the used methodologies are *combination of chemical enhancers* – as some chemical enhancers work at a specific concentration whereas some work at a specific area. The combination of two or more penetration enhancers can cause more aggressive penetration, thereby enhancing deeper delivery of drugs without causing any unwanted side effects [33].

3.3.1 Electroporation

In this methodology, short, high-voltage pulses are employed for milliseconds duration to reversibly disrupt the lipid bilayer structure in the stratum corneum. As the resistant power of the stratum corneum is higher than its successive lower layers, the application of electrical energy causes a disturbance in the lipid bilayers of the stratum corneum. Therefore, the drug applied to the above area diffuses directly to the inner layers for its therapeutic use. The disadvantage of this technique is that the motor and sensory neurons present in the dermal layers can cause extreme muscle pain and stimulation to the patient [5].

3.3.2 Microneedles

This is a direct technique of transferring the drugs through the stratum corneum using very small needles in a negligibly invasive way. Through this method insulin, vaccines, antibodies, nanoparticles, and drug-encapsulated formulations can be directly delivered to the inner layers of skin. The advantage of using microneedles

is that the drug can be targeted directly to the inner layers of the dermis with an increased rate of efficacy [34].

3.3.3 Thermal Ablation

In the thermal ablation technique, a very high amount of heat is applied to the selected area of the skin surface for a period of milli-/microseconds which causes the formation of punctures (in micrometer) in the stratum corneum. As the applied heat energy is very high, the underlying water present in the stratum corneum vaporizes leaving large pores/craters in the surface of the skin. This facilitates the diffusion of the drug molecule to its targeted area [5].

3.3.4 Microdermabrasion

In this method sandpaper is employed to mechanically scrub and remove the stratum corneum. This methodology is used extensively for cosmetic purposes. Delivery of lidocaine and 5-fluorouracil across the skin has been reported using this technique [9].

The delivery vehicle should be decided based upon applicability, production cost, drug stability, site of action, and patient compliance. For the delivery of drugs for skin cancer, ethosomes show a promising application for delivery of selected drugs by disturbing the barrier function of the stratum corneum [35, 36].

3.4 Vehicles for Transdermal Drug Delivery

Drug delivery vehicles are an essential component for designing strategies for enhanced and effective delivery of active pharmaceutical drugs or compounds necessary for targeting a disease or therapy. In terms of preferability of patients, the oral route of drug delivery is the preferred mode of administrating active pharmaceutical drugs owing to its ease, patient convenience, dosage compatibility, and overall safety [37, 38]. However, the oral route possesses some drawbacks such as drug degradability due to acid and enzymes in the gastrointestinal tract and quick clearance from the body [39]. Hence, the transdermal drug delivery route proposes various innovative tools for the effective delivery of various hydrophilic and hydrophobic pharmaceutically active drugs and molecules for site-specific delivery.

Innovative and target-oriented design of transdermal vehicles is a very important criterion for targeted drug delivery with proper encapsulation of pharmaceutically active drugs without exhibiting any possible side effects when applied through the skin surface [40, 41]. Hence, various vehicles for the transdermal delivery route were developed for enhancing the proper penetration of drugs, pharmacologically active ingredients, and synthesized compounds through them.

3.4.1 Liposomes

Liposomes were first conceptualized in 1965 by Dr. Alec D. Bangham, at Babraham Institute. Dr. Bangham worked as a hematologist, where he experimented with lipids and their interaction with water [42]. He found that when dried lipids were immersed in water solution, they rearranged spontaneously due to the unfavorable hydrophobic interactions between water molecules and lipids which generated repulsive effects [43]. Hence, liposomes can be defined as spherical vesicles composed of amphiphilic molecules characterized by a thin bilayer of phospholipid lipid core having an inner closed aqueous layer. The arrangement of the hydrophilic polar head and two lipophilic tails encourages the encapsulation of both hydrophilic and hydrophobic pharmacologically active molecules [44, 45].

Based on the preparatory methodology applied, liposomes can be further classified into unilamellar and multilamellar liposomes, which can again be tuned accordingly for their size from macromolecular to nanoparticle range. Liposomes can be synthesized from various sources of phospholipids and cholesterol such as phosphatidylcholine, phosphatidylethanolamine, phosphatidyl serine, phosphatidylinositol, and phosphatidylglycerol [46, 47]. Liposomes have been explored in various fields of drug delivery, and hence their popularity is based on their biocompatibility with the phospholipids of our biological membranes, biodegradability, nontoxicity, and tremendous interaction with the pharmacologically active molecules [48]. However, liposomes demonstrate some potential drawbacks such as poor drug storage stability, leakage, rapid systemic clearance, and a higher rate of oxidation due to the presence of organic solvents during the preparatory phase [49].

3.4.2 Glycerosome

Effective and controlled distribution of drugs through the skin surface provides various challenges; hence, various methods are employed by different researchers for transdermal delivery of drugs. In 2013, Manca's group reported a new methodology to proliferate the physiochemical properties of liposomes by substituting various concentrations of glycerol to the core phospholipids used during the preparatory phases [50]. They evaluated the basic physiochemical parameters of the novel glycerosomes with conventional liposomes; further, their in vitro toxicity was studied using normal keratinocyte cell lines. The formulations were designed to deliver diclofenac, which is one of the most commercially used nonsteroidal anti-inflammatory drugs and the ideal and potential drug for transdermal drug delivery [51].

Glycerol is a nontoxic compound with fully acceptable short-chain alcohol that is abundantly used in various commercially available skin-related applications [52]. Glycerosomes can be defined as modified liposomal bilayer vesicles containing glycerol as the primary compound with cholesterol or other phospholipids for active delivery of pharmacologically active ingredients through the topical surface with higher efficacy. The presence of a high concentration of glycerol provides additional

flexibility, malleability, and deformability of the bilayer lipid vesicles, thereby enhancing the penetration rate through the skin surface during transdermal drug delivery [53].

3.4.3 Transferosomes

The potentiality of liposomes for transdermal delivery was first exploited by Mezei and Gulasekharam in 1980 for the effective delivery of a steroid compound triamcinolone acetonide. However, later it was observed that liposomes generally accumulate in the tough stratum corneum layer of the skin with minimal rate of penetration, thereby promoting researchers to design new models for effective transdermal delivery of drugs. In 1992, Cevc and Blume reported the development of a new class of liposomal vesicles, transferosomes which were highly elastic, flexible, and highly deformable vesicles that can infiltrate as intact vesicles through the stratum corneum of the skin layer and reach the systemic circulation [54].

The basic composition of transferosomes remains the same as traditional liposomes were prepared from phosphatidylcholine with additional components such as sodium cholate, deoxycholate, tween 80, di-potassium glycyrrhizinate, and ethanol (<10%) [55]. Cevc's group suggested that as transferosomes were smaller in size aided with higher rate flexibility and difference in osmotic gradient, they could squeeze and penetrate through the stratum corneum and reach the targeted site of action for delivery of active pharmaceutical drugs effectively [56]. Further, to validate their findings, Cevc's group used confocal laser scanning microscopy to observe the lipid vesicles using murine skin [57]. They reported that the transferosome vesicles penetrate through intercorneocyte routes to reach the inner layers of the skin surface, thereby reaching the systemic pathways.

3.4.4 Ethosomes

Ethosomes were first conceptualized by Elka Touitou in 1996 during her research on lipid vesicles for the delivery of drugs for the treatment of skin diseases. Ethosomes can be defined as soft malleable vesicles primarily composed of phospholipids (phosphatidylcholine/phosphatidylserine/phosphatidic acid), ethanol, and water [58]. Phospholipids being non-immunogenic owing to their biological origin are suitable for skin delivery. They are extensively found in soy oil, corn oil, egg yolk, liver, and marrow [59]. Ethanol is a very important component of the ethosomal formulations as it provides the penetration enhancer effect to the delivery vehicle, thereby surpassing the barrier function of the stratum corneum [60] (Fig. 2). The higher concentration of ethanol (10–45%) not only helps in the deeper penetration of the drugs but also in maintaining the required nano-size and malleable property of the ethosomes [35, 61].

Ethosomes are considered to have a higher efficiency rate than liposomes owing to the presence of ethanol, water, and phospholipids that combine to provide a

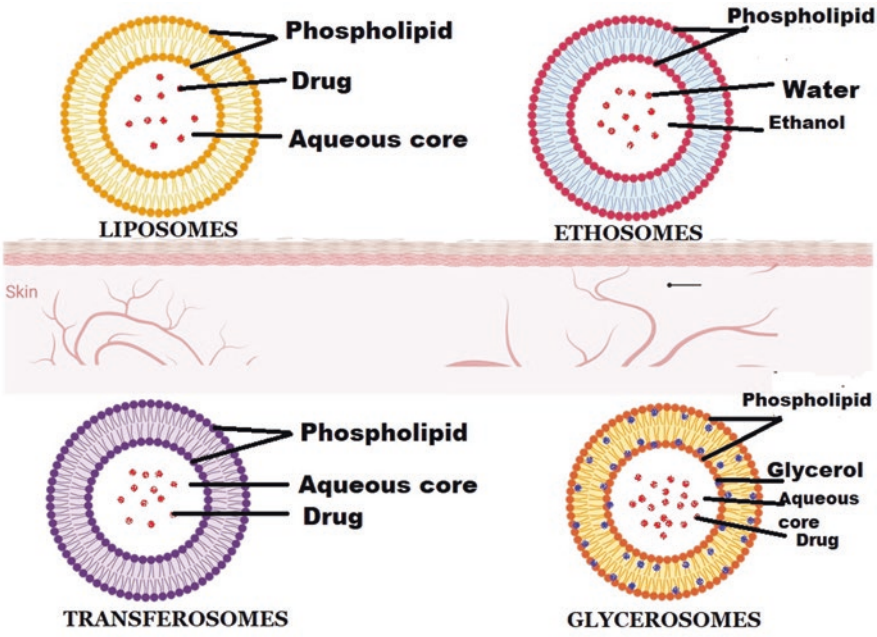

Fig. 2 Various drug delivery vehicles for transdermal drug delivery

synergistic effect for deeper penetration of the drug [60]. The phospholipids provide the hydrophilic and lipophilic properties to the ethosomes and thus are considered effective for targeted delivery through the skin surface. Some of the advantages associated with ethosomes are their nontoxic and noninvasive properties and low production cost with high production capacity without using any sophisticated instruments [35].

Ethosomes can be prepared very easily because it does not require any costly chemicals or any sophisticated instrument. Conventionally, ethosomes are prepared by the "hot" or "cold" method [58]. In the "cold method," the phospholipid, drug, and ethanol are mixed in a covered flask in a magnetic stirrer. After getting a clear solution, water (30 °C) is added separately and the solution is mixed until a milky solution is visible. To obtain the desirable size, the solution can be sonicated using a probe sonicator [62]. In the "hot method," the phospholipid and water are heated on a hot plate at 40 °C and are mixed until a colloidal suspension is obtained. In a separate flask, ethanol, propylene glycol, and drug are mixed and heated up to 40 °C. When both the solution reach 40 °C, the organic phase is injected into the aqueous phase with the help of a syringe. The solution is mixed properly, and to get the desired size, the ethosomal solution can be sonicated by using a probe sonicator [63].

Unlike liposomes that deliver drugs mostly to outer layers of the skin, ethosomes have displayed enhanced permeation by disrupting the barrier function of the stratum corneum assisted by ethanol [64]. The presence of phosphatidylcholine (soya

lecithin) and ethanol facilitates the encapsulation of a wide variety of hydrophilic, lipophilic, and amphiphilic drugs [62]. Bhalaria et al. reported that fluconazole encapsulated ethosomes possessed better fluidity and demonstrated enhanced antifungal activity when compared with liposomes, marketed formulations, and hydroethanolic solution of the same encapsulated drug [65]. The prepared ethosomal formulations also demonstrated increased skin residence time in the in vitro experiments with reduced side effects within a shorter therapy duration. Similarly, Dubey et al. reported that an ethosomal formulation containing indinavir increases the half-life of the encapsulated drug proved through in vivo experiments (Dubey et al. 2010). Dayan and Touitou reported effective formulation and skin permeation potential of the psychoactive drug trihexyphenidyl hydrochloride which is a commonly used drug for treating Parkinson's disease [67]. Similarly, Lodzki et al. reported the increased skin permeation, accumulation, and overall increased biological activity of cannabidiol encapsulated ethosomal formulation [68]. Apart from the encapsulation of many essential drugs, ethosomes have been reported to successfully encapsulate antigens and hormones. In 2007, Mishra et al. prepared HBsAg-loaded ethosomes for transcutaneous immunization against hepatitis B [69]. The ethosomal formulations showed efficient uptake by murine dendritic cells in vitro, higher skin penetration in human cadaver skin, and greater protective immune response when applied topically in mice models. Besides transdermal drug delivery, the role of ethosomes in topical drug delivery is also well documented [60].

4 Preparation of Ethosomes for Transdermal Delivery of Drugs

The preparatory steps employed for the synthesis of ethosomal vesicles are very critical as they provide the basic phytochemical characteristics for the targeted transdermal drug delivery. Various classes of phospholipids such as soya phosphatidylcholine, egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine are some of the basic choices of researchers as the main vesicle-forming component of the ethosomes. Apart from them, propylene glycol being nontoxic has been used abundantly as a penetration enhancer along with ethanol in various concentrations for providing flexibility and deformability to the designed ethosomal vesicles [61]. Two techniques are presently used for the preparation of ethosomes which are very simple, cost-effective, and convenient methods as they don't use any high-end sophisticated equipment and apparatus or don't have any potential biohazardous end products formed during the preparatory steps (Fig. 3).

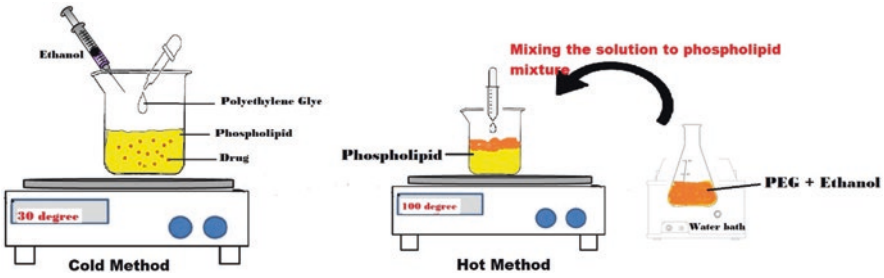

Fig. 3 Methods of ethosome preparation: cold plate method and hot plate method

4.1 Cold Method

In this method, the drug of interest and the phospholipid of our choice are mixed together using a magnetic stirrer with dropwise addition of propylene glycol at 30 °C. When the organic phase of phospholipid, drug, and propylene glycol are completely mixed, warm distilled water (30 °C) is added to the organic phase using a syringe under continuous stirring. Based on the requirement of vesicle, size ethanol is added in various concentrations followed by probe or water bath sonication [65].

4.2 Hot Method

In this method, the phospholipid of our choice is dispersed in water using a magnetic hot plate with continuous stirring at a high temperature. In a separate beaker, propylene glycol and ethanol are mixed using a water bath. When both the solutions have reached equal temperature, both the solutions are mixed together. Depending upon the solubility of the drug of interest, it is mixed with ethanol or water, and then it is added dropwise to the mixture solution. Finally, the reaction mixture is subjected to probe or water sonication depending upon the requirement [70].

4.3 Penetration Enhancers

The *permeation enhancers* are nothing but chemicals that help in increasing the influx of drugs to the deeper layers of skin. The ideal properties of penetration enhancer are that they should be nontoxic, non-irritating, and non-allergic, should not cause damage to the healthy neighboring cells, should change the permeability of the stratum corneum in a reversible reaction, should not have any pharmacological effect on the body, should be compatible with the enclosed drug, and should not react with the drug of interest [71].

Some of the popularly used penetration enhancers include:

(a) *Water*: This is the most commonly used penetration enhancer in ointments and patches. As the stratum corneum contains around 15–20% water, increasing the water content causes swelling of the underlying cells, and thereby hydrophilic and lipophilic drugs are taken up along with the water. However, the exact mechanism of action through which water increases the influx rate of drug through the transdermal route is still unclear.
(b) *Dimethyl sulfoxide (DMSO)*: This is the most widely studied penetration enhancer owing to its "universal solvent" property. It is a component of a commercially available drug used for the treatment of *Herpes simplex*. Despite being an excellent penetration enhancer, it has certain drawbacks such as at higher concentrations it can denature proteins and cause skin irritation and erythema and wheals of the stratum corneum.
(c) *Azone*: Azone (1-dodecylazacycloheptan-2-one) was the molecule that was specifically designed as a penetration enhancer. It acts by interaction with the lipid domains of the stratum corneum and disrupts its arrangement. It has been observed that azone is very effective in lower concentrations of 0.1–5%.
(d) *Ethanol*: This is another widely used penetration enhancer that is popularly used in various formulations and transdermal patches. In many cases, ethanol is mixed with water to increase the flux rate. Ethanol works by disturbing the polar head groups of the lamellar lipids present in the stratum corneum and thereby increases the efflux of the drug to the deeper layer of skin. Eventually, the ethanol evaporates the concentration of the drug increase, thereby making it supersaturated and allowing the diffusion of the drug from higher concentration to lower concentration.

4.4 Characterization Studies of Ethosomes

4.4.1 Scanning Electron Microscopy (SEM)

The surface morphology of the ethosomes can be observed through scanning electron microscopy (SEM) (Touitou, 1990). The ethosomal solution is made with proper dilution and placed onto the grid and allowed to dry. The images are recorded on a scanning electron micrograph at different magnification (magnification, 60x; accelerating voltage, 12.0 kV; at 25 ± 2 °C) [72].

4.4.2 Transmission Electron Microscopy (TEM)

Vesicular morphology of ethosomes can also be evaluated by using transmission electron microscopy (TEM). The ethosomal solution is placed onto a carbon or copper grid and then negatively stained by adding 2% phosphotungstic acid, followed by drying at room temperature. Then the grid is visualized in a transmission electron microscope with an accelerating voltage of 80 to 120 kV [72].

4.4.3 Ethosomal Size and Charge Distribution

The vesicular size and size distribution can be evaluated by various techniques such as size exclusion chromatography (Sec), microscopy techniques, or dynamic light scattering (DLS). A newly developed technique, atomic force microscopy (AFM), is a rapid, noninvasive, and effective tool to evaluate the vesicular size, morphology, and stability [73]. The physical stability of ethosomes is determined by measuring zeta potential using a Zeta meter. The vesicular size ranges from micrometer to nanometer that is mainly regulated by the ethosomal composition, e.g., the average size of ethosomes formulated with 30% ethanol was 153 ± 4 nm, while the vesicular size of ethosomes formulated with 20% ethanol was 193 ± 8 nm. When ethanol concentration was increased from 20 to 45%, the size of ethosomes was found to be decreased. The ethosomes formulated with 45% ethanol were found to be 103 ± 9 nm. The phospholipid content has minimal influence on ethosomal size as observed in various studies. The ethosome size was doubled only when the phospholipid concentration increased eight times. The ethosomal formulation containing 30% ethanol and 0.5–4% phospholipid has minimal impact on vesicular size [74].

4.4.4 Encapsulation Efficiency

The entrapment efficiency of the drug in the ethosome can be measured by using either the ultracentrifugation technique or the dialysis method.

Ultracentrifugation

The drug entrapment in ethosomes is determined by the total amount of drug added to the formulation and the amount of drug that is not encapsulated in ethosomes and remained free in the supernatant after ultracentrifugation. The formulated ethosomal solution is kept overnight and subjected to ultracentrifugation at optimized conditions (speed and time). The concentration of pure drug in the supernatant is evaluated either by a simple spectrophotometric method or a sophisticated developed method, high-performance liquid chromatography (HPLC).

The entrapment efficiency is calculated as per below-mentioned formula:

$$\text{Entrapment Efficiency}(\%) = \frac{\text{Total amount of Drug}(Dt) - \text{Free Drug in the Supernantant}(Ds)}{\text{Total amount of Drug}(Dt)} \times 100$$

Dialysis

The drug-loaded ethosomes or free drugs in an aqueous solution are placed into the dialysis bag made up of cellulose acetate and then shifted into an appropriate buffer (phosphate buffer saline, pH 7.0) with stirring conditions. The aliquots with equal amounts are withdrawn at a fixed time from the outer medium and replaced equal volume of buffer to maintain the perfect sink conditions. The samples were further assayed for drug content using either spectrophotometer or HPLC methods. The entrapment efficiency can be then evaluated using the formula mentioned above [75].

4.4.5 In Vitro and Skin Drug Permeation Study

The drug release from ectosomes is studied in vitro using diffusion cells [76]. The effective permeation area of the diffusion cell and receptor cell volumes is 1 cm^2 and 20 ml, respectively. The receptor cell contains 20 ml of buffer at a specific temperature and constant stirring. The synthetic semipermeable membrane or the mice's skin is mounted between the donor and receptor compartments. The ethosomal solution is applied to the abovementioned membrane. The sample from receptor cells is withdrawn at a specified time interval and evaluated for the drug content by either UV spectrophotometer or high-performance liquid chromatography (HPLC). The receptor cell is immediately replenished with an equal volume of buffer to maintain the sink condition. The in vitro release is employed to evaluate the drug content deposited on the skin. The degree of drug penetration in the skin can also be visualized by using advanced microscopy SEM, TEM, and confocal laser scanning microscopy (CLSM) [1] (Table 1).

Table 1 Physicochemical characterization of ethosomes

Sl. No.	Study parameter	Techniques	Significance
1	Size and morphology	SEM, TEM, AFM, DLS	Determine skin penetration
2	Charge distribution (zeta potential)	DLS	Stability
3	Entrapment efficiency	Ultracentrifugation, dialysis, UV spectrophotometry, or HPLC	Determine drug content
4	Stability of ethosomes	SEM, TEM, UV spectrophotometry or HPLC	Determine the shelf life of vesicle formulation
5	In vitro drug permeation	UV spectrophotometry, HPLC	Determine drug release kinetics
6	Skin permeation	CLSM	Determines rate of drug transport through the skin

4.5 Mechanism of Action of Ethosomes

Cevc's group proposed that transdermal delivery of drugs through ethosomes may occur due to the elasticity of the deformed vesicles and the osmotic gradient across the skin. According to this theory, the driving force for the movement of the ethosomes is presumably generated by the hydration gradient across the skin which varies from 15% water content in the stratum corneum to 60% in the stratum granulosum. When the elastic vesicles are applied to the skin and allowed to dry, they are attracted through their moisture content in the epidermis and, due to their flexible nature, they penetrate the skin. The osmotic gradient caused by the differences in water concentration between the skin surface and the interiors has been proposed as the major driving force for the penetration of vesicles [77].

Two simultaneous mechanisms of action have been proposed by Touitou et al.:

1. "Ethanol effect" which affects lipid fluidity of the skin because ethanol acts as a penetration enhancer that effectively enhances its capacity to penetrate the stratum corneum of the skin [8]. Ethanol interacts with the lipid molecules in the polar head group regions stimulating the reduction of the transition temperature of the lipids in the stratum corneum, thus resulting in the increase of its fluidity and decrease in density of lipid bilayer.
2. "Ethosome effect" which helps in the penetration of the lipid by opening a route due to the malleability and fusion of ethosomes with the skin's lipid layer resulting in the release of the drug into deep layers of skin and/or systemic circulation (Fig. 4). It is a known fact that ethanol is a relatively volatile solvent which evaporates when it comes in contact with the skin temperature, thus making the encapsulated drug highly concentrated which will influence drug flux across the membrane [30].

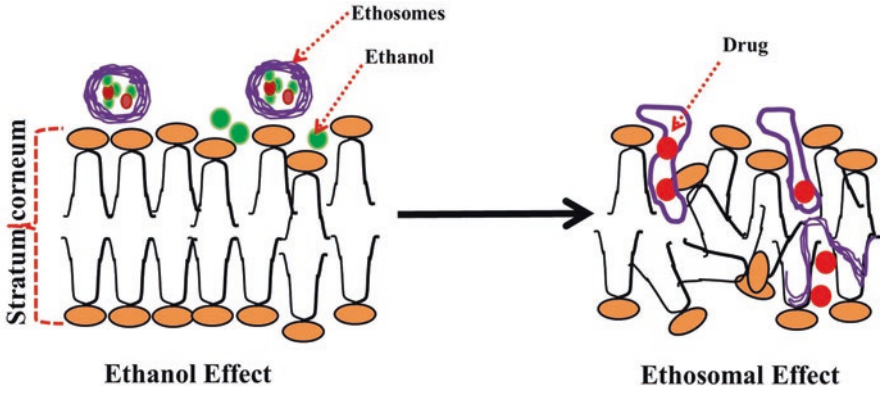

Fig. 4 Mechanism of drug penetration using ethosomes due to the combined effect of ethanol and phospholipids

5 In Vivo and Clinical Trials on Ethosomes

In comparison to traditional lipid-based vesicles, ethosomes exhibit enhanced therapeutic applications in transdermal delivery. Following Touitou's group novel innovation, ethosomes have overcome many hurdles, and subsequently, they have experimented with various combinations of penetration enhancers, ethanol concentration, and methods of preparation [58]. In 2000, Touitou's group prepared testosterone ethosomes and compared their efficacy against commercially marketed testosterone transdermal patches using rabbit pinna skin. It was observed that testosterone ethosomal formulations demonstrated a higher rate of skin permeability of the inbound testosterone compared to the marketed patch. Further in another study by the same group, the systemic circulation and plasma concentration of testosterone were found to be comparatively less than the commercial gel (AndroGel, USA) formulation with respect to the ethosomal gel formulation [78]. In 2001, Dayan's lab developed an ethosomal formulation for the treatment of Parkinson's disease containing the drug trihexyphenidyl hydrochloride (THP). Basically, THP is used as a psychoactive drug that is supplemented orally to the patients; however, its half-life is only 3 hours. When Dayan and Touitou compared the THP formulated ethosomes with classical liposomes using mouse skin, it was observed that the ethosomal formulations exhibited a higher rate of transdermal delivery of active THP molecules up to 18 h of treatment [67].

In 2003, Lodzki's group synthesized cannabidiol-loaded ethosomal formulation for the treatment of rheumatoid arthritis through the transdermal route. In vivo studies using a mice model exhibited significant accumulation of cannabidiol in the skin and underlying muscles, thereby increasing its penetration and accumulation to the targeted region for rheumatoid arthritis [68]. In 2004, Maiden's laboratory developed minoxidil containing ethosomal formulation for its enhanced delivery for treating baldness in men [79]. Using a nude mice model, it was observed that the accumulation and penetration of minoxidil enhanced in comparison to the conventional topical ointment. In 2005, Paolino's group examined the efficacy of ammonium glycyrrhizinate-loaded ethosomal formulations for the treatment of inflammatory skin diseases through the transdermal route [80]. In a clinical trial, they observed that after 48 h of application, the ammonium glycyrrhizinate-loaded ethosomal formulations exhibited excellent skin tolerability and enhanced biological anti-edema activity in human volunteers. In 2007, Mishra's group reported the development of antigen-loaded ethosomal formulation for transcutaneous immunization against hepatitis B [81]. When compared to conventional lipid-based formulations, the antigen-loaded ethosomes exhibited excellent encapsulation efficiency and physiochemical characteristic studies. In vitro studies using murine dendritic cells demonstrated efficient uptake of the ethosomes followed by higher skin penetration in the human cadaver skin model. In vivo studies in a mice model exhibited enhanced systemic and humoral immune response in comparison to intramuscular vaccine administration. In 2008, Koli's group reported the development of vitamins A, C,

and E containing ethosomal formulation as a synergistic antioxidant formulation [82].

Clinical studies against the herpes virus using acyclovir ethosomal formulations were reported by Horwitz et al. in comparison to commercial acyclovir cream which demonstrated significant improvement in patient compliance with relation to the time of cure and pain relief without any additional side effects using 40 random human volunteers [83]. Another clinical trial was conducted using clindamycin phosphate and salicylic acid containing ethosomal gel in 40 random volunteers having severe acne problems [84]. The volunteer patients were treated by topical application of the prepared ethosomal gel daily twice for a duration of 8 weeks. When the trial was completed, the patient volunteers exhibited improved acne condition when compared to the control group. Further, in vivo safety studies and human clinical trials must be conducted to evaluate the efficacy and long-term side effects of differently prepared ethosomes which are currently investigated for nanocarriers and targeted drug delivery tools.

6 Ethosome-Based Marketed Products

In recent years with the advancement of innovations and technological expertise, various ethosomal formulations have evolved to address the smooth and effective delivery of drugs through the skin. Most importantly, the barrier effect of the stratum corneum has been addressed for quick and efficient delivery. In the case of ethosomes, the investigator gets ample autonomy during the formulation and designing stage for the selection of a diverse range of starting materials, penetration enhancers, drugs, and various concentrations of ethanol to overall change its property based on its efficacy. Hence, ethosomal formulations containing various pharmaceutically active molecules and compounds have been reported for their incorporation in gels, creams, and sprays in different cosmetic and skin care products [85]. Various physicochemical parameters were observed and several stages of clinical trials were conducted for understanding the viability and utility of the proposed ethosomal formulations. Though the commercialization of ethosomes started in early 2000, very few products are available which are produced by some companies in Israel (Novel Therapeutics Technologies, Osmotics, and Trima), Germany (Sinere), the UK (Physonics), and the USA (Hampden Health and Genome Cosmetics). The formulations were precisely used for wrinkles, anti-aging creams, Anti-cellulite lotion, hair growth promoters, and delivery of antifungals and antibiotics [86].

Various research groups have reported the effective delivery of active ingredients through the skin and topical ointments and gels such as 5-aminolevulinic acid, ammonium glycyrrhizinate, epigallocatechin gallate, erythromycin, isoeugenol, matrine, minoxidil, methotrexate, azelaic acid, bacitracin, colchicine, finasteride, fluconazole, ibuprofen, ligustrazine, salbutamol, sotalol, amphotericin B, econazole nitrate, ketoconazole, lopinavir, losartan, meloxicam, testosterone, tretinoin, valsartan, 5-fluorouracil, aceclofenac, methoxsalen, vinpocetine, clotrimazole, psoralen,

benzocaine, black tea extracts, buspirone hydrochloride, capsaicin and capsicum tincture, ligustrazine phosphate, repaglinide, valsartan, cetirizine, griseofulvin, felodipine hydroxypropyl, and vitamins A, C, and E. Hence, from the above-reported results with a wide variety of active pharmaceutical compounds, it was observed that gels exhibited more prominent efficacy in ethosomal formulations in comparison to creams which were mostly preferred for cosmetic and beauty supplements [79].

7 Future Perspectives and Conclusion

Delivery of potential drugs without any proficient side effects is an important criterion for the transdermal route. The stratum corneum is the primary hindrance to transporting the pharmaceutically active molecule/compound to the deeper layers of the skin. The success of any transdermal delivery vehicle is measured by the effective and systemic release of the drug to the concerned site of interest. Ethosomes owing to their soft and malleable property aided by the presence of ethanol have gained much appreciation for the delivery of drugs without affecting their chemical property. The presence of a diverse range of penetration enhancers and ethanol concentration has provided an undue advantage to the ethosomal vesicles to simultaneously disrupt the lipid bilayer as well as malleability to squeeze through the small opening to their its site of action. Hence, this extremely proficient delivery vehicle has owned its position as an emerging and promising contender for the administration of pharmacologically bioactive molecules/compounds in various ethosomal gels, creams, and sprays through the topical and skin route.

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Liposomes and Niosomes for Targeted Drug and Gene Delivery Systems

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1 Liposomes

Liposomes are tiny spherical structures that may completely enclose single or multiple aqueous sections with hydrophilic and hydrophobic compounds like phospholipids and cholesterol (Fig. 1). Liposomal properties are influenced by various factors like their composition, vesicular size, the ionic charge on the surface, and synthesis techniques. They may be composed of one or several lipid bilayers. Liposomes are commonly used as a means of delivery or as a carrier for a variety of bioactive compounds. The positively or negatively charged lipids, along with the stabilizing agents present, decide the surface charge reported on the liposome (Fig. 1b). When compared to the freely available medicines in solution, medications bound to liposomes exhibit significantly different and enhanced pharmacokinetic features. Liposomes are widely utilized to manage systemic toxicity and to keep the encapsulated medicines from deteriorating rapidly once they have been ingested. PEGylated or stealth liposomes have been developed with polymers like polyethylene glycol (PEG) and can demonstrate prolonged blood circulation [1]. Certain

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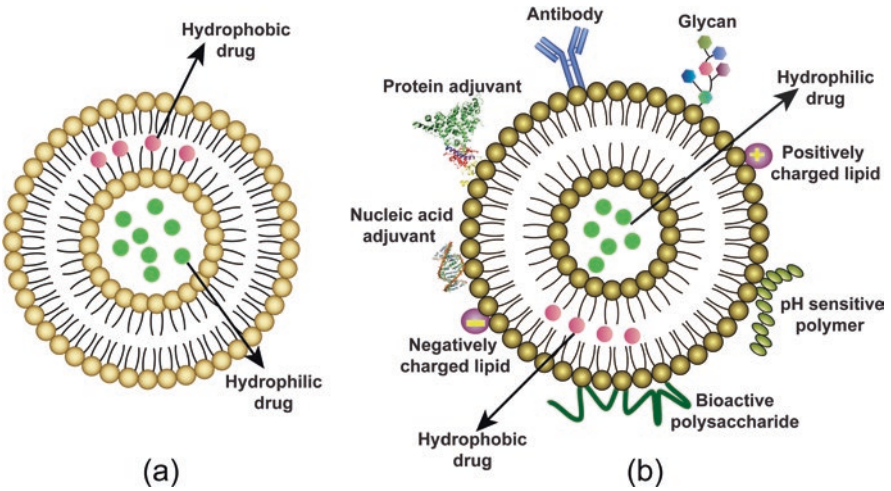

Fig. 1 Liposome. (**a**) Conventional liposome with a hydrophilic and hydrophilic core. (**b**) Multifunctional liposome

liposomal antibodies or ligands can also be attached to liposomes to improve target-specific medication treatment.

Visser et al. investigated the targeting of PEGylated liposomes tagged with transferrin and loaded with horseradish peroxidase (HRP) [2]. The possibility of effectively targeting liposomes laden with proteinaceous or peptide drugs to the endothelial cells of the brain capillaries was demonstrated by the team, and it was posited that this method is an appealing approach for brain medication delivery [2]. By applying the liposomes in a non-occlusive manner to rat skin, Lopez-Pinto and his colleagues investigated the dermal administration and delivery of a lipophilic medication, minoxidil, from ethosomes as compared to the traditional liposomes [3]. Different liposomal systems were examined to study their pattern of permeation, the depth of permeation across several layers into the skin, and the major penetration pathways [3].

Leibiger et al. explored the utilization of liposomes bearing the expression vector pRSVneo which codes for neomycin phosphotransferase-II for an in vivo gene transfer into rat liver cells [4]. Non-integrated vector DNA was identified in isolated nuclei of hepatocytes of Wistar rats after the administration of liposomes in them, intravenously [4]. Cirli and Hasirci created reverse-phase evaporation vesicles enclosed by calcein, and in the lipidic bilayer, suprofen was incorporated as a photoactive destabilizer [5]. The group investigated how the UV photoactivation of the photoactive destabilizer, suprofen, might affect the integrity of the bilayer of the liposome. Additionally, its impact on the release of encapsulated calcein, as a model active agent, was studied [5].

The role of liposomes as genes, DNA fragments, and cell transporters is also being probed by scientists. Kunisawa et al. developed a method for the encapsulation of nanoparticles within the liposomes, which were later developed to form

fusogenic liposomes by the fusion of the former with the UV-inactivated Sendai virus. It was realized with further studies that these fusogenic liposomes exhibit an excellent ability to transfer nanoparticles containing DNA molecules into the surrounding cytoplasm [6]. The adjuvanticity of two gamma inulin/liposomes/vitamin E combinations in the contraceptives administered to mice was investigated by Fuentes et al. by employing sperm protein extracts or a synthetic HE2 peptide as an antigen [7]. They discovered that a mixture of gamma inulin, liposomes, and vitamin E, as well as sperm protein extracts, outperformed Freund's adjuvant [7].

Vierling and co-investigators provide a review of fluorinated liposomes and fluorinated lipoplexes [8]. The properties of fluorinated lipoplexes like their stability as well as their in vitro cell transfection were elucidated [8]. The integration of activators or surfactants into the liposomes showed an enhanced skin delivery of estradiol [9]. The negatively charged ion of diclofenac interacts with the ammonium group of the phospholipid present in the lipid bilayer. It was also suggested in the established model for sodium diclofenac (SD) interaction with liposome phosphatidylcholine that the dichlorophenyl ring occupied a site near the phosphate group which was more internal on the bilayer [10]. They showed that hydrophilic medications may be loaded into liposomal vesicles with an average diameter of less than 300 nm without any difficulties [10]. When the vesicles were administered intravenously into rats, they discovered that the liposomes whose surface was modified circulated for an extended period than the untreated liposomes. Koynova and MacDonald researched the lipid exchange using differential scanning calorimetry between model lipid systems, vesicles of the cationic lipids ethyl dimyristoylphosphatidylcholine, ethyl dipalmitoylphosphatidylcholine, or their combinations with zwitterionic lipids and DNA [11]. Cationic ethyl phosphatidylcholines were significantly easier to exchange via lipid monomers than zwitterionic phosphatidylcholines or the positively charged liposomes [11].

It was observed that when serum was present in the dispersion media, the lipid transfer between cationic liposomes enhanced significantly; however, no effect was seen on the zwitterionic liposomes [11]. The phenomenon described above has been put forward as a significant feature in the nonviral gene delivery technique using cationic liposomes. The administration of sodium ascorbyl phosphate (SAP) which is an efficient scavenger of the oxygen species, as a way of preventing the deteriorating effects of UV radiation on our skin, was examined by Foco et al. [12]. SAP was encapsulated in liposomes to allow it to pass through the epidermal barrier and into the deeper layers [12]. They created two forms of cholesterol-containing multilamellar vesicular structures, the first one was made using non-hydrogenated soybean lecithin, while the second one was made using hydrogenated soybean lecithin [12]. Sinico et al. looked at how the liposomal architecture, size ranges, lamellarity, and charge affected the transdermal distribution of tretinoin. They studied anionic and cationic liposomes of various forms, including multilamellar and unilamellar vesicles, synthesized from either hydrogenated or non-hydrogenated soy phosphatidylcholine and cholesterol, along with the addition of stearylamine or dicetyl phosphate [13]. Negatively charged liposomes were found to significantly increase hydration and tretinoin retention in newborn pig skin [13]. Arcon et al. managed to

encapsulate cisplatin, an anticancer drug within stabilized liposomes, and examined the complexes using the prolonged X-ray absorption fine structural (EXAFS) method. They finally concluded with the studies that the medicinal drug contained in liposomes is highly stable and does not hydrolyze [14].

1.1 Therapeutics Applications of Liposome

During the early developments in the study of liposomes, it was believed that a way to enhance the selectivity of the liposome and diseased cell interaction was necessary. Liposomes are acknowledged to be perhaps one of the most prosperous drug-carrier systems identified hitherto due to their various biological and technical benefits as ideal delivery vehicles for biologically active compounds in vitro as well as in vivo. Momentous advancements made over the past few years have helped us push various novel biomedical applications of liposomes into clinical trials, while some are already available in the market for public use. The liposomal formulation can be used for many parenteral delivery methods, including intramuscular, intravenous, and intraperitoneal routes. When matched against other existing routes of administration, the oral route is found to be more convenient for patients and so improves compliance. Numerous studies show liposomes can be used effectively for oral delivery of medication. Liposomes improve lymphatic circulation, which increases medication bioavailability in liposomes [15]. Similarly, liposomes like dry-powder inhalers, nebulizers, etc. have many pros for specifically the pulmonary drug delivery since it has remarkable stability, tiny particle size, and a high affinity to control the delivery of drugs to the lung tissue along with good absorption and local action in the respiratory tract [16]. Lipids let medications penetrate and diffuse through the skin more effectively, avoiding first-pass metabolism. It gives a long-acting dosage form of a drug with a comparatively shorter half-life and limited solubility, improving the bioavailability of medicines like aceclofenac and nicotine [17].

1.2 Liposomal Small Therapeutic Agent

One of the first therapeutic applications of traditional liposomes was the administration of small molecule medicines [18]. In comparison to free medicines, liposomal-mediated delivery of medications exhibited an enhanced pharmacokinetic profile and therapeutic efficacy. To preferentially cause the aggregation of chemotherapeutics at the site of the uncontrolled cell growth, a method of passive targeting moderated by the increased permeability and retention (EPR) effect is utilized by the liposomes (conventional/PEGylated) [19]. Other chemicals being probed for their potential therapeutic activities in association with liposomes include aptamers, antibodies, folic acid, etc. [20–22]. Xing, along with his colleagues, discovered that a PEGylated liposome system functionalized with a DNA aptamer can bind to an

mRNA-stabilizing protein called nucleolin (NCL) which tends to get overexpressed on the cell membrane of many cancerous cells, selectively. On analyzing the interactions found between PEG backfilling and DNA aptamers on the surface of liposomes, Lu et al. put forward that there was a need for a long spacer for the targeting of the aptamer sequence to differentiate from the nearby PEG molecules and generate an optimal targeting impact [23]. Tumor targeting may be improved if we focus on targeting the aspects related to the microenvironment of the tumor-like elevated activity of protease enzyme, etc. Suitably, matrix metalloproteinase 2, whose levels are heightened in the tumor microenvironment, has been targeted by the attachment of anti-nucleosome monoclonal antibody 2C5 to PEGylated liposomes for boosting specific delivery [24]. Other disorders have also been treated with liposomal small molecule medicines. Linoleic acid was successfully loaded into liposome lipids phosphatidylcholine and cholesterol and showed substantial antibacterial activity against antibiotic-resistant *Helicobacter pylori* [25]. The effect of the liposomal surface alterations and the content present inside them, on cardiovascular disorders, was investigated by Szebeni et al. with an array of phospholipid constitutions like EPC, HSPC, etc. [26].

1.3 Liposomal DNA Vaccines

DNA vaccines are a promising substitute for traditional vaccines to trigger the immune response against antigens which are difficult to be generated in a recombinant form. Nucleic acid vaccines are a potent alternative for weakened bacterial antigens/protein or peptide vaccines. Rodriguez and colleagues used MLVs (multilamellar vesicles) as inexpensive carriers to conduct the delivery of DNA to mice along with plasmids encoded with type 1 bovine herpesvirus. Specific IgG responses were developed by vaccinated mice [27]. Liu and colleagues used the M1 gene of influenza A virus to generate a positively charged liposome called DNA vaccine by M1-encoding plasmid using the oral route of administration. As a result, the expression of the M1 gene in the vaccinated mice's intestine was seen along with powerful immune responses and protection against various severe diseases [28].

For the treatment of pulmonary fungal infection called paracoccidioidomycosis liposomes loaded with plasmid DNA encoded with heat shock protein, 65 (hsp65) were used. It may decrease the fungal burden and increase the immune response [29]. The concept of artificial microbes as liposomes was proposed by Amidi and colleagues, programmed to design and synthesize specific antigens intended for vaccination. On a liposome, a bacterial translation and transcription system was laden along with a gene coding for β-galactosidase or a luciferase-nucleoprotein (NP) fusion epitope as antigens. On administration of this vaccine, it was observed that such antigen-generating liposomes triggered greater specific immune responses to counter the produced antigen as compared to the control vaccine [30].

1.4 Liposome-Loaded Peptides and Proteins as Antigens

The antigen site of liposomes is affected by immunogenicity. The immunogenic responses of T-cells can be induced by both entrapped and surface antigens present in the cell. However, surface antigens are more accessible for B-cell recognition than the endogenous antigens that need disruption of the vesicle for recognition by the B-cell. The important role of CD4+ cells in increasing the amount of memory CD8+ cells was studied in mice that were administered with liposome-loaded surface-coupled OVA peptides. The response of CD4+ T-cells was not binding for the increased production of CD8+ cells as CTL response had been reported in mice missing CD4+ cells as well [31]. Phosphatidylserine (PS) liposome conjugated with antigens results in rapid detection by APCs which will amplify the TH cell stimulus and can be listed as an adjuvant intended for peptide vaccines [32]. Liposome-loaded protein antigen has been used recurrently hitherto; recently, Nagill and his team contrasted an encapsulated antigen of *Leishmania donovani* and antigen plus monophosphoryl lipid A (MPLA), and it resulted in decreased parasite load after challenge [33]. Again, studies on encapsulated OVA and the TLR ligand Pam3CysSK4 or CpGs in dioleoyl-3-trimethyl ammonium propane (DOTAP) liposomes revealed that there was no prevention of TLR-transfected cell activation by the encapsulation of both of the ligands [34]. Gupta and Vyas had developed hepatitis B surface antigen (HBsAg) encapsulated liposomes combined with Ulex europaeus agglutinin 1 used in latinized liposomes which principally targeted the M cells of the intestinal Peyer's patches after orally immunizing the model [35]. The encapsulated *Streptococcus Equi* antigens in cholesterol/PC/stearyl amine liposomes or chitosan nanoparticles had been developed by Figueiredo and colleagues as a mucosal vaccine, which had been administered to mice via intranasal immunization and lead to cellular, humoral, and mucosal responses with elevated levels of serum IgA due to increased mucoadhesive properties of the chitosan nanoparticles [36]. In another study, liposome was altered with pH-sensitive 3-methyl-glutarylated hyperbranched poly (glycidol) (MGlu-HPG) which had been encapsulated with OVA and showed strong immune responses when suppressed with anti-MHC-I/MHC-II antibodies (Hebishima et al. 2012). RAFTsomes arose from OVAprimed DCs by the isolation of membrane microdomains that contain MHC-I and I-Ab-restricted epitopes and reconstituted on liposome surfaced by Ding and colleagues which shows antiOVA IgG1 levels and protection against EG.7 tumor challenge caused by the expression of OVA [37].

1.5 Liposomes as Carriers for Adjuvants

CpGs are adjuvants that are comprised of unmethylated CpG dinucleotide sequences that are alike the DNA seen in bacteria which had been found to activate DC maturation, trigger TLR9, and enhance the expression of antigens and induce TH1

immune responses [38]. For the generation of desirable immune responses, CpGs and antigen must be colocalized in one APC [39]. Encapsulation of CpG in different properties of liposomes had shown altered encapsulation of antigen efficiency, delivery rates, and release which affect the immune response [40]. The immune response can be shaped by using encapsulated OVA and Pam3CysSK4 or CpGs in cationic liposomes from IgG1/IgG2a to IgG2a [34]. Nuclease-sensitive phosphodiester CpGs (PO-CpGs) or nuclease-resistant phosphorothioate CpGs (PS-CpGs) encapsulated in DOTAP liposomes can also be used as adjuvants, which have been studied in mice inoculated with soluble liposomal leishmania antigens (SLA) integrated with PO-CpGs or PS-CpGs which has shown no major shift in immune response from what had been studied in a leishmaniasis model by Shargh and colleagues [41]. However, CpGs assimilated in cationic DOTAP liposome give complete protection against *Burkholderia pseudomallei* in a mouse model but not in neutral 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes [42].

1.6 Cationic Liposome Adjuvant Vaccines (CLDC)

Currently, the use of cationic compounds to modify the properties of liposomes is a relatively widespread technique that is repeatedly utilized in cell transfection reagents and vaccine adjuvants. Although numerous positively charged lipids can form bilayer liposomes, frequently other lipids are also required. The positive charges have a high density on the surface, which attracts and thus increases the adsorption of negatively charged cell surfaces on it. Cationic liposomes move through cells in a variety of ways and activate multiple cellular pathways, relying on the nature of the cationic lipid, the type of cell, and the liposome formulation and size [43, 44]. An extensively studied cationic liposome complex comprises cationic lipid 1-[2-(oleoyloxy)- ethyl]-2-oleyl-3-(2-hydroxyethyl) imidazolinium chloride and cholesterol. CLDC is formulated by combining DNA with liposomes. CLDCs promote APC absorption, stimulate IFN production and TLR activation, and trigger the adaptive immune response. A large number of CLDC vaccines have already been evaluated in a variety of animal models. Gowen and coworkers investigated the liposomal transport of plasmid DNA and its CpG content using CLDCs [45]. They examined the ability of CpG-free or CpG-containing plasmids with and without liposomes, as well as poly (I: C), to protect hamsters from the deadly Punta Toro virus [45]. CLDCs containing the CpG plasmid dramatically increased survival, decreased hepatic damage, and reduced the viral loads [45].

CLDC augmented immunological responses against simian immunodeficiency virus (SIV) generated by SIV vaccinations. Rhesus macaques inoculated with CLDC developed stronger SIV-specific T- and B-cell responses as compared to the controls, causing an increased persistence and memory responses [46]. Hong and coworkers evaluated an influenza A virus vaccine in which the adjuvant was CLDC or alum. CLDC generated stronger adaptive immune responses characterized by increased levels of virus-specific IgG2a/c, CD4+, and CD8+ T-cells, as well as

Table 1 Clinical trials of some liposome adjuvant vaccines against infectious disease

Name	Disease	Route of administration	Description	Status	Sponsor	References
TVDV with Vaxfectin	Dengue fever	Intramuscular injection	Tetravalent dengue vaccine (TVDV) with Vaxfectin cationic lipid-based adjuvant	Phase 1	US Army Medical Research and Materiel Command	[49]
Ag85BESAT-6 with CAF01	Tuberculosis	Intramuscular injection	Subunit protein antigen Ag85B-ESAT-6 with a two-component liposomal adjuvant system composed of a cationic liposome vehicle (dimethyl dioctadecyl ammonium (DDA)) stabilized with a glycolipid immunomodulator (trehalose 6,6-behenate (TDB))	Phase 1	Statens Serum Institute	[50]
Biocide with lipid A	HIV	Intradermal	Recombinant envelope protein rgp120/HIV-1SF2 combined with lipid A	Phase 1	National Institute of Allergy and Infectious Diseases	[51]
ID93 with GLA-SE	Tuberculosis	Intramuscular injection	Four *M. tuberculosis* antigens (ID93) recombinant fusion protein formulated in a glucopyranosyl lipid adjuvant (GLA-) stable emulsion (SE)	Phase 1	National Institute of Allergy and Infectious Diseases	[52]
Maxime with CCS/C	Influenza	Intramuscular injection	Commercial split influenza virus and polycationic liposome as adjuvant (CCS/C)	Phase 2	NasVax Ltd.	[53]
Fluzone with JVRS-100	Influenza	Intradermal	JVRS-100, a cationic lipid-DNA complex adjuvant, was used to administer an inactivated trivalent influenza vaccine	Phase 2	Colby Pharmaceutical Company and Juvaris BioTherapeutics	[54]
RTS/S with AS01	Malaria	Intramuscular injection	Repeated sequences of the *Plasmodium falciparum* circumsporozoite protein (RTS/S) fused to the hepatitis B surface antigen using a liposome-based adjuvant system containing monophosphoryl lipid A (MPL) and *Quillaja saponaria* Molina, fraction 21	Phase 3	KEMRI-Wellcome Trust Collaborative Research Program and GlaxoSmithKline	[55]
Amphenol	Visceral leishmaniasis	Intramuscular injection	Amphotericin B lipid emulsion	Phase 3	Bharat Serums and Vaccines Limited	[56]

cross-protection against deadly viral challenges [47]. Jones and colleagues studied the efficacy of an oral CLDC-adjuvanted vaccination to fight against fatal pneumonic plague. Oral inoculation with the *Y. pestis* F1 antigen in combination with CLDC resulted in significant anti-F1 antibody titers and enduring CD4+ T-cell-dependent protection against deadly lung challenges with *Y. pestis* [48]. Liposome adjuvant vaccines against infectious diseases are listed in Table 1.

2 Niosomes

Niosomes are vesicles made of nonionic surfactants that were created to replace liposomes as a controlled drug delivery method owing to sterilization, large-scale production, and stability difficulties. Nonionic surfactants are monoalkyl or dialkyl polyoxyethylene ether, hydrated cholesterol, and a charge-inducing chemical accumulate in liposome-like vesicles. These vesicles do not form on their own in most cases. The presence of the correct mixtures of surfactants and charge-inducing substances indicates the creation of thermodynamically stable vesicles. After encapsulation of hydrophobic material, niosomes can entrap/bind hydrophilic medications and other bioactive compounds by separating these molecules into hydrophobic domains/regions (Fig. 2). The structure of these vesicles might be unilamellar or multilamellar. Additionally, niosomes have a high degree of stability, pricing, and simplicity in terms of regular and vast-scale production without the use of damaging solvents. However, niosomes are utilized in various medical and nonmedical fields (food technology, cosmetics, medicine, diagnostics). The role of the noisome in drug distribution can be regarded from a variety of perspectives, including the method of administration and the active substance for which the noisome is a

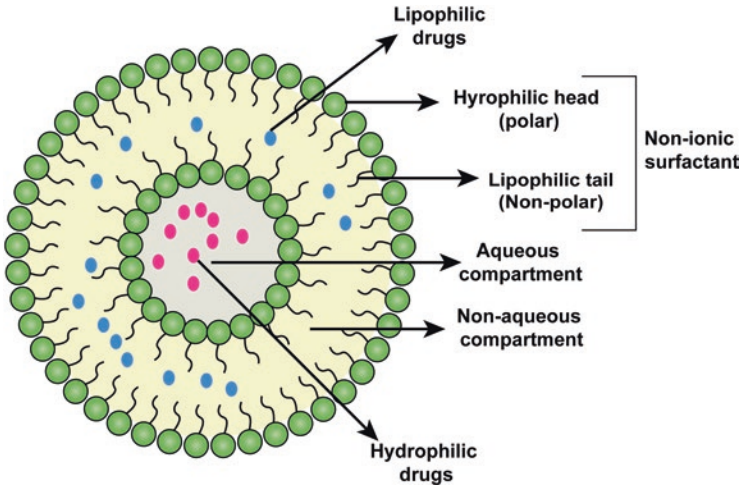

Fig. 2 Representative image of niosome

carrier. The various means of administration employed in niosomes were explored, and it was observed that the route of administration had a significant impact on the pharmaceutical formulation. The superiorities and advantages of niosomes, compared to other micro- and nanoencapsulation technologies, are first compared to phospholipid molecules used in liposome formulations, the surfactants used in the formation of niosomes are more stable, and basic procedures are mandatory for manufacturing and huge-scale manufacture of niosomes, which is the second most significant factor.

2.1 Transdermal Applications of Niosomes

Transdermal applications are thought to provide a substantial advantage in terms of circumventing hepatic first-pass metabolism. The skin's stratum corneum layer functions as a barrier, causing absorption to be delayed at the application site. In the synthesis of noisome, nonionic surfactants are commonly used to create vesicles; hence, they are attractive candidates for transdermal medication administration [57]. The cosmetic industry has produced and treated niosomes containing urea formulations as practically supernatural compounds. For transdermal vesicle absorption, two methods have been proposed. The first is the overall dispersion of niosomes from the stratum corneum layer of skin as a whole, and the second is the reformation of vesicles by each component. The latter occurs exclusively in particular areas of the stratum corneum with high water content.

The key benefit of transdermal drug delivery above the other types of medication delivery is that the patch facilitates a controlled release of medication into the patient, usually through a porous membrane covering a medication reservoir or by body heat melting thin layers of medication implanted in the adhesive. The stratum corneum intercellular lipid barrier is so porous that vesicles like niosomes can pass through it. Passive diffusion is utilized to absorb a drug into and across skin whenever the driving force is a concentration gradient. Throughout the preceding decade, several studies employing niosomes to improve medication delivery through the skin were done. Transdermal or dermal delivery is one of these approaches of ellagic acid [58], salidroside [59], clomipramine [60], gallidermin [61], and curcumin [62].

2.2 Parenteral Applications

For parenteral delivery, submicron-sized niosomes are employed through I.p. or i.m. administration of up to 10 microns. Florence and Cable created 59 Fe-deferoxamine trioxyethylene cholesterol vesicles for intravenous injection and discovered that vesicle size affects distribution, as indicated by enhanced distribution in the liver and spleen [63]. Uchegbu et al. examined the effect of dosage on drug concentration in the plasma. The findings demonstrated that dose affects

plasma drug concentration [64–66]. Niosomes assist in increasing the concentration of a drug in circulation. They also performed toxicity studies and discovered that dosage and toxicity had a beneficial connection [64–66]. However, Florence and Cable discovered that intravenously administered doxorubicin in the form of niosomes reduces cardiac toxicity [63].

2.3 Peroral Applications

In a study utilizing 100 nm methotrexate C16G3 niosomes, Azmin and colleagues demonstrated the oral use of niosomal formulations [67]. Significantly higher levels of methotrexate were found in the blood, liver, and brain of PKW mice after orally administrating the niosomal formulation [67]. As a result, it appears that certain niosomal formulations improve medication absorption [67]. Rentel et al. (1996) used two distinct types of surfactants to make niosome-based ovalbumin vaccines, which they then gave to mice p.o [68]. The antibody titer of niosome-based vaccinations was higher than that of conventional vaccines [68]. The type of surfactant, on the other hand, did not affect antibody formation [68, 69]. Drugs are administered by this route to address issues such as acid and digestive enzymes in the stomach and small intestine, poor absorption, and variable bioavailability. As a result, reliable and innovative medications, such as niosomes, were used to increase the bioavailability of drugs [68, 69]. Ganciclovir could be delivered orally using a niosome formulated using Span 40, Span 60, and cholesterol. In vivo experiments conducted in rats reveal a significant increase in medication bioavailability after oral administration of an optimized formulation in comparison with a tablet dosage form [70].

2.4 Ocular Delivery

It is challenging to attain higher bioavailability from traditional ocular doses like ointment, suspension, and ophthalmic solution, because of corneal epithelial impermeability and precorneal tear film barriers which hinder drug absorption [71]. Various ocular medication delivery systems, such as niosomes, have been investigated to alleviate the problems associated with standard eye formulations. Since the size of the noisome is big enough to survive draining by reflex tearing and eye blinking, and because the structure of the niosomes is such that it remains on the eye surface, niosomes are favored for ocular medicine delivery [72]. Niosome formulations containing tacrolimus [73] and gatifloxacin [74] have been tested for ocular drug delivery. The biological assessment of a niosomal cyclopentolate delivery system for ophthalmic distribution was published by Saettone et al. [75]. The niosome formulations contained polysorbate 20 and cholesterol [75]. Within these niosomes, cyclopentolate was found to permeate the cornea in a pH-dependent way [75]. The result shown to be the best pH 5.5 was for peak permeation values [75]. In vivo

findings, however, showed that the niosomal formulation improved mydriatic response regardless of pH [75]. In summary, changing permeability features of the conjunctival and scleral membranes may be the cause of enhanced cyclopentolate absorption. Niosomes larger than 10 microns can be used to provide drugs to the eyes [75].

2.5 Pulmonary Delivery

In recent years, researchers have started studying drug delivery via the respiratory system since it enables direct targeting of the drug to the lung for both systemic and local therapies. Due to the high permeability and large surface areas of the alveolar region, the concept of the pulmonary pathway seemed to have sparked experts' interest. Regardless of the potential benefits of pulmonary administration, there are more than a few disadvantages, such as limited inhalation systems, decreased drug exposure per puff, and drug formulation constancy. Niosomes may be used to address these difficulties, resulting in more efficient drug delivery to the respiratory system. To date, physicians have recommended a variety of niosome formulations for pulmonary drug delivery [76, 77]. The anti-asthma steroid beclomethasone dipropionate proniosomes were formulated to produce niosomes that could be aerosolized using either an air-jet or a vibrating mesh nebulizer [78].

2.6 Cancer Therapy

Anticancer medicines could benefit from new novel drug delivery systems that boost bioavailability and reduce drug degradation and adverse side effects. Niosomes have long been known to be efficient carriers of genetic material. The majority of anticancer drugs have a slew of negative side effects. Niosomes can minimize pharmacological side effects by altering metabolism (prolonging circulation and half-life of drugs). If niosomes carrying anticancer drugs are formed successfully, they should be able to aggregate efficaciously within the tumors. One of the many anticancer drugs such as paclitaxel (PCT) has been successfully entrapped in many noisome formulations. Niosomes containing optimized ratios of Span 40, cholesterol, and diacetyl phosphate increased PCT stability and entrapment efficiency against gastrointestinal enzymes. The delayed release found in these formulations might be beneficial in lowering PCT's harmful side effects [79]. Niosomes encapsulating cisplatin efficiently averted loss of weight and lowered bone marrow toxicity when compared with free medicines [80]. Niosomes encapsulating methotrexate are engineered to have a significantly greater area exposure compared to methotrexate solution [81].

Paolino et al. developed bola-niosomes, which contain 5-fluorouracil, to treat skin cancer [82]. The transport greatly increased medication penetration when

compared to an aqueous solution of 5-fluorouracil [82]. Rogerson and associates investigated the niosomal delivery of doxorubicin to mice having S-180 tumors, which is commonly employed in combination chemotherapy and is classified as an anthracycline antibiotic [83]. After just one injection, doxorubicin bolus injection into the tail vein of mice boosts drug concentration in S180 sarcoma [83]. After the first peaks, the levels of niosomal-DOX created by surfactant alone were slightly higher than those generated by pure drug administration, but only to a significant degree following treatment in cholesterol-containing niosomes [83]. The enhancement in drug concentration in plasma is attributable to the fact that niosome entrapment and sluggish release are more likely than vesicle entrapment and accumulation in the liver [83]. However, recent research in mice demonstrated that niosomes may be gobbled up by the reticuloendothelial system, and methotrexate was deposited in the liver after niosome administration [67]. In one study, niosomal formulations based on dodecyl glucuronamide surfactant were employed to entrap doxorubicin and 5-fluorouracil as model medicines, and it was discovered that dodecyl glucuronamide surfactant could create niosomes with or without cholesterol [84]. To analyze the physicochemical properties of a medicine, Tavano et al. used magnetized niosomes to transport doxorubicin. This study implemented the surfactants Tween 60 and Pluronic L64 to show that magnetized noisome formulations have been consistent over time and performed a controlled drug release [85]. Tamoxifen citrate (TMC) niosomes were utilized to treat localized cancer therapy based on in vitro breast cancer cytotoxicity and in vivo solid antitumor efficacy. In comparison to the free medication, niosomal TMC demonstrated a higher reduction in tumor size in the in vivo experiment [86].

2.7 Immunological Applications

Immune niosomes are vesicles that have antibodies linked to their surfaces which operate as a potent adjuvant with great immunologic specificity, minimal toxicity, and long-term stability. According to in vivo investigations, niosomes encapsulating *Leishmania major* (ALM) showed a modest impact in the anticipation of cutaneous leishmaniasis in BALB/c mice [87]. The immune-stimulating efficacy of niosomes made from Span 85/cholesterol for encapsulating DNA encoding and HBsAg has been examined by topical treatment of niosomes in Balb/c mice. The results were compared to both bare DNA and DNA contained in liposomes [88]. Pardakhty et al. studied sorbitan esters, cholesterol, and CTAB in a series of positively charged micron-sized niosomal formulations for the trapping of autoclaved *Leishmania major* [89].

2.8 Delivery of Peptide Drugs/Peptide Drug Administration

Peptide drugs are usually challenging to administer orally due to issues such as gastrointestinal enzyme digestion. The ability of niosomes to protect this class of medications against breakdown has been demonstrated in several investigations. PEG 6000, Span 80, and Tween 80 were used in one investigation to make a niosomal carrier for hemoglobin (Hb) [90]. Their findings reveal that Hb can be adsorbed and disseminated on the noisome membrane surface, where it can be stabilized [90]. Researchers created insulin encapsulated in niosomes that was stable in bile salt solutions. The findings of this study suggested that niosomes could be used as oral insulin carriers since they could successfully prolong insulin release in both simulated gastric fluid and simulated intestinal fluid [91]. According to the researchers, the insulin was encapsulated in niosomes and was stable in bile salt solutions. The outcomes of this study revealed that niosomes can be utilized as oral insulin carriers since they successfully prolonged insulin response in both simulated gastric and intestinal fluid.

2.9 Radiopharmaceuticals

Erdogan et al., in 1996, were the first to use niosomes as radiopharmaceuticals. They made positively charge 131I-labeled iopromide niosomes to improve contrast during CT in rats [92]. Gel and liquid crystal compositions were used. They were identified in greater numbers in the kidneys and remained active for more than a day [93]. 99mTc-labeled DTPA-containing niosomes in another study and discovered that considerable quantities of DTPA were collected in the liver and spleen. 99mTc-DTPA niosomes produced improved gamma scintigraphic pictures in mice. Similarly, 99mTc-labeled DMSA niosomes accumulated in the liver, kidneys, and spleen of mice for 24 h and maintained activity. In addition, because noisome formulations become less vulnerable to light, temperature, and oxidation than traditional DMSA solutions, they provide superior stability. Iobitridol, a diagnostic chemical used in X-ray imaging, is carried by niosomes. Polyoxyethylene glycol 4000 stearate, sorbitan monostearate, cholesterol, D-alpha tocopherol polyethylene glycol 1000 succinate, and dicetyl phosphate were used to create these niosomes [94].

Luciani et al. examined a magnetic resonance (MR) imaging contrast dye for tumor identification based on a mixture of PEG (polyethylene glycol) and glucose conjugates to the surface of niosomes for the targeting of overexpressed glucose receptors [95]. In a human carcinoma xenograft model, they discovered that niosomal system talked about earlier dramatically enhanced tumor targeting of an encapsulated paramagnetic drug tested by MR imaging [95].

2.10 Niosomes as DNA Targeted Drug Delivery

Non-ionic surfactant vesicles (NSVs), niosomes, are the hydrated lipids that consist mainly of various classes of nonionic surfactants, familiarized in the 1970s as a cosmetic vehicle. Currently, numerous researchers are using niosomes as major novel drug delivery methods, as well as useful immuno-adjuvants in which certain commercial versions are available on the marketplace. Later, these vesicles are utilized as vectors for gene transfer.

2.11 Niosomes as Gene Delivery Vectors

As bilayer vesicles are decomposable, lesser immunogenic, and less toxic and can activate lower levels of a complement as compared to the viral vectors; hence, using this type of gene carrier is more suitable and harmless than the viral vectors. Sorbitan monoesters cationic niosomes were used by Huang et al. (2005), for the administration of antisense oligonucleotides (OND) using COS-7 cell line in which Span 40 and span 60 vesicles demonstrated a more substantial effect [96]. Generally, positively charged particulate matter is more susceptible to nonspecific interactions through plasma proteins that may result in dissociation, destabilization, and speedy clearance of gene/carrier complexes [97]. Huang Y et al. (2008) proposed using PEGylated cationic niosomes to create an efficient non-phospholipid vesicular gene delivery vector [98]. They employed DSPE-mPEG 2000 for PEGylation of cationic liposomes in which resultant OND-vesicle complexes presented a neutral zeta potential having a particle size of about 300 nm [98]. The discovered complexes have low serum protein binding affinity and aggregate stability in serum [98]. Alternatively, the PEGylated niosomes presented more efficiency toward OND cellular uptake in serum on comparing with cationic niosomes. Manosroi et al. reported a new rising problem that there was lower stability of luciferase plasmid (pLuc) loaded either in Span 60 or Tween 61/dimethyl dioctadecyl ammonium bromide (DDAB)/CHOL than to cationic liposomes [99]. However, DDAB/Tween 61/CHOL nanovesicles were reported as a potential cationic vector for pLuc delivery ensuing the use of iontophoresis on the stratum corneum of rat skin [100]. These researchers reported lately the fruitful gene expression, transdermal absorption, and stability of tyrosinase plasmid (pMEL34)-loaded DDAB/Tween 61/CHOL nanovesicles as a challenging topical delivery for vitiligo therapy [101]. Scientists also effectively articulated a human tyrosinase plasmid (pAH7/Tyr) and increased melanin synthesis by knocking out the tyrosinase gene in human melanoma (M5) cells and tyrosine-producing mouse melanoma (B16F10) cells utilizing plasmid loaded in elastic cationic niosomes [102].

2.12 Niosomes in Vaccine Delivery

2.12.1 Niosomes in Protein Subunit Vaccines

The major goal of many researchers throughout the world is to create unique, safe, and effective vaccinations. Although due to very limited effectiveness, DNA or subunit protein from many species is safer to administer than live attenuated vaccines. Adjuvanted systems have been shown to improve the immunogenicity of these subunit vaccines by preventing antigen degradation and enhancing antigens targeting professional antigen-presenting cells [103]. Niosome antigen delivery for vaccination was first applied by Brewer and Alexander against bovine serum albumin (BSA) [87]. They reported that niosomes were effectively improved stimulators for the Th1 lymphocyte subset and potent stimulators for cellular insusceptibility [87]. According to Hassan et al., there is better immunogenicity in mice for herpes simplex virus 1 antigen-loaded l-mono palmitoyl (MP) glycerol/CHOL/DCP niosomes [104]. Furthermore, a challenging infection offered for mice by HSV-2 antigen-loaded niosomes (80) indicated the efficacy of the technique and composition of niosomal formulations for partial protection against type 2 herpes simplex virus HSV-2 [104]. Span/CHOL/DCP niosomes were fabricated by Yoshioka et al. (1995), consisting of a tetanus toxoid (TT) in an external oil phase. Cottonseed oil, when utilized as an external oil phase, demonstrated more immune activity than unbound antigen/vesicles [105]. Murdan S et al. (1999) reported the application of encapsulated BSA or haemagglutinin (HA) in the case of v/w/o emulsion. From the immunogenicity studies, it was observed that water-in-oil (w/o) gel and v/w/o gel both as control showed immunoadjuvant properties and can accelerate both the primary and secondary antibody titers, i.e., total IgG1, IgG, IgG2b, and IgG2a, to HA antigen [106]. According to Chambers MA et al. (2004), a subcutaneous dose of the killed *Mycobacterium bovis* BCG vaccine in Brij® 52-based nano-niosomes (Novasome ™) can protect guinea pigs from tuberculosis [107]. According to Vangala et al. (2007), DDA niosome formulations containing TDB demonstrated markedly enhanced proliferation of hepatitis B surface antigen-specific splenocytes and encouraged cytokine making in relation to a durable T-cell-driven response, representing the benefit of the use of the formulations for evaluation of their clinical effects [108].

2.12.2 DNA Vaccines

DNA encapsulation in liposomes may be owing to the biological milieu shielding genetic material, supporting improved humoral and cell-facilitated resistance responses against the encoded antigen in vaccinated mice [109]. The ability of niosome to deliver by topical [88], parenteral [110], and oral administration makes them novel nontoxic and active vaccine delivery tools. According to Perrie et al., subcutaneous injection of a nucleoprotein containing plasmid of the H3N2

influenza virus in niosomes led to improved vaccination of treated mice as compared to bare DNA [110]. Encapsulating plasmid pRc/CMV-HBs(S) expressed coding sequence for the minor proteins of hepatitis B virus, HBsAg, in mannosylated niosomes indicated the effectiveness of the formulations as DNA vaccine carrier as well as an adjuvant for beneficial oral immunization of hepatitis B [111]. Vyas et al. fabricated niosomes of Span 85/CHOL encapsulating DNA encoded with HBsAg for topical application in Balb/c mice. An increase in the level of serum anti-HBsAg titer, as well as cytokines level (IL-2 and IFN-γ), specified the efficiency of its use as topical delivery of the vesicular vaccine [88].

3 Conclusions

The road from the discovery of therapeutic applications for liposomes to their recognition as a conventional drug delivery method has been long and winding, spanning more than four decades. Liposomes, with both "traditional" and "stealthing," have become ubiquitous as effective drug delivery systems for everything from small molecule therapies to nucleic acids in vivo. The formulations have undergone clinical trials for many applications, including imaging tumors and infection sites, administering vaccines and gene therapy, curing infections and cancer, and controlling respiratory illness and dermal problems. Liposomal medications have been demonstrated to be the most beneficial in clinical applications due to their capacity to "inactively" accumulate at locations of higher vascular permeability and to lessen the negative effects of encapsulated pharmaceuticals when compared to free drugs. The therapeutic index and bioavailability improvements have favored reduced toxicity over increased effectiveness. When compared to free unentrapped medicines, liposomes exhibit poor extravasation into tissues with tight endothelial connections, which can lead to a large reduction in adverse effects. When free doxorubicin is encapsulated in liposomes, the drug's irreversible cardiotoxicity is significantly reduced. In a limited investigation with a limited group of patients, PEGylated is also known as long-circulating liposomes that were found to accumulate extensively in Kaposi's sarcoma and head and neck tumors, with lung cancer showing intermediate accumulation and breast cancer showing less accumulation.

For transdermal, parenteral, oral, and ophthalmic administration, niosomes have been proved to be excellent controlled drug delivery systems. They can incorporate anti-infective, anticancer, anti-inflammatory, and, more recently, vaccine adjuvants. Niosomes have the potential to be used as diagnostic imaging agents by allowing them to target specific parts of mammalian animals. When it comes to stability, toxicity, and cost-effectiveness, niosomes outperform other carriers. Although numerous unique approaches have been developed to address the issue of drug loading, it is still critical to increase encapsulation efficiencies to maintain the biological potential of formulations. Surfactants with a greater phase transition should be employed because they have better permeability and toxicity profiles and have the largest influence on vesicle formation, toxicity, and stability. Niosomal applications

can be delivered via transdermal, peroral, parenteral, and ocular methods. Recently, the use of niosomes as vaccines and radiodiagnostic agents has been evaluated and shown to be a promising application area. Because niosomes can encapsulate both hydrophobic and hydrophilic medicines, selecting an appropriate niosome delivery medication should consider this.

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Dendrimers as Targeted Systems for Selective Gene and Drug Delivery

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1 Introduction

Dendrimers are considered as novel drug delivery systems. For the last 15 years, this type of branched architectural nanosystems has been studied and mainly focuses on design parameters such as shape, architecture, size, elemental composition, surface chemistry, and flexibility [1]. Dendrimers have been proposed as a platform for vaccines, for delivering drugs or genes.

In the field of therapeutics, these three-dimensional polymers have demonstrated potential as intratumoral or intracellular delivery vectors. They have also been widely studied as targeted and controlled systems for delivering anticancer agents, nucleic acids, or antiviral drugs. Drugs encapsulated in dendrimers include anti-inflammatory, antiviral, nonsteroidal, or antimicrobial compounds. The most studied dendrimers are poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI)-based.

In dendrimer chemistry, click and orthogonal chemistries have been useful tools for their synthesis. Although dendrimers have been used to contain drugs inside, many approaches fail to fulfill their specific function or their response is very limited since only part of them reaches the site of interest. Effective targeted drug delivery systems require the following properties: drug *retention*, immune system *evasion*, specific *targeting*, and selective cargo *release*. The most explored

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application of dendrimers is targeted cancer therapy, particularly in the use of combined therapies, where dendrimers' multifunctional properties can potentially improve therapeutic responses [2].

Dendrimer-based nanoapproaches are also promising for clinical translation. However, enabling them more functionalities implies more challenges for their chemical and/or biological characterization.

Since dendrimers exemplify one of the most studied scaffolds for anticancer drugs, this chapter focuses on reviewing dendrimer-based systems developed for modalities that combine controlled and sustained drug or gene delivery for biomedical applications in anticancer therapies.

2 Chemical Properties

Dendrimers are highly branched three-dimensional polymers, similar to a tree arrangement. These are monodispersed, with nanometric size (1–100 nm) and well-defined structure and molecular weight [3]. The general architecture of dendrimers (Fig. 1) shows three main components: (a) inner core, composed of a central atom or group of atoms, (b) inner layers, consisting of repeating branching units, and (c) multiple external moieties attached to the branches, which play a determining role in their physicochemical and biological properties. Their shape, size, and surface charge can be modified by the chemical nature and level of the repeating units.

The functional entity of the dendrimer is called "dendron" and multiple molecules can be attached to their surface or trapped on its branches.

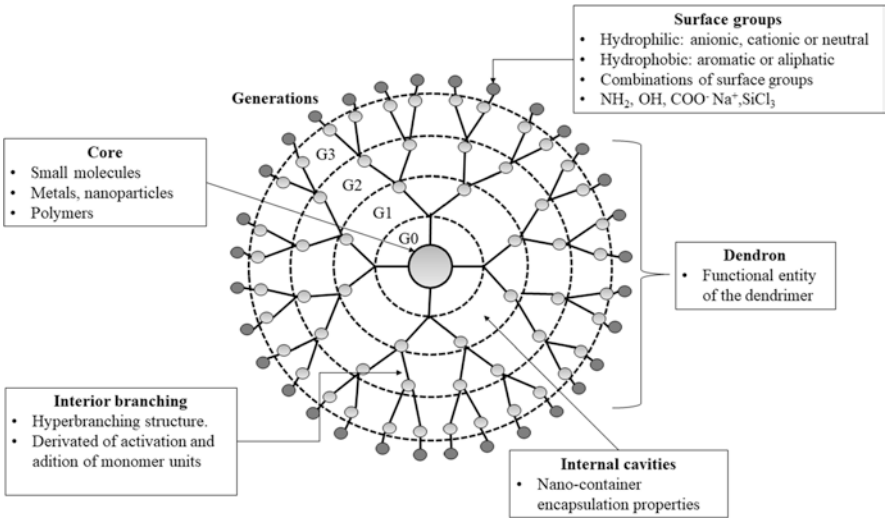

Fig. 1 Schematic representation of dendrimers

As the size of the dendrimers increases, they adopt a spheroidal structure to minimize repulsion between their branches. The dendrimer properties differ from their linear analog and are influenced by the exterior functional group. The superficial groups can be selected or modified to impart distinct characteristics to the dendrimer structure, such as solubility, lipophilic or hydrophilic properties, miscibility, and acidity [4].

In general, there are two main strategies for dendrimer synthesis: divergent and convergent. The synthesis in the divergent method begins from the central core to the periphery. This growth process starts with nucleus activation and the first monomeric unit linkage; subsequently, growth depends on the repetition of two essential steps, namely, (a) activation of the monomer end group and (b) coupling of new monomers, until the acquisition of the desired dendrimer. The addition of a new generation of branches requires that each step of the reaction be fully completed allowing the synthesis of highly symmetric dendrimer molecules. This method is not a limitation to search for dendrimers with different types of peripheral groups. The divergent process allows symmetric internal branches but is possible to incorporate heterogeneous terminal monomers for better applications [5, 6].

On the other hand, the convergent approach does not start from the core but from what will make up the external structure of the dendrimer. It also requires repetitive steps of activation and incorporation of new structures. The growth process starts when the peripheral groups bind a monomer unit. Posteriorly, this fragment is activated and the reaction with other monomers is promoted to produce the initial dendron. The activation reaction and monomer addition can be repeated until reaching specific dendrons and, finally, coupling with the inner core, to provide the expected dendrimer structure [6].

Compared to the divergent method, the convergent method yields dendrimers of higher purity due to fewer coupling reactions at each growth step. However, steric hindrance makes it difficult for the dendrimer arms to react with the inner core. Synthesis of symmetric and asymmetric dendrimers is possible because of the capability of controlling the addition of homogeneous or heterogeneous dendrons, providing several active sites and versatile dendrimers for different applications.

In both methods, specific and successive reaction steps allow the incorporation of new branches, and the number of repeated steps or a new level of branches is called dendrimer generation (G). Normally, the first dendrimer-core activation and modification is called generation 0, and there are intermediate generations, beginning with G0.5, until achieving the most common generations, such as G1 to G10.

An increase in G produces an increase in dendrimer volume and confers greater density and the number of terminal groups. For example, lower-generation dendrimers possess more open structures and, occasionally, an asymmetric shape. However, as the dendrimer generation increases, the dendrimers become globular and densely packed structures. When hyperbranching becomes critical, dendrimers cannot grow uniformly because of steric impediments.

The physicochemical properties such as solubility, miscibility, glass transition temperature, and chemical reactivity are mainly influenced by the features of the peripheral chemical motifs and not by their core or branches. Dendrimer solubility

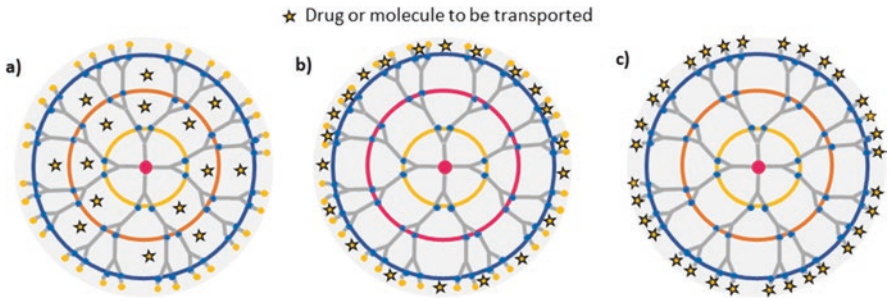

Fig. 2 Representation of possible ways of loading molecules in the dendrimer. (**a**) encapsulation in internal cavities, (**b**) electrostatic interactions, and (**c**) covalent bonding

also varies with the change of superficial groups. Hydrophilic superficial groups allow high solubility in polar solvents, whereas the hydrophobic groups are responsible for the solubility in nonpolar solvents. Additionally, dendrimers show some improved physicochemical properties compared to analogous linear polymers. For example, dendrimer solutions have a lower viscosity than the corresponding linear polymer [6].

Dendrimers exhibit cytotoxicity dependent on generation, charge, and concentration. In general, toxicity increases in high generations, and cationic structures exhibit high toxicity levels compared to neutral or anionic dendrimers. To reduce the toxicity levels, the selection of neutral or anionic biocompatible dendrimers and their surface modification with more biocompatible groups have been explored [7].

Therefore, the specific control over dendrimer architectures, such as shape, the presence of internal cavities, and specific peripheral groups, makes dendrimers an ideal pharmaceutical per se, or as ideal carriers of drugs, metals, and imaging moieties, among others, for biomedical application [6, 8]. Dendrimers can load and store diverse molecules by encapsulation of molecules into their cavities, absorption on the surface by electrostatic interactions, or conjugation (through covalent bonding) with the surface groups (Fig. 2).

Among the most-studied dendrimer families, there are the poly(amidoamine) (PAMAM, the first synthesized dendrimers), poly(propylene imine) (PPI), polyether-copolyester (PEPE), poly-L-lysine (PLL), polyester, triazine, polyether, citric acid, phosphorous, peptide dendrimers, and PEGylated dendrimers, which garner a wide interest for their innumerable potential applications in the medical field [9].

3 Biological Properties and Uptake Mechanisms

Dendrimers have proven to be molecules with good biocompatibility that can be used as therapeutic agent carriers so that they represent promising vectors for different biological and pharmaceutical applications. Thus, the dendrimers must be nontoxic and must not activate the immune system [10].

3.1 Cytotoxicity

The factors that determine the cytotoxicity of dendrimers depend on their generation, charge, and type of functional groups on their exterior. Toxicity levels increase as dendrimer along with the generation. The amine type and substitution level give them different peripheral charges (positive, negative, or neutral charges) that interact differently with the biological membrane. Positively charged dendrimers show higher toxicity when compared with that neutral or anionic dendrimers, and this is because the positive charges interact with the lipid bilayer, weakening membrane integrity, and increased permeability, with the consequent cell lysis. Different studies have shown that dendrimers possessing surface amine groups such as PAMAM and PPI are characterized by higher toxicity (generation- and concentration-dependent) in contrast with grafted carbosilane-poly(ethylene oxide) dendrimers and other dendrimers with anionic or neutral surface groups [9, 10]. PAMAM dendrimers with amino groups on the surface have lower cytotoxicity compared to the linear amino-terminated polymer, causing hemolysis, due to the cationic superficial groups on the PAMAM [10, 11]. Similar to PAMAM with amino-terminal groups, PPI dendrimers show hemolytic effects and generation-dependent cytotoxicity [12].

One way to improve dendrimers' biocompatibility and reduce toxicity is through surface modification of the conjugated dendrimer with functional moieties such as targeting ligands, imaging agents, drugs, and radionuclides. Generally, PAMAM, PPI, and PEI (polyethyleneimine) dendrimers possess modified positively charged groups on their surface with anionic or neutral residues to reduce their toxicity and hepatic accumulation. This is why PAMAM dendrimers show differences in toxicity when functionalized with hydrophobic end groups, polyethylene glycol (PEG), OH, and pyrrolidine [13, 14].

3.2 Cellular Internalization and Action on the Mononuclear Phagocyte System

Like cytotoxicity, dendrimer cellular uptake mechanisms vary considerably due to generation, functionalization, surface charge, concentration, and cell type. Dendrimers can pass through the cell membrane via an energy-independent process, but the main internalization mechanism is endocytosis, an energy-dependent process [13, 14]. There are different endocytosis mechanisms; the main is shown in Fig. 3.

Phagocytosis is the uptake of particulate with a size range of micrometer by vesicles inside the cell, and it is a mechanism rarely used by dendrimers. Pinocytosis consists of the uptake of fluids by vesicles smaller than those present in phagocytosis. This internalization mechanism is classified into macropinocytosis and receptor-mediated endocytosis.

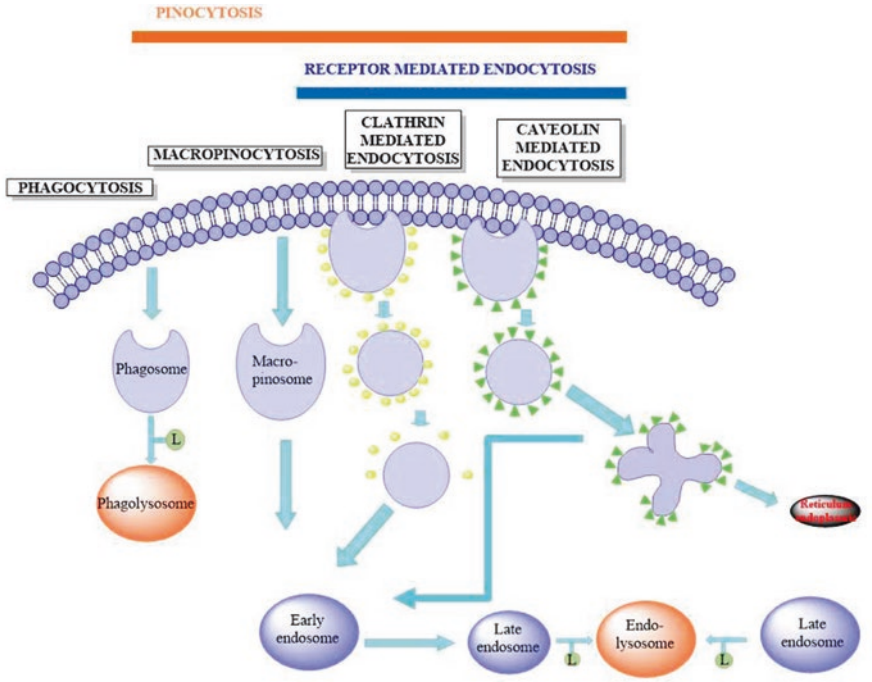

Fig. 3 Endocytosis mechanisms. Macropinocytosis, clathrin-mediated endocytosis (CME), and caveolin-mediated endocytosis (CVME) are the main receptor-mediated endocytosis (RME) mechanisms

- Macropinocytosis involves the uptake by vacuoles called macropinosomes. After the internalization of macropinosomes, there is a decrease in pH, and endosomal markers appear. Finally, macropinosomes can fuse with late endosomes, with lysosomes, or recycle their cargo to the membrane [15].
- Receptor-mediated endocytosis, the most frequent pathway for nanoparticle internalization, can be through the following:
  - Clathrin-mediated endocytosis (CME) occurs in regions where clathrin is recruited in the plasma membrane. First, clathrin-coated endocytic vesicles (70–150 nm) are formed, as a response to the interaction of an agonist with its receptor. Then, the internalized vesicle loses its clathrin coat and fuses with other vesicles to form an early endosome, which becomes a late endosome that eventually fuses with a lysosome [16].
  - Caveolin-mediated endocytosis consists of invaginations (60–80 nm) of the plasma membrane, which incorporate extracellular fluid content. Proteins like caveolin-1 bind to cholesterol in lipid rafts, which do not dissociate from the vesicles after uptake. Caveolin vesicles are formed and fused with other caveolin vesicles, giving rise to multicaveolar structures called caveosomes, which fuse with early endosomes [17].

Table 1 Main endocytic pathways of PAMAM dendrimers considering factors such as size and charge

Dendrimer	Endocytic pathway	Generation	Charge	Ref
PAMAM-NH_2	CME CME and CVME	G4 (5–150 nm) G2	Positive Positive	[20–23]
PAMAM-OH	CVME	G4	Positive	[24]
PAMAM-COOH	CVME CME	G3.5 G1.5	Negative Negative	[24, 25]
PAMAM	CME- and CVME-independent	G4	Neutral	[24]

The pathway by which dendrimers enter the cell is broad and dependent on the type of cell and dendrimer. Concerning size, dendrimer internalization is greater in nonphagocytic cells with a size around 50 nm, while for a size between 150 and up to 200 nm, internalization has been observed mainly through CME or CVME, and above 250 nm and up to 3 μm showed better in vitro uptake through phagocytosis and macropinocytosis [18].

Regarding the generation of PAMAM dendrimers, it has been reported that dendrimers higher than the fourth generation show the highest internalization rate, due to their ability to form pores and eliminate lipids from the membranes, while dendrimers <G-5 are intercalated or adsorbed onto the membrane surface. The charge has also a large impact, as this behavior is governed by electrostatic interactions between charged dendrimeric structures and the lipidic membranes, which give rise to greater membrane disruption compared to uncharged dendrimers [13, 14].

- Chemical modification can influence the uptake pathway. Authors have reported that amino-ended PAMAM dendrimers (cationic) stimulate effective cellular uptake by endocytosis and through membrane pore formation [13, 14, 17, 19] (Table 1).

4 Dendrimers for Imaging

Molecular imaging has provided the opportunity for obtaining high-resolution physiological information. Molecular imaging modalities comprise positron emission tomography (PET), single-photon emission computed tomography (SPECT), optical tomography imaging, computed tomography (CT), and magnetic resonance imaging (MRI) [26].

Molecular images are acquired from fluorescent molecules, γ-emitting radionuclides, or metal oxides. Radiolabeled imaging probes allow their noninvasive in vivo biodistribution and pharmacokinetics monitoring by the PET or SPECT nuclear modalities [27]. Dendrimers can be radiolabeled with diagnostic, therapeutic, or theranostic isotopes through several strategies, for example, using bifunctional chelating agents [28]. Technetium-99 m (^{99m}Tc) and Indium-111 (^{111}In) are the most

common SPECT radionuclides employed for this aim, while for PET applications, Gallium-68 (^{68}Ga) and Copper-64 (^{64}Cu) are the most-studied radionuclides [29].

4.1 Dendrimers for PET Imaging

Targeted dendrimers have been synthesized and successfully labeled with ^{64}Cu to obtain PET images of tumors overexpressing receptors (Fig. 4). G5 PAMAM dendrimers conjugated to folic acid (FA) and labeled with ^{64}Cu-DOTA show specific recognition of cancer cells and tumor xenografts that overexpress folate receptors. Since the DOTA chelator is suitable for radioactive labeling with other radiometals, these dendrimers can also be employed for SPECT nuclear imaging and radiotherapy with yttrium-90 or lutetium-177 [30]. Furthermore, dendrimers can penetrate the tumor via the enhanced permeability and retention (EPR) effect, due to dysfunctional vascularization and lymphatic drainage in the tumors. The EPR also called the "passive effect of the tumor target" enables the imaging agent for increasing its concentration in the tumor, improving in this way the resolution and sensitivity of the image [31]. Another example of an imaging system based on dendrimers is the triazine dendrimer functionalized with DOTA, which presents the characteristic of reacting with the urea-based ligand (DUPA). DUPA has an affinity for the prostate-specific membrane antigen (PSMA). Targeted uptake of ^{64}Cu-labeled dendrimers, G1-(DUPA)64 and G5-(DUPA)64, was systematically assessed using positive PSMA PC3-PIP and negative PSMA PC3-FLU cell lines. From these studies, the G1-(DUPA)64 showed the highest uptake for PC3-PIP, while G5-(DUPA)64 exhibited the highest affinity for PSMA. PET studies showed that nontargeted uptake increased as a function of the size, despite the good multivalence of larger dendrimers [32].

4.2 Dendrimers for SPECT Imaging

To acquire SPECT images, G5 PAMAM dendrimers, terminated by an amine (G5 NH_2), have been modified using 3-(4′-hydroxyphenyl)propionic-OSu acid and folic acid (FA), coupled to polyethyleneglycol. Additionally, these dendrimers were modified through acetylation of the remaining surface amine motifs and radiolabeled with iodine-131 (^{131}I). Multifunctional NHAc-HPAO-PEG-FA G-5 dendrimers radiolabeled with ^{131}I were also reported as effective approaches for targeted SPECT imaging and concomitant therapy of a xenograft tumor model overexpressing folate receptors [33]. In addition to being excellent platforms for SPECT images, dendrimers are also used for SPECT-CT imaging applied to post-chemotherapy evaluation on cancer cell apoptosis. These platforms are PAMAM dendrimers (G-5) loaded with gold nanoparticles, conjugated to DOTA, PEG-duramycin, fluorescein isothiocyanate, and radiolabeled with ^{99m}Tc. In vivo micro-CT imaging data

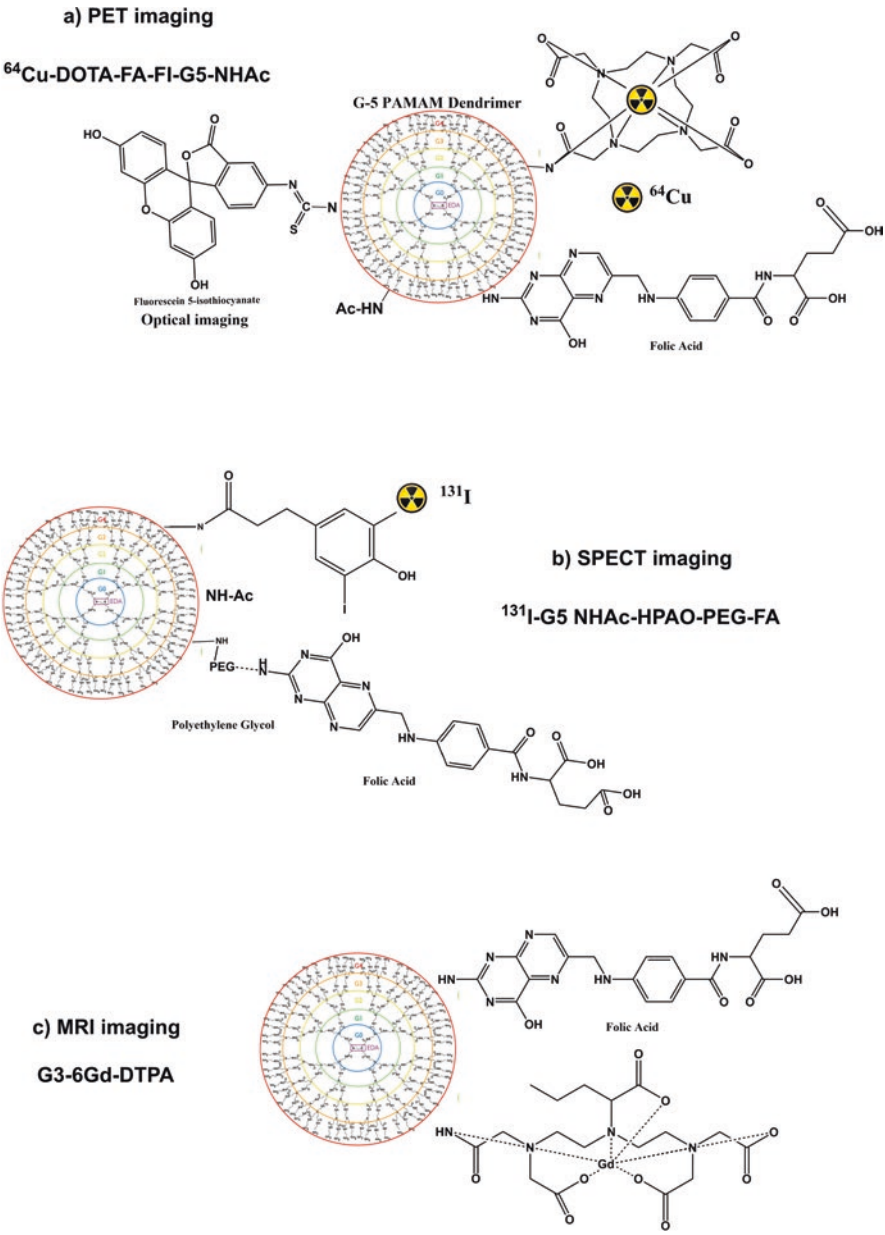

Fig. 4 Examples of dendrimer-based nanosystems for imaging. (**a**) G5 PAMAM dendrimers conjugated to folic acid (FA) and labeled with ^{64}Cu-DOTA for PET imaging. (**b**) G5 PAMAM dendrimers, terminated by an amine (G5 NH_2) modified with 3-(4′ hydroxyphenyl)propionic-OSu acid (HPAO) and FA, coupled to PEG, modified by acetylation of the remaining dendrimer surface amines and labeled with radioactive iodine-131 for SPECT. (**c**) Folate receptor (FR)-targeted dendrimer, PEG-G3-(gadolinium-DTPA)11-(folate)5 for magnetic resonance imaging

indicates that these dendrimers can be used to obtain specific CT images of the premature tumor reaction to treatment [34]. The ^{99m}Tc-PAMAM-Tyr^3-Octreotide multimeric nanosystem was designed for neuroendocrine tumor imaging. The [Tyr^3-Lys(Boc)^5]-octreotide was conjugated to the –COO$^-$ motifs on the surface of PAMAM dendrimer (G3.5). The radioactivity distribution on tissue 2 h after ^{99m}Tc-PAMAM-Tyr^3-Octreotide administration showed specific tumor uptake of 4.12 ± 0.57%ID/g and high pancreas uptake which overexpresses high density of somatostatin receptors. The dendrimer peptide displayed suitable features to be used as a target imaging agent for tumors overexpressing somatostatin receptors [35].

4.3 Dendrimers for CT Imaging

Dendrimers are an essential and stable nanoparticle system for developing contrast agents for X-ray medical imaging [36]. Gold nanoparticles, encapsulated in dendrimers, are an example of contrast agents since gold nanoparticles exhibit high X-ray attenuation in comparison with conventional contrast agents based on molecular iodine [37]. For improvement of CT imaging, dendrimers with gold nanoparticles and conjugated to an iodinated small molecule can be prepared. These two radio-dense gold and iodine components enhance the sensitivity of CT images [38]. Moreover, to provide specificity to contrast agents based on nanoparticles, ligands (e.g., FA, Arg-Gly-Asp (RGD) sequence, and lactobionic acid) can bind covalently to a dendrimeric structure. For example, FA has been bound to the surface of dendrimers, with entrapped gold nanoparticles, through a 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride coupling reaction for CT imaging of human cancers that overexpress folate receptor [39]. Moreover, for dual CT and magnetic resonance imaging (MRI) multifunctional tecto dendrimers, encapsulating gold nanoparticles, have been designed. Gold nanoparticles are encapsulated in β-cyclodextrin-modified G5 PAMAM dendrimers to obtain a dual probe. Dendrimers were sequentially modified with RGD peptide via a PEG spacer, gadolinium chelator, and 1,2-propane sultone, followed by chelation of Gd (III) ions. These dual systems, based on dendrimers, show suitable colloidal stability, high X-ray attenuation, relaxivity, suitable anti-fouling properties, and good cytocompatibility. In addition, these nanoparticles target cancer cells that overexpress the integrin αvβ3, due to their functionalization with RGD [40].

4.4 Dendrimers for Magnetic Resonance Imaging

Dendrimers have been shown to significantly affect magnetic resonance relaxivities and physiological properties of magnetic nanoparticles. Therefore, dendrimer magnetic nanoparticle agents are desired to supply sharper images with physiologically

relevant contrast, longer retention times in blood, and specific organ uptake. Fine-tuning the size and functionalities of the final group of dendrimers supply an additional advantage in this regard (Fig. 4) [41]. For developed injectable contrast agents in MRI, dendrimers have been used to incorporate gadolinium chelates, including paramagnetic iron oxide particles (*magneto-dendrimers*), to label and track cells [42]. Dendrimers act as a central platform for the transport of small molecules such as Gd ions or Gd (III) chelates for magnetic resonance contrast. Such molecules must be on the dendrimer surface to allow them to interact freely with water molecules. PEGylated dendrimers exhibit a much higher Gd(III) concentration in blood than other dendrimer formulations [43]. These dendrimers have been modified with folate or antibodies, poly-L-lysine, and PEG to improve the in vivo circulation time [42–44].

5 Targeted Drug Delivery Based on Dendrimers

Although dendrimers have been used to transport drugs, to increase their bioavailability and their active fraction, it has been shown that functionalization with biomolecules is the best strategy to improve the delivery to the tissues of interest. Dendrimers without functionalization are likely to be insufficient to produce significant clinical benefits. Therefore, special emphasis has been directed toward delivering cytotoxic drugs used as chemotherapeutic agents for cancer treatment [2]. Targeted drug delivery involves the transport of a therapeutic agent to a specific tissue without affecting healthy tissues in the body.

Effective targeted drug delivery systems need to be retained, evade healthy organs or tissues, target specific tissues, and be released in the selected regions [2]. The most-explored use of dendrimers is the targeted therapy of cancers. Besides, dendrimers have the capability of being grafted with two or more targeting biomolecules to enhance selectivity.

The targeted delivery of therapeutic agents can be systemic, through blood circulation and extravasation (ligand-receptor mediated), or intracellular (by self-triggered cargo delivery) [2]. Regarding the size of nanoparticles, their accumulation occurs faster when compared to larger molecules; larger molecules are retained longer inside the tumor but can also be diffused back into the systemic vascular bed.

Active targeting is generally achieved through biomolecules which improve the accumulation in cancer cells, and intracellular organelles, within the tumor or a tumor-bearing organ [45]. Retention, evasion, targeting, and release features should be considered when designing effective targeted drug delivery nanosystems [46].

Targeting cell-surface receptors enable nanoparticles to be internalized at the cell surface or into the microenvironment. When nanoparticles are decorated with tumor-targeting biomolecules, these nanoparticles can target cancer-specific receptors or the tumor microenvironment with great specificity. In this context, several approaches have shown synergistic effects when targeted drug delivery systems, based on dendrimers, are used. The drug inside dendrimers can be achieved through

encapsulation or conjugation of drugs by covalent bonds. Drugs encapsulated in dendrimers include nonsteroidal anti-inflammatory drugs and antiviral or antimicrobial compounds; however, the most-explored targeted drug delivery systems are for anticancer therapy. Several receptors are overexpressed in cancer. Therefore, anticancer drugs can be concentrated in target sites by encapsulation or conjugation of drugs to the dendrimer surface with biomolecules that possess affinity toward these receptors.

Different types of targeting biomolecules, such as RGD, FA, aptamers, and other biomolecules, have been used to functionalize dendrimers encapsulating or bound to cytotoxic agents, against several cancer types.

The camptothecin SN38 (7-ethyl-10-hydroxy-camptothecin), a replication and transcription inhibitor, was encapsulated in PAMAM dendrimers functionalized with BR2 and CyLoP1 cell-penetrating peptides. A family of synthesized derivatives was evaluated and demonstrated a significant tumor growth inhibition when they were compared against their commercial counterparts, on colon carcinoma cells [47].

For colon cancer, PAMAM dendrimers were functionalized to target laminin receptors through the YIGSR sequence peptide, and gemcitabine was encapsulated within these [48]. After exposure to treatment, the targeted nanoapproach was internalized to the cytoplasm and the nucleus of the HCT-116 cell line. The gemcitabine-loaded dendrimer showed greater mortality at 24 h when compared to normal fibroblasts.

Also, natural compounds like curcumin have been encapsulated within dendrimer structures to provide mitochondrial anticancer therapy for hepatocellular carcinoma through TPP (triphenylphosphonium) to target the mitochondria [49]. In this specific case, the curcumin and TPP were conjugated to the PAMAM structure. As a result, selective toxicity against cancer cells was observed.

5.1 Folate Receptors

The presence of FA on the dendrimer surface has demonstrated improvement of the specific therapeutic response. FA has been attached to PPI dendrimers, as carriers for methotrexate (MTX). The MTX was covalently bound to the dendrimer surface, by EDC activation, and in vitro and in vivo characterization was carried out [50].

Doxorubicin has been conjugated to PAMAM dendrimers (G.5) and functionalized with FA. The nanosystem was conjugated to fluorescein isothiocyanate for fluorescence microscopic imaging. This approach did not exhibit important toxicity. The NAHAc-FI-FA-DOX (G-5) demonstrated stability and showed a sustained-release profile. The nanosystem specifically targets folate receptors on KB cells, with the consequent high toxicity toward this cell line [51]. Recent nanoapproaches based on triazine dendrimers grafted with a photoluminescent FA derivative, which showed a pH-dependent DOX release, were proposed as a suitable photoluminescent nanoapproach antineoplastic drug delivery [52]. The stability of FA dendrimers

G3 and G4 was increased along with the size, and the loading efficacy was higher for doxorubicin when compared to tetracycline and tamoxifen [53].

Also, to monitor cells positive for folate receptors via imaging techniques, radionuclides have been attached too. These nanoapproaches will be studied in the "Imaging" section.

MTX has been encapsulated by or conjugated to PPI dendrimers targeted at folate receptors and evaluated at the preclinical stage in the MCF-7 breast cancer cell line [50].

Some reports have demonstrated that PEG formulations also exhibit enhanced responses against cancer cells when loaded with 5-fluoroacyl (5-FU) [54]; FA-conjugated PEG dendrimers loaded with 5-FU (31% encapsulation) showed important uptake with a sustained drug release profile and high uptake when compared to a non-PEGylated approach [55]. ^{99m}Tc was bound to PEG-PAMAM-FA, loaded with 5-FU, to provide specific therapy on MDA-MB-231 cells [56].

Molecular dynamics simulations have demonstrated that increasing the degree of PEGylation produces an improvement over the total 5-fluoroacyl loading capacity, while a 25% PEGylated system was proposed as the best choice for drug delivery procedures [46]. In the case of MTX release, the simulations suggest that a high PEGylation ratio limits MTX diffusion toward inner cavities, with the consequent decrease of MTX release [57].

5.2 Epidermal Growth Factor Receptor (EGFR)

To target EGFR, the specific sequence peptide ARSHVGYTGAR was conjugated to poly-lysine dendritic nanoplatforms, to deliver MTX for preclinical evaluation. The preliminary studies showed a suitable in vivo therapeutic efficacy [54]. EBP-1 (EGFR-binding peptide 1) was also used to functionalize PAMAM dendrimers loaded with DOX, which improved the antiproliferation effect of DOX in breast cancer cells [58]. Gefitinib and hematoporphyrin were encapsulated within a fluorinated dendrimer functionalized with an aptamer, to recognize EGFR-positive cells in non-small cell lung cancer [59].

5.3 Integrin Receptors

Integrin receptors overexpressed in neovasculature have also been targeted via RGD-functionalized PAMAM G-5 dendrimers and modified with fluorescein isothiocyanate and PEG, which were loaded with approximately six molecules of DOX each [60, 61].

Chemotherapy in gliomas represents a big challenge due to the low penetration through the blood-brain and blood-tumor barriers. Thus, a PEGylated PAMAM dendrimer (G-5) was loaded with arsenic trioxide (As_2O_3, ATO) to produce

RGDyC-mPEG-PAMAM/ATO, as a promising nanosystem for the treatment of gliomas. Results showed specific targeted drug delivery, with long circulation time and an improved antitumor efficacy (by RGD), when compared to As_2O_3 alone [62].

A recent study demonstrated that functionalization with RGD improves the internalization of the synthesized nanosystem in resistant cells, allowing the efficient delivery of paclitaxel within KB CHR8–5 cells, thus improving apoptotic mechanisms [63].

Another kind of therapy incorporates targeted radio- or photodynamic therapy, for example, by using PAMAM-DOTA-cRGDfK, which was prepared and radiolabeled with lutetium-177, to produce targeted integrin αvβ3 radiotherapy [64]. Multiple chorine e6 molecules were covalently bound to a PAMAM dendrimer (G-7) to produce specific cell death in cells positive for integrins, at nanomolar concentrations, under photo-irradiation [65].

5.4 CXCR4 Receptors

CXCR4 is a chemokine receptor involved in the progression and metastatic process of cancers. They are highly expressed in several cancer types [9, 10]. The efficacy of dendrimers functionalized with the LFC131 (Tyr-Arg-Arg-Nal-Gly) peptide sequence, covalently bound and loaded with DOX, significantly improved binding when compared to dendrimer-DOX (without targeting moiety) and presented enhanced cytotoxicity, with regard to BT-549-Luc and T47D, attributed to the binding of the LFC131 peptide to the CXCR4 receptor [55].

5.5 Somatostatin Receptors

PAMAM dendrimers have also been designed and synthesized for somatostatin receptor binding and MTX delivery in MCF7 cells and demonstrated enhanced cytotoxicity when compared to free MTX [56].

6 Dendrimers for Gene Therapies

Gene therapy is an emerging therapeutic strategy that consists in modifying the target gene expression through nucleic acids release into the cell, such as antisense oligonucleotides (AO), small interfering RNA (siRNA), microRNA (miRNA), or short hairpin RNA (shRNA) [66–68]. These nucleic acids present diverse modes of action to achieve their therapeutic effect (Table 2).

The nucleic acids can be ex vivo or in vivo delivered to the target cell. In the ex vivo method, the stem cells removed from the patient are transfected with the

Table 2 Therapeutic nucleic acids used in dendrimers for gene therapy applications

Therapeutic nucleic acid	Description	Biological mechanism
Antisense oligonucleotides (AO)	AO are short 15–30-base (~4–10 kDa) long DNA or RNA molecules designed to selectively bind to target RNA (pre-mRNA and mRNA) through Watson-Crick base-pairing [69, 70].	AO binding to target DNA or RNA induces the downregulation of the target gene by different mechanisms, including RNase-H-mediated mRNA cleavage, inhibition of polyadenylation, inhibition of 5′ cap formation, steric hindrance of ribosomal activity, and modulation of the pre-mRNA splicing [69, 71, 72]
Small interfering RNA (siRNA)	A non-coding RNA containing 21–23 nucleotides (~14 kDa) hybridized to each other, so that the two bases at the 3′ end of each strand are single-stranded [73, 74]	siRNA executes its function when its antisense strand is completely base-paired to the target messenger RNA (mRNA). This process occurs in an RNA-induced silencing complex (RISC), which degrades the corresponding mRNA, resulting in gene silencing [73, 74]
MicroRNAs (miRNAs)	They are non-encoding RNAs containing around 22 (~600–10,000 kDa) [74, 75]. miRNA structure has internal mismatches, called *bulges*, in the secondary structure [76]	miRNA interacts through base paring with the 3′ untranslated region of its target mRNAs to suppress expression. miRNA can also interact with 5′ UTR, coding sequences, and gene promoters. The gene silencing initiates with the formation of a miRNA-induced silencing complex (miRISC) followed by the miRISC: target mRNA interaction. Next, PAN2/3 initiates deadenylation while CCR4-NOT concludes the process, giving rise to the removal of the m7G cap on the target mRNA. Decapped mRNA may then undergo 5′-3′ degradation via the exoribonuclease XRN1 [75]
Short hairpin RNA (shRNA)	shRNA is composed of a sense and antisense sequence connected by a loop of unpaired nucleotides with 19–22 bp. *shRNA structure* has completed internal base-pairing and no bulging, a difference with regard to miRNA [76]	shRNA expression in cells is obtained by plasmid or viral delivery. Once the vector has integrated into the host genome, the shRNA is then transcribed in the nucleus. shRNA is transported to the cytoplasm by Exportin 5 and recognized by Dicer, which processes the shRNA into the siRNA duplexes [76–78]

therapeutic gene in vitro. The genetically modified cells are introduced into the patient, where the stem cells can differentiate and the gene can be expressed. In contrast, with the in vivo method, a therapeutic gene is directly administered to the patient via systemic or localized injection [79, 80]. The latter is less efficient in reaching the target site, due to multiple factors, such as enzymatic degradation, immune activation, undesired protein interactions, and limited cellular uptake [80, 81]. To counteract and increase the probability of delivery of the genetic material

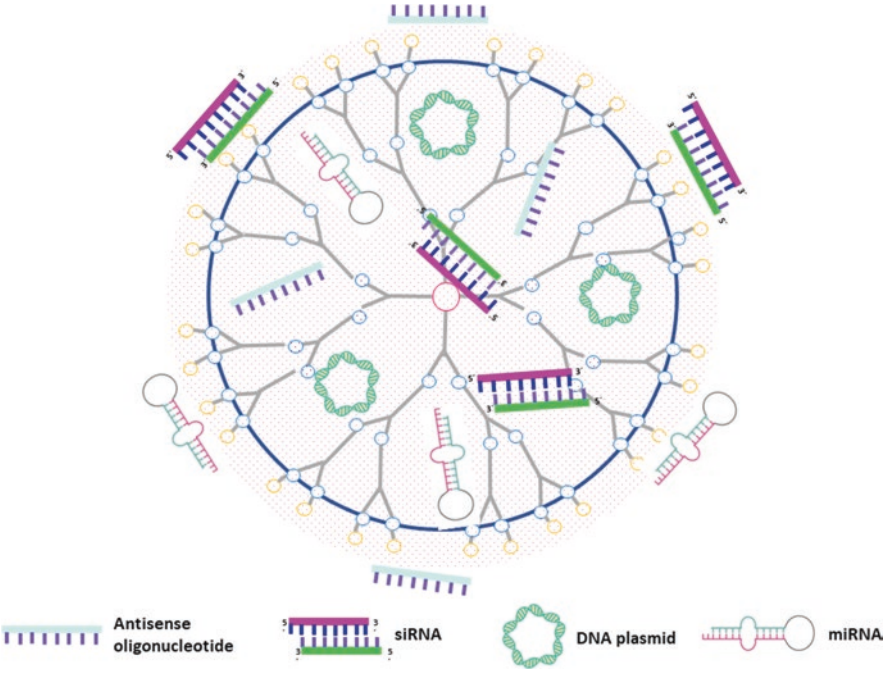

Fig. 5 Schematic representation of several nucleic acids transported by dendrimers

into the target cell, different transport systems, based on organic nanoparticles, have been evaluated [67, 70, 81–88]. Dendrimers represent an efficient nucleic acid delivery strategy (Fig. 5). PAMAM dendrimers are currently the most studied. These dendrimers form stable complexes with RNA or DNA employing electrostatic interactions, giving rise to *dendriplexes* [83, 89]. Dendrimers also protect nucleic acids from enzymatic degradation and facilitate endosomal escape [87, 88]. Endosomal escape occurs when tertiary amines in dendrimer structures get protonated within endosomes. This induces the flux of negatively charged ions (Cl^-), which disrupts the endosome membrane, with the consequent release of nucleic acids [88]. Pedziwiatr-Werbicka et al. (2011) mention that the nature of the dendriplexes depends on the concentration and stoichiometry of the DNA phosphates and dendrimer-amines, as well as on the solvent properties (salt concentration, pH, buffer strength) and even the dynamics of the mixing process [90]. The dendrimers' clinical applications are still restricted, due to their biocompatibility and cytotoxicity. To counteract the above, chemical modifications on the surface or in the core of the dendrimers have led to the development of dendriplexes with a greater capacity to transport and release nucleic acids into target cells. Particularly, PAMAM, PPI, PLL, phosphoric, and carbosilane dendrimers are the most-used cationic dendrimers for the release of DNA or RNA [87].

Since amine-ended PAMAM is positively charged at physiological pH, complex formation with nucleic acids is favored [89, 91]. There is evidence that PAMAM

transfection efficiency increases with the increase in dendrimer generation [87]. Low-generation PAMAM dendrimers, such as G1 and/or G2 dendrimers, have few primary surface amines (i.e., low positively charge density), which limits nucleic acid complexation and transfection [88, 92]. The most efficient transfection is seen with intermediate and high PAMAM generations, including G3–G10, which is associated with the high density of surface primary amines [88, 89, 92]. However, these dendrimers have more rigid structures and are highly toxic. PAMAM toxicity is related to nanohole formation in cell membranes, by the effect of the interaction with positively charged primary amines [88, 89]. Chemical modifications of PAMAM dendrimer surfaces and core are a strategy for reducing cytotoxicity and promoting gene delivery [87, 92–95].

The partial acetylation or PEGylation of the surface groups is recommended to increase the biocompatibility of PAMAM dendrimers [89, 96, 97]. As a result of these reactions, a charge density reduction is produced. Fant et al. (2010) found that acetylation and PEGylation of G4 and G5 PAMAM dendrimers reduce cytotoxicity. However, the transfection efficiency is lower than that of the nonmodified dendrimer [89]. Shakbazau et al. (2010) proposed that decrease in transfection efficiency in acetylated G4 dendrimers is due to an increase in hydrophobicity [97]. Froehlkish (2011) examined DNA interactions with mPEG-PAMAM (G-3), mPEG-PAMAM (G-4), and PAMAM (G-4), keeping DNA concentration constant. The structural analysis demonstrated a strong dendrimer-DNA interaction via major and minor grooves and the backbone phosphate group with binding constants of $K_{\text{mPEG (G-3)}} = 1.5 \pm 0.5 \times 10^3\ \text{M}^{-1}$, $K_{\text{mPEG (G-4)}} = 3.4 \pm 0.8 \times 10^3\ \text{M}^{-1}$, and $K_{\text{mPAMAM (G-4)}} = 8.2 \pm 0.9 \times 10^3\ \text{M}^{-1}$. The reported dendrimer-DNA complexation stability was PAMAM (G-4) > mPEG (G-4) > mPEG (G-3); this effect was associated with the neutralization of charges [98].

The conjugation of bromoalkylcarboxylates (of different chain lengths) with different percentages of primary amines of dendrimers is another strategy that has been used for the modification of the drug delivery efficiency in terms of drug solubility, release profile, and cytotoxicity [99, 100]. Alkylated PAMAM dendrimers were synthesized for targeted siRNA release to lung blood vessels, to treat chronic asthma and obstructive pulmonary disease. Using a combinatorial approach, the free amines on PAMAM dendrimers (of increasing generations) were substituted for alkyl epoxides of various carbon chain lengths. Such modifications improved the avidity of dendrimers for Tier2-positive endothelial cells in the lung [100].

Studies have shown that peptide functionalization in PAMAM dendrimers improves cell-penetrating ability, useful for targeted gene delivery. For example, a siRNA delivery system, based on a G4 PAMAM dendrimer, was conjugated to cell-penetrating peptides, oligo-arginine, and a transactivator of transcription, through a PEG crosslinker. The siRNA in this dendrimer carried out effective gene silencing of AT1R in cardiomyocytes [101].

Arima et al. (2011) prepared siRNA complexes employing a G3 PAMAM conjugate loaded with α-cyclodextrin and studied the intracellular distribution and in vitro RNAi effects on endogenous gene expression. Cyclodextrins interact with cholesterol and phospholipids of the cell membrane, resulting in augmented membrane

permeability of hydrophilic compounds (i.e., siRNA). The dendrimer delivered fluorophore-labeled siRNA only into the cytoplasm and showed the efficient gene silencing of Lamin A/C and Fas expression after transfection [102]. Recently, Hersh et al. (2021) modified a G5 PAMAM dendrimer-DNA complex with a skeletal muscle-targeting peptide, a DLC8-binding peptide for enhancement of intracellular transport, and a nuclear localization signaling peptide for nuclear uptake complexed to plasmid DNA (containing the microdystrophin gene). This nanocarrier was able to induce microdystrophin protein expression, with the potential to be a therapeutic agent for Duchenne muscular dystrophy [83].

To obtain more flexible dendriplexes, some PAMAM dendrimers have been designed with an ethylenediamine [87] or triethanolamine (TEA) core [103]. The branching units in TEA core-based dendrimers are characterized by being less-densely packed, allowing optimal interaction with siRNA and increasing release properties. The TEA core can effectively protect siRNA from degradation and facilitate cellular uptake of siRNA via micropinocytosis [103].

Several strategies to inhibit the expression of target genes, at the mRNA level, have become popular recently. For example, mRNA or pre-mRNA containing specific nucleotide sequences allows the design of antisense oligonucleotides specific to target genes [69]. The first in vivo applications of AO showed limited clinical potential because of unfavorable properties such as rapid degradation by nucleases, poor uptake through cell membranes, and suboptimal binding affinity for complementary sequences [72, 81, 104]. Such characteristics increased the possibilities for using dendrimers as nanocarriers for therapeutic antisense oligonucleotides. Table 3 shows some examples of PAMAM dendrimers for the delivery of antisense oligonucleotides.

PPI dendrimers, composed of a 1,4-diaminobutane nucleus and branching units of propylene imine monomers, comprise only tertiary amine groups in the inner structure and primary amines on the surface. Ethylenediamine and other molecules

Table 3 Examples of PAMAM dendrimers for the delivery of antisense oligonucleotides

PAMAM dendrimer	Therapeutic antisense	Ref
Cholesteryl G5 PAMAM dendrimer	Phosphorothioate 2′-O-methyl oligonucleotide (5′-CCUCUUACCUCAGUUACA-3′)	[105]
G5 PAMAM dendrimer grafted with Oregon green 488	Phosphorothioate 2′-O-methyl oligonucleotide (5′-CCUCUUACCUCAGUUACA-3′)	[106]
G5-PAMAM conjugated to cationic TAT peptide and BODIPY fluorophore	Phosphorothioate 5995 antisense, targeting AUG start codon of the MDRI gene and siRNA	[107]
G5 PAMAM dendrimer conjugated to FA	Antisense oligodeoxynucleotide 5′-GTC-CAC-TCT-TGT-CCT-CAA-TG-3′ corresponding to the rat EGFR	[108]
G5 PAMAM dendrimer	c-Src antisense oligodeoxynucleotide (5′-GGG-CTT-GCT-CTT-GCT-GCT-CCCCAT-3′)	[109, 110]
G4 PAMAM-NH_2 G4 PAMAM-OH	Oligonucleotide sequence 5′-TGT-GCTATT-CTG-TGA-ATT-3′ targeting the protein Survivin	[111]

can also be used as a dendrimer core [95, 112, 113]. The presence of alkyl chains in their branching units provides a more hydrophobic interior compared to PAMAM dendrimers [112]. Similar to PAMAM dendrimers, chemical modifications on the surface of PPI have been carried out to reduce toxicity and increase colloidal stability and uptake by target cells. The addition of hydrophobic moieties and/or cross-linker fragments containing dithiol, followed by PEG coating of PPI dendrimers, has led to enhanced DNA or RNA transfection [95, 112]. For example, Hashemi et al. (2015) evaluated the effect of the conjugation of bromoalkanoic acids with different side chains lengths (6, 10, and 16 carbons) and three different substitution degrees of substitution (10%, 30%, and 50% of surface amines) onto G5 PPI dendrimers, for DNA transfection. The hydrophobic modifications improved transfection activity, exhibiting higher DNA delivery for 30% and 50% grafting with decanoate moieties when compared to native G5 PPI [112]. For targeted dendriplexes, tyrosine modification of PPI shows high efficacy of gene knockdown with regard to EGFP activity mediated by siRNA [94]. Jugel et al. (2021) have reported the targeted delivery of a therapeutic siRNA specific for BIRC5/Survivin in tumor cells expressing the prostate stem cell antigen (PSCA). The single-chain antibody fragments (specific for PSCA) were conjugated to siRNA/maltose-modified PPI dendriplexes; these targeted polyplexes induced the knockdown of firefly luciferase and Survivin expression in prostate cancer cells and PC3/PSCA xenograft-bearing mice with significant anticancer effects [114]. Other modifications on PPI dendrimer surfaces, for enhancement in the accumulation of therapeutic nucleic acids in target cells, have also been made using the anti-CD44 antibody [115], maltose/anti-EGFRVIII single-chain antibody [116], folate [117], and luteinizing hormone-releasing peptide [94, 118]. Table 4 shows some examples of PPI dendrimers for gene delivery.

Table 4 Examples of PPI dendrimers for gene delivery

PPI dendrimers	Application	Ref
G2 and G3 PPI dendrimers with antisense oligonucleotides, targeting the EGFR	Potential evaluation of PPI dendrimers (G-2 and G-3) for antisense oligonucleotides targeting EGFR cellular delivery	[113]
Multifunctional siRNA delivery system based on G5 PPI dendrimer with superparamagnetic iron oxide nanoparticles and incorporation of PEG coating and luteinizing hormone-releasing peptide	For enhancement of in vivo antitumor activity of cisplatin	[118]
siRNA delivery system based on PPI dendrimer conjugated to anticancer drug paclitaxel and synthetic analog of luteinizing hormone-releasing peptide	For delivery of paclitaxel and siRNA targeted to CD44 mRNA (specifically, to metastatic ovarian cancer cells)	[119]
shRNA-encoding plasmid DNA delivery system based on G3 PPI dendrimer conjugated to anti-CD44 antibody and Pluronic P123	To resensitize doxorubicin-resistant breast cancer cells to the anticancer agent through the selective inhibition of P-GP	[115]

PLL dendrimers are a type of peptide dendrimer, whose core and branching units are composed mainly composed of lysines linked via peptide bonds [88, 120]. The surface lysine of PLL dendrimers has two primary amines that are frequently modified to allow transfection and the cytotoxic effect of nucleic acids [120]. The use of different amino acids as replacements for lysine residues can change the distribution and flexibility of charged groups, allowing additional interactions with nucleic acids. The addition of charged aliphatic amino acids, such as Arg, between inner Lys branching units, confers more charge and more hydrophilic character inside the dendrimer, increasing the affinity for DNA phosphate groups. For example, Gorzkiewicz et al. (2020) studied the formation of three types of G-3 PLL dendrimers with siRNA molecules; the G3 PLL dendrimers were modified, at the same points, with two lysine, histidine, or arginine residues between each pair of neighboring branching points of the standard PLL dendrimer. Such modifications changed binding stoichiometry and strength of dendrimer-siRNA interactions, electrostatic surface potential, and size, as well as cytotoxicity. For example, the low zeta potential of PLL dendrimers with His-His residues suggests a lower tendency to interact with the cell membrane and, thus, lower transfection efficiency, when compared to Lys-Lys or Arg-Arg modified dendriplexes [120]. Dendrigraft poly-L-lysine (DGL) polymers are a type of dendritic PLL derivative without tertiary amine groups, which have presented characteristics suitable for gene delivery (e.g., high density of amine groups on the surface) [121]. Li Ye et al. (2022) evaluated the potential of G2 y G5 DGL dendrimers for gene delivery. These dendriplexes were prepared with different siRNAs: fluorescein amidite (FAM)-siRNA, anti-polo-like kinase 1 (PLK1), siRNA (siPLK1), and anti-EGFR siRNA (siEGFR) for the evaluation of gene transfection, cellular uptake, endosomal escape of polyplexes, cytotoxicity and gene silencing efficacy in vitro, as well as treatment with polyplexes in vivo. These results provide an effective approach for improving the endosomal escape and transfection effectiveness of dendrimers.

Carbosilane dendrimer architecture includes silicon-carbon (Si-C), silicon-silicon (Si-Si), carbon-carbon triple (Si-C ≡ C-), and siloxane (Si-O) bonds. These bonds can be added to the surface or core of dendrimers [85, 122]. Carbosilane dendrimers are divided into two types: cationic and anionic dendrimers; there is evidence that cationic carbosilane dendrimers show high toxicity in comparison with anionic dendrimers. To decrease the cationic dendrimer cytotoxicity, surface modifications with negatively charged or neutral moieties and PEG modifications are recommended [82, 123, 124]. For example, to enhance the release of genetic material in target cells and reduce the cytotoxic effect, dendrimers with high water solubility, such as azide-terminated carbosilane dendrimers with two different propargyl amines, were applied [125]. Additionally, different degrees of PEGylation on cationic dendrimers loaded with miRNAs have shown to be successful, particularly in therapy against HIV/AIDS [82]. A study demonstrated that carbosilane and PAMAM dendrimers preserved anticancer siRNA cocktails better than phosphorous dendrimers [126]. Functionalization of cationic carbosilane dendrimers to nano-emulsions, through the carbodiimide reaction, favored the electrostatic

attachment of antisense oligonucleotides to the surface of the nanoparticles, as well as the gene-silencing effect [127]. A nanosystem based on mesoporous silica nanoparticles, covered with carbosilane dendrimers, is an excellent transport of single-stranded oligonucleotides into the cells [128]. A nanosystem based on gold nanoparticles, conjugated to cationic carbosilane dendrimers (G1-G3) and loaded with siRNA, penetrated the target cells more efficiently with an increase in the generation of the dendrimers [129].

7 Dendrimers for Combined Therapies

The excellent capability of dendrimers to carry both individual molecules (drugs as antineoplastic agents, anti-inflammatory drugs, antibiotics, antibodies, genes, metal ions, metal nanoparticles, or radionuclides) and combined molecules, to a specific target, offers a broad spectrum to conventional therapy, radiotherapy, or combined therapy, with several medicinal and practical applications [130]. Specifically, in the anticancer medical research field, combined therapy has been reported in the use of carbosilane ruthenium dendrimers complexed with conventional anticancer drugs (methotrexate and doxorubicin), evaluated with human leukemic cells. The presence of ruthenium within the structure expands the anticancer properties of nanosystems containing antineoplastic compounds and reduces the viability of leukemia with regard to 1301 and HL-600 cancer cells [131].

On the other hand, G3 dendrimers bearing conjugated copper(II) on their surface have been reported to have antiproliferative activity related to their capacity to activate Bax translocation. The activity of multivalent Cu-conjugated dendrimers with different chemotherapeutic agents has been evaluated, showing an additive effect with taxanes, such as paclitaxel and the proteasome inhibitor MG132, and synergy with the topo II inhibitor doxorubicin [132].

Another application of combined therapy has been reported with the synthesis of multifunctional nanocarriers based on PAMAM dendrimers (G5), fixed to polydopamine (PDA)-coated magnetite nanoparticles (Fe_3O_4), allowing applied chemo- (doxorubicin) and photothermal therapy of liver cancer cells in vitro [133].

PAMAM-based dendrimers have been widely used to simultaneously carry both therapeutic molecules as radionuclides for dual therapy. G4 PAMAM dendrimers, modified in their surface with targeting ligands for the tumor microenvironment (e.g., bombesin, CXCR4, folate), have been radiolabeled with lutetium-177, a predilected radionuclide. These dendrimer structures combine radiotherapeutic effects with chemotherapy (paclitaxel), small molecules (C19), and metallic structures (gold nanoparticles), which produce a better cytotoxic effect in comparison with elements alone [8, 134, 135].

8 Radiolabeled Dendrimers as Theranostic Approaches

Dendrimers represent favorable choices as diagnostic, therapeutic, or theranostic probes. The variety in core materials and surface modification, as well as several targeting and radiolabeling strategies, have allowed dendrimers to be used as multifunctional platforms for multimodal imaging and therapeutics [27].

8.1 Radiolabeling

There are different strategies for radiolabeling, among them, the formation of complexes through chelating agents, the direct incorporation of radionuclides (via electrostatic interactions, adsorption or covalent bonds), or confinement strategies (trapping or encapsulation) [136]. The advantages and selection of these strategies will depend on the physiological environment and/or radiochemical parameters, such as radiolabeling efficacy, specific activity, and radiochemical purity. In general, they can be classified into *direct* and *indirect* radiolabeling techniques, which are described below [136, 137].

Indirect Radiolabeling The selection of a bifunctional chelator agent (BFC) has the highest priority since the in vivo stability of the radiolabeling is highly dependent on the coordination chemistry between the radionuclide and the BFC. In indirect radiolabeling methods, a bifunctional chelating agent is used to conjugate dendrimers with a radionuclide through chemical linkers. A disadvantage of the addition of bifunctional groups to the dendrimer surface is that it can negatively affect its particle size, charge, and solubility [138]. The impact on the in vivo behavior is that it can cause dissociation of the radionuclide from the dendrimer, and this can result in erroneous image output and unwanted side effects [136]. Therefore, for indirect radiolabeling to be successful, the selection of a BFC with high in vivo stability is paramount.

BFCs consist of a metal-chelating unit and a reactive functionality; the former binds to metal radionuclides, and the latter is covalently conjugated to the surface of the dendrimers. Conjugation of BFCs with the dendrimer generally requires surface modification of the dendrimer. The chelator selection depends on the radionuclide chosen and the desired physicochemical properties [136, 139].

Figure 6 shows the structures of some chelators, such as diethylenetriaminepentaacetic acid (DTPA), 6-hydrazinonicotinyl, tetraazacyclododecanetetraacetic acid (DOTA), and 2,2′-(7-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic (NOTE) and derivatives that can be conjugated on the surface of PAMAM dendrimers to label with radionuclides such as ^{99m}Tc and ^{64}Cu for SPECT and PET images, respectively. To label with other radionuclides such as iodine-131(^{131}I), the phenol groups of 3-(4′-hydroxyphenyl)propionic acidOSu (HPAO) are employed to modify the surface of the PAMAM dendrimer and can be used for SPECT image-guided RT of tumors [140].

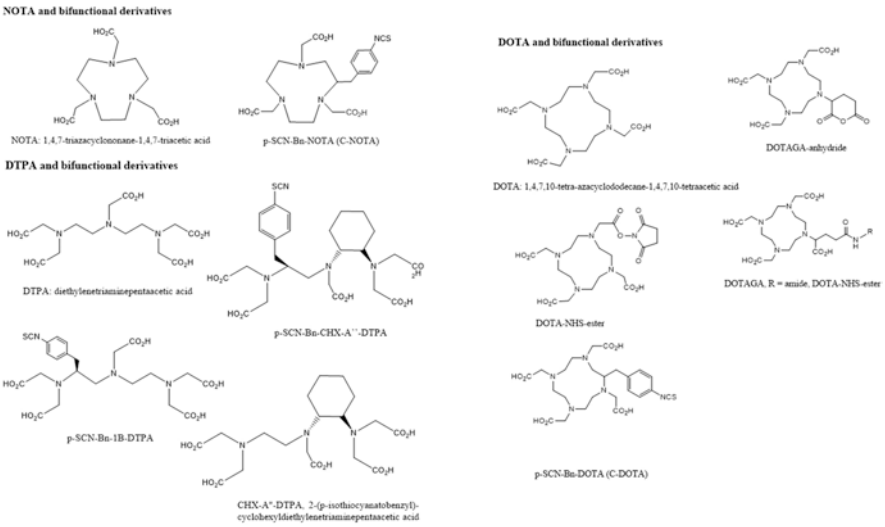

Fig. 6 Chemical structure of some chelating agents used for the radiolabeling of PAMAM dendrimers

Direct Radiolabeling One of the advantages of this technique is that in vivo dissociation of radiolabeled dendrimers can be minimized; however, some technical issues need to be addressed, such as radiation exposure of personnel during synthesis and reproducibility of radiolabeling. Surface modifications of radiolabeled dendrimers, using various polymer coatings, are commonly used to minimize interactions in vivo [136].

To prepare multifunctional dendrimers, the most common method is to covalently modify its surface and subsequently, several BFCs and different radionuclides can be attached to it. Some PAMAM structures have been radiolabeled through this technique, for example, ^{131}I can be successfully attached to the surface of G5 PAMAM dendrimers prefunctionalized with HPAO (3-(4′-hydroxyphenyl) propionic acid-OSu) via phenol groups, using the chloramine T (tosylchloramide sodium) method [140]. Complexation of paramagnetic metal ions such as Mn(II) and Gd(III) has also been achieved through preconjugated chelators at the periphery of the dendrimer [141]; ^{99m}Tc or ^{64}Cu radionuclides can also be linked to the dendrimer surface by chelation, for SPECT [142] or PET imaging applications [143]. To achieve dual-mode SPECT/MR imaging applications, Luo et al. placed Mn(II) and ^{99m}Tc onto the surface of G5 dendrimers via DOTA chelation [141].

Another radiolabeling technique used for dendrimers is *interior trapping*, in which the highly branched molecular structure of PAMAM dendrimers, with sufficient interior cavities to trap radiometallic NPs such as Au NPs, is exploited, exhibiting a superior X-ray attenuation property when compared to commercial iodinated contrast agents for CT.

8.2 *Radionuclides*

Depending on their medical applications, radionuclides can be classified as *diagnostic* or *therapeutic*. Diagnostic radionuclides used for SPECT imaging are gamma-emitters (energy range: 75–360 keV), while for PET imaging are positron-emitter radionuclides, which generate two 511 keV photons, via annihilation, for PET imaging [27]. Therefore, the selection of radionuclide depends on the intrinsic characteristics of each radionuclide. A variety of radionuclides, such as ^{67}Ga, ^{123}I, ^{131}I, ^{111}In, and ^{201}TI, are suitable for SPECT imaging applications. In the case of ^{99m}Tc, it has been used for different applications, such as SPECT/optical, SPECT/MR, and SPECT/CT imaging (based on dendrimers) [27, 139] (Table 5).

9 Limitations of Dendrimers in Biomedical Applications

Liposomes, micelles, and dendrimers are cationic macromolecules with a positive charge on the surface, which tends to destabilize cell membranes and promote cell death [144]. The cytotoxicity of dendrimers relies on their charge, generation, and concentration. Positively charged products show increased toxicity compared to their negatively charged or neutral counterparts, while cytotoxicity increases with increasing generation and concentration [145]. The cytotoxicity of cationic dendrimers is due to the interaction between their positively charged dendrimer amines and negatively charged cell membrane compounds. This interaction promotes structural damage to the cell membrane via the formation of nanopores and the following leakage of cell content leading to cell death [12].

Another critical limitation of dendrimers for their biomedical application is their retention by the reticuloendothelial system. The administration of macromolecular anticancer drugs mainly depends on the permeable nature of the tumor's vasculature, compared to the healthy vessels of normal organs. When administered

Table 5 Radiolabeling strategies of dendrimers [136, 139]

Technique	Radionuclide	Dendrimer	Strategy of radiolabeling
SPECT	^{99m}Tc	PPI	Direct, with $SnCl_2$
		PAMAM	Indirect, nicotinic acid as coligand
			Indirect, EDDA as coligand
			Indirect, tricine as coligand
	^{111}In		Direct, via [99mTc-(CO)3(H2O)3]+
			Indirect, with DTPA
			Indirect, with DOTA
			Indirect, with Lys(DTPA)
PET	^{64}Cu		DOTA
	^{68}Ga		DOTA
	^{76}Br		Tyrosine moieties

intravenously, dendrimers tend to circulate for long periods, unless they are sufficiently small to be excreted by the kidney or stealthy enough to evade the phagocytic system of macrophages (reticuloendothelial system) [146]. Dendrimers can treat cancer because they can get trapped and accumulate in tumors. This EPR effect has explained the reason why macromolecules and nanoparticles are found in higher proportions in tumors than in healthy tissues. Although tumor accumulation is observed, cell uptake and intracellular release of encapsulated drugs in dendrimers have been questioned because PEG is employed for protecting dendrimers from the reticuloendothelial system uptake but prevents also the cell absorption and, as a consequence, the intracellular drug release [147]. Another limitation of dendrimer platforms is their use for the controlled release of drugs, due to the diversity of release mechanisms and the spectrum of release kinetics. For example, dendrimer-encapsulated drugs are quickly released, discharging their payload prematurely before the dendrimers reach a target location. In contrast, the release of drugs from functionalized dendrimers depends mainly on the bond character between the drug and the dendrimer periphery [148].

Nanoparticles as carriers of cancer drugs represent one of the fastest-growing areas of medical research and are considered one of the most favorable strategies to treat cancer. Liposomes and polymer conjugates were the first nanoparticles approved by the FDA. However, there are only five commercial formulations based on liposomes [149]. Due to the narrow emerging success in clinical translation, the FDA and the Nanotechnology Characterization Laboratory (NCL) stimulate a regulatory review of nanopharmaceuticals. The European Technology Platform on Nanomedicine created the European Nanocharacterization Laboratory in the framework of the Horizon 2020 project. Moreover, the FDA emitted the Industry Guide ("Pharmaceuticals, including biological products, containing nanomaterials") to contribute providing legal certainty to this field [150].

10 Prospects for Clinical Translation

The goal of nanomedicine is to address specific clinical problems via the development of accurate diagnostic and therapeutic nanosystems. Several targeted and non-targeted nanosystems have emerged as strategies for delivering recombinant proteins, aptamers, therapeutic nucleic acid (siRNA, miRNA, or shRNA), and traditional cytotoxic drugs [14, 31, 84, 86, 107, 151, 152]. These nanosystems display excellent features such as high stability, biocompatibility, and efficient therapeutic effects, making them suitable as drug delivery scaffolds with high translational potential [14, 31]. Although many dendrimer formulations have been designed and evaluated over the years, there has been little done in clinical trials (Table 6) [14, 31, 84, 153]. Most of these nanosystems are based on fifth-generation PEGylated polylysine dendrimers conjugated with chemotherapeutic agents: docetaxel (DEP®-docetaxel), cabazitaxel (DEP®-cabazitaxel), or irinotecan (DEP®-irinotecan). For example, DEP®-docetaxel is found in phase II, in which the administration in

Table 6 A summary of the clinical status of dendrimers

Gene therapy	Description	Clinical trial
Dendrimer-enhanced product (DEP®) 1. DEP®-docetaxel 2. DEP®-cabazitaxel 3. DEP®-irinotecan	It is a nanosystem based on G5 PEGylated poly-l-lysin dendrimers conjugated with chemotherapeutic agents	DEP®-docetaxel is in Phase II [154] DEP®-cabazitaxel is in Phase II [155] DEP®-irinotecan is in the recruitment stage for phase II [162]
DEP®-Bcl2/xL conjugates (AZD0466)	It is a Bcl2/xL inhibitor based on poly-l-lysin dendrimers	It is currently in a Phase I clinical trial [156]
VivaGel®	It is a G4-poly(L-lysine) polyanionic dendron with 32 naphthalene disulfonate groups on its surface	Phase III trials for the treatment of bacterial vaginosis (SPL7013 Gel, astodrimer sodium) [153]
OP-101	It is a fourth-generation hydroxyl PAMAM dendrimer, linked to N-acetyl cysteine (NAC) via a disulfide bond [14, 158]	Phase I of clinical trials [14, 158]
ImDendrim	It is a platform based on poly-l-lysin dendrimers with a ^{188}Rh ligand (nitro imidazole-methyl-1,2,3-triazol-methyl-di-[2-pycolyl]amine) [159]	An interventional study involving ten patients with inoperable liver cancer, resistant to chemotherapy [161]

patients with pancreatic, oesophageal, and gastric cancer has shown tumor reduction and prolonged stable disease, as well as notable lack of bone marrow toxicity and reduction of other common side effects (e.g., hair loss, mouth ulcers, anaphylaxis) [154]. DEP®-cabazitaxel is in Phase II with substantial tumor biomarker reductions (e.g., prostate-specific antigen) in prostate cancers [155]. In 2019, Starpharma in association with AstraZeneca announced that DEP®-Bcl2/xL started Phase 1 clinical trials. DEP®-Bcl2/xL is a dendrimer formulation conjugated to the AZD0466 inhibitor, which is a dual Bcl2 and Bcl/xL inhibitor, with excellent anticancer activity [156]. Another dendrimer in clinical trials is VivaGel®; it is a G4-PLL polyanionic dendrimer with 32 naphthalene disulfonate groups on its surface. It has potent topical vaginal microbicidal activity and is currently in Phase III, for the treatment of bacterial vaginosis (SPL7013 gel; astodrimer sodium) [153]. This same dendrimer showed a potent antiviral agent against the respiratory syncytial virus before and/or after exposure, using nasal spray technology [157]. Recently, the SPL7013 gel has also been shown to inactivate >99.9% of SARSCoV-2 within 1 min [157]. OP-101 is a G4 hydroxyl PAMAM dendrimer linked to N-acetyl cysteine (NAC) via a disulfide bond. NAC is released in the cell via cleavage of the disulfide bond by interaction with glutation [14, 158]. OP-101 is in Phase I of clinical trials. The safety, tolerability, and pharmacokinetics of OP-101 were evaluated

after intravenous administration in healthy volunteers [158]. ImDendrim is an adendrimer in clinical trials composed of a 5G PLL dendrimer, combined with nitro imidazole-methyl-1,2,3-triazol-methyl-di-[2-pycolyl]amine and labeled with ^{188}Rh [159]. ^{188}Rh is beta emitter with a physical half-life of 16.9 h, $E_{\beta max}$ of 2.12 MeV, Eγ of 155 keV (15%), and mean tissue penetration of approximately11 mm; these properties make it suitable for theranostic applications [159, 160]. ImDendrim initiated a Phase I clinical trial in 2017, for the treatment of nonresponsive and inoperable liver cancers. Currently, there is no information concerning the status [161].

With regard to dendrimers that show potential to be used as prospects for clinical translation, there are targeted dendrimers for therapy and/or diagnosis. The advantage of these systems over nontargeted dendrimers is that solubility, bioavailability, and pharmacokinetic/pharmacodynamic (PK/PD) profile of the drugs (e.g., chemotherapeutic agents) can be improved. At the same time, these dendrimers can recognize specific sites on target cells, with high affinity, reducing side effects in healthy tissues. Low polydispersity and biocompatibility are two properties that must also be considered for the use of dendrimers in human trials. Several investigations highlight the in vitro and in vivo studies of dendrimers. Table 7 shows examples of prospects of dendrimers conjugated or complexed with targeting agents. An interesting study was performed by Huang et al. (2011), who developed a PEGylated PAMAM dendrimer conjugated to angiopep-2 (PAMAM-PEG-Angiopep) and complexed with DNA plasmid, to deliver tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) to glioma cells and brain tumors. Angiopep-2 is a ligand for lipoprotein receptor-related protein 1 and TRAIL. It is a cytokine that activates apoptosis through binding to death receptors 4 (TRAILR1) and 5 (TRAIL-R2/KILLER). PAMAM-PEG-Angiopep/DNA, complexed with TRAIL DNA plasmid, displayed excellent blood-brain barrier penetration ability and a favorable biodistribution and pharmacodynamic profile in vivo, thus emerging as a promising dendrimer for targeted therapies [163]. Another prospect, as far as dendrimers go, is the PEG-cored PAMAM dendrimer conjugated to the Flt-1 antibody and loaded with gemcitabine; Flt-1 is a receptor for vascular endothelial growth factor (VEGF). This system has shown enhanced cytotoxicity of gemcitabine and increased the accumulation of the chemotherapeutic agent with satisfactory in vivo anticancer efficacy [151].

The G2-S16 water-soluble polyanionic carbosilane dendrimer (G2-S16 PCD) is another prospect for clinical translation. This is a polyanionic carbosilane dendrimer that has shown great potential as an antiviral agent to prevent HIV-1 sexual transmission, by blocking gp120/CD4/CCR5 interaction and providing a barrier against infection for long periods. The dendrimer has the capability to inhibit cell-to-cell HIV-1 transmission and is active against exposed mock and semen [164].

Dendrimers, in addition to being used for therapy, have been prepared as contrast agents for imaging techniques such as magnetic resonance, CT, and SPECT or PET [31]. In MRI, dendrimers have been used as carriers for the delivery of gadolinium ions. For example, biodistribution studies of PAMAM dendrimers conjugated to FA and dithylenetriamine pentaacetic acid (DTPA)-gadolinium have shown strong MRI signals in tumors, with negligible toxic effect [165].

11 Conclusion

Dendrimers have demonstrated their excellent capability to transport a broad variety of medically relevant molecules. For this reason, the current research focuses on modifying and improving dendrimeric structures to obtain new and innovative

Table 7 Examples of prospects of dendrimers conjugated or complexed with targeting agents

Dendrimer prospect	Target/ targeting agent	In vitro assays	In vivo assays	Ref
PEG-PAMAM dendrimer conjugated to angiopep-2 and complexed with DNA plasmid to deliver tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Lipoprotein receptor-related protein 1 (LRP1)/ angiopep-2	The cellular uptake mechanism showed that the DNA of PAMAM-PEG-Angiopep/DNA entered into nuclei through the endosome/lysosome pathway	Excellent blood-brain barrier penetration ability Favorable biodistribution and pharmacodynamic profile in vivo	[163]
PAMAM dendrimers conjugated to PSMA specific aptamer	PSMA/PSMA specific aptamer	High uptake and cytotoxicity in target cells	Tumor shrinkage	[152]
Dendrimers conjugated to MUC1, AS1411, and ATP aptamers and loaded with epirubicin	MUC1, AS1411 and ATP specific aptamers	High delivery of epirubicin after 72 h in lysosomal pH High cytotoxicity and internalization in target cells	Tumor growth blockage	[166]
Dendrimers entrapping AuNP, conjugated to PEG and loaded with miR-21 and gemcitabine	miR-21	High therapeutic effect and induction of apoptosis	Inhibition of tumor growth	[167]
PAMAM-grafted halloysite nanotubes loaded with siRNA-VEGF	siRNA-VEGF	High cellular uptake knockdown of cellular VEGF mRNA Induction of apoptosis	Enhancement of anticancer efficacy Negligible toxic effect	[168]
PEG-cored PAMAM dendrimer with PEG2000 surface modification. PAMAM was conjugated with Flt-1 antibody and loaded with gemcitabine	Flt-1 antibody /Flt-1 receptor	Anti-Flt-1 increased the intracellular accumulation of dendrimers in cells with high Flt-1 surface expression	Tumor size reduction Improvement of the anticancer efficacy of gemcitabine	[151]
PAMAM dendrimer conjugated to FA and diethylenetriamine pentaacetic acid (DTPA)-gadolinium	FA/folate receptor	High specificity for cancer cells positive to folate receptor	Increase MRI signal of the tumor – negligible toxic effect	[165]

therapeutic alternatives in personalized medicine. Improved knowledge of the properties of dendrimers as drug- and gene-carrying macromolecules could lead, in the medium term, to clinical trials to introduce multifunctional drug delivery dendrimeric systems, leading to improved therapeutic efficacy over drugs alone. However, to a more considerable number of molecules in the functionalized dendrimers, there is a greater challenge to accurately assess their physicochemical and biological properties.

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Polymersomes for Targeted Drug and Gene Delivery Systems

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1 Introduction

With the development of nanotechnology in the last decades, several structures have been used as candidates to improve drug and gene delivery systems, mainly in the correct targeting of these molecules to a target compartment. Among these innovations are polymersomes, defined as self-assembling polymeric vesicles widely used as nanocarriers due to their high colloidal stability and long circulation time in the blood [1–3]. The assembly process of these vesicles involves the use of amphiphilic copolymers, which, when organized into triblocks, give them a morphological arrangement similar to a lipid bilayer enclosed by an aqueous center, allowing the transport of hydrophilic or hydrophobic molecules, or both, depending on whether inserted into the center or peripheral bilayer [4]. For the drug or gene carried to be delivered on a specific substrate, the polymersome structure must be planned, using copolymers and other specific elements, to disaggregate from stimuli, such as variations in pH, redox action, temperature, luminosity, and magnetic field, among others [5]. Thus, the specificity for delivery of the defined carrier molecule gives polymersomes an important space in the study of new polymeric systems for the delivery of drugs and genes, which can optimize the treatment of pathological situations and the implementation of gene therapies. In addition, the amphiphilic copolymer blocks used in the production of polymersomes can have varying lengths and molecular weights [6], interfering with the stability of vesicles and, consequently, their ability to trap drugs, for example. Thus, the choice of copolymers to be used in the assembly of the polymeric vesicle must also consider the physicochemical characteristics of the molecule to be loaded. Discher et al. reported the encapsulation of

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drugs, as well as nucleic acids and vaccines, in polymersomes to improve their targeting to predetermined sites and reduce the effects caused by the interaction of these substances with the living organism, reiterating the importance of a system polymeric for delivery of molecules destined for distinct therapeutic targets [7]. The main characteristics of this polymeric system, specifically in relation to the use of polymersomes as carriers, include biocompatibility, biodegradability, low aggregation rate, extensive circulation half-life, and greater uptake in target cells when compared to normal cells [8], which favors the good performance of polymersomes when associated with pharmacological and genetic systems. A relevant application is the use of polymeric vesicles for the transport of nucleic acids, since, in this case, the blocks of copolymers protect the molecules during their journey through the body, directing the delivery and preventing the loss of genetic material [9, 10]. In addition, part of the approaches involving polymersomes also refers to the use of these structures as carriers of antineoplastics, which are mainly characterized by a high incidence of side effects. In this perspective, Ahmed et al. [11] reported that multiple loading of doxorubicin (DOX) and Taxol (TAX) in polymeric vesicles resulted in intense apoptosis of tumor cells, confirmed in just 1 day of treatment through immunofluorescence assays. In addition, it was found that this apoptotic rate was twice as high as that measured for the free drug, that is, without the polymeric transport mechanism, proving the effectiveness of polymersomes in optimizing the cancer treatment, as well as the need for to broaden the notions about the development, characterization, and application of these structures in therapeutic systems, with emphasis on the delivery of drugs and genes, which will be the main points addressed in this chapter.

Figure 1 illustrates, on the left, the morphological constitution of a polymersome from an amphiphilic copolymer, which has a hydrophilic and a hydrophobic portion, enabling the transport of molecules of both chemical natures. On the right, the structure of a polymersome, in which it is possible to visualize a hydrophilic center,

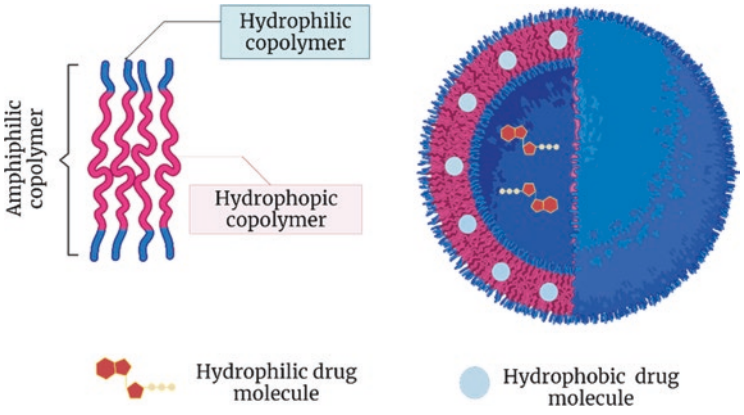

Fig. 1 Amphiphilic copolymer (left) and polymersome carrying hydrophobic and hydrophilic drugs (right). (Created with BioRender.com)

capable of carrying molecules (in this example, of drugs) that are also hydrophilic, as well as a peripheral and hydrophobic membrane, where drugs whose nature is similar are inserted.

2 Self-Assembling of Block Copolymers and Polymersomes

Most polymersome preparation methods have been adapted from liposome preparation, such as mass hydration, film rehydration, electroforming, and precipitation techniques [12]. Polymerization methods, such as radical atom transfer polymerization (ATRP), reversible fragment-chain transfer (RAFT), and ring-opening polymerization, allow controlled polymerization to generate polymersomes with specific properties, such as controlled molecular weight [13]. Block properties, such as molecular weight, and hydrophobic-hydrophilic moiety ratio are important in the applicability of polymersomes, and, to increase their versatility, other components can be attached to the blocks, such as low molecular weight biomolecules (peptides, phospholipids, proteins). These are targeting portions, used especially to change permeability, which is an important feature since the hydrophobic part of the membrane makes it difficult to release compounds encapsulated in the hydrophilic core. To control permeability, specific approaches must be adopted in the assembly of polymersomes [14, 15]. A limited number of polymersomes have good permeability intrinsically, as is the case with polystyrene-b-poly(isocyanoalanine(2-thiophene-3-yl-ethyl)amide) (PS-b-PIAT), as the packaging of the molecules that form the coating promotes this characteristic, allowing small molecules to diffuse into the structure and poly(ethylene glycol)-b-poly(2-hydroxypropyl methacrylate) (PEG-b-PHPMA) [16]. Blackman et al. [17] produced PEG-b-PHPMA vesicles using self-assembly induced by visible light polymerization (photo-PISA). The hydration of these membranes is what allows the selective passage of molecules. A fluorescent protein was encapsulated and fluorescence was used to calculate its loading efficiency. The results showed that the protein was stored in pockets within the core of the polymersomes and that the empty vesicles did not fluoresce. Furthermore, horseradish peroxidase (HRP) was encapsulated; its catalytic activity, for converting 3,3′-dimethoxybenzidine (DMB) to a colored dimer product, was evaluated; the enzyme activity was maintained even after entrapment; and no activity was observed for the empty vesicles. Another enzyme, glucose oxidase (GOx), was encapsulated and its interaction with HRP, through a catalytic cascade reaction, was evaluated. The permeability of the membrane allowed that even being in the nuclei of different vesicles the reaction occurred, as the substrates and products could pass from one compartment to another. Another strategy to modulate low permeability is the insertion of channel proteins into the membrane to selectively transport molecules into and out of the cell. Langowska et al. [18] developed polymersomes loaded with the enzyme penicillin acylase, used as a catalyst to synthesize an antibacterial compound, cephalexin, in situ. To the vesicular membranes, bacterial porin outer membrane protein F (OmpF) was added. Free and encapsulated acylase penicillin was

tested in the presence of protein denaturers, to ensure that the catalyzing enzyme was protected in the vesicles, and the conversion to cephalexin only occurred in the encapsulated form, due to the presence of OmpF that allowed the entry of substrates. Polymersomes with encapsulated enzyme but no membrane proteins were also tested and no activity was observed. In addition, the polymersome with protein added to the membrane was also the only one to present antibacterial activity, which shows the effectiveness of membrane proteins in enabling the conversion of substrate into antibiotic by promoting membrane permeability, also promoting the therapeutic effect. Figure 2 shows an example of membrane-coupled channel proteins. The studies demonstrate the importance of adapting the characteristics of the membrane during assembly to the characteristics of the compound to be encapsulated. The increase in membrane permeability, for example, allows the selective passage of components from one medium to another without substances subject to degradation suffering this action in the external environment.

2.1 Techniques Applied in Obtaining Polymersomes

Obtaining polymersomes can be associated with several methodologies, depending on the copolymers used and the type of therapeutic activity to be performed by these structures. The main techniques applied in the preparation of polymeric vesicles involve film rehydration, solid rehydration, electroformation, and nanoprecipitation [19], whose viability can be analyzed from preclinical and clinical studies that require the preparation of vesicles on a reproducible scale, as well as the use of

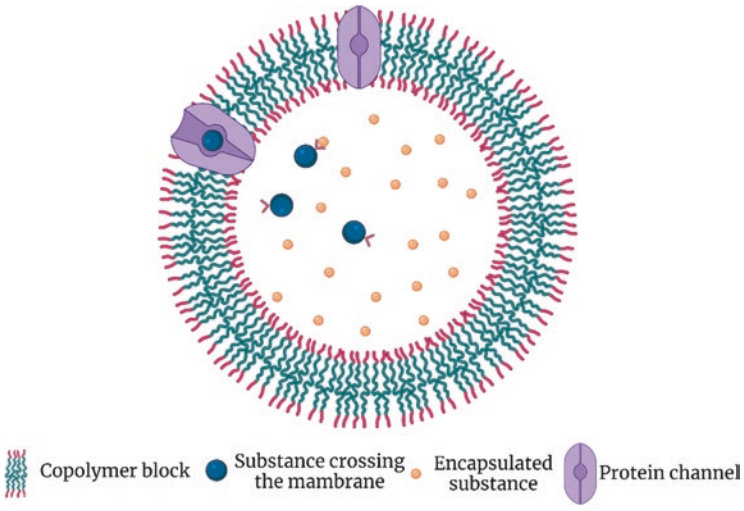

Fig. 2 Channel protein coupled to the copolymer block structure allowing substances to cross the membrane. (Created with BioRender.com)

adequate amounts of organic solvents and degradation products [20]. From this perspective, the main methods for obtaining polymersomes, together with their basic characteristics, will be described below.

2.1.1 Film Rehydration

In this method of obtaining, an amphiphilic film is prepared on a solid surface, which can be a glass bottle or a rough Teflon. This procedure is done, initially, from the dissolution of amphiphilic copolymers in a mixture of organic solvents or in the isolated solvent. Then, the liquid fraction is evaporated and, as a result, a thin film is obtained, which will later be hydrated by a buffer solution, allowing the formation of vesicles on the glass or Teflon vial and dispersion in the solution [21].

2.1.2 Solid Rehydration

The method of obtaining polymersomes by solid rehydration presents operations similar to that of film rehydration, especially in relation to the use of amphiphilic copolymers. However, one of the differences between the techniques is that, in solid rehydration, the hydration of the copolymer does not occur as a film on a solid surface but from an energetic process of strong and prolonged agitation [22]. Thus, initially, the amphiphilic copolymer undergoes a dissolution process in tetrahydrofuran (THF) at 1 wt% to obtain a polymeric solution with a concentration in the range of 0.5–1%. Then, a specific amount of this solution is sucked through a pipette and transferred to a glass container, where the solvent evaporates. Finally, the remaining solid is rehydrated with deionized water and bubbling nitrogen for 1 h [19].

2.1.3 Electroformation

Electroforming represents another way to prepare polymersomes, mainly characterized by the dissolution of amphiphilic copolymers in a 20 mg/L chloroform solution. The result of this mixture can be poured onto indium tin oxide (ITO) sheets, where the drying process will take place under a vacuum or nitrogen. In addition, to create a solution reservoir, a silicone gasket is attached to one of these two blades. Subsequently, the electrodes are immersed in a buffer solution employing a spaced Teflon, while the silicone gasket previously fixed between the blades is glued between the electrodes, which are connected to a generator with specific voltage and frequencies for the formation of the polymeric vesicles. The voltage defined as optimal for obtaining the polymersomes was 5 V [23].

2.1.4 Nanoprecipitation

Nanoprecipitation, also known as the solvent-switch method, is the main technique used for the preparation of polymersomes, whose methodology is based on the dissolution of amphiphilic copolymers in a solvent or mixture of organic solvents. An example is N, N-dimethylformamide (DMF), which is widely used because of its affinity for both hydrophobic and hydrophilic components. Thus, initially, the copolymers can be dissolved in DMF, forming a first solution (DMF + copolymers). Then, to this solution, deionized water is added, which is a solvent intended only for the hydrophilic components, by stirring. This procedure makes it possible to reduce the proportion of solvent to the hydrophobic block, which forms aggregates among themselves and provides turbidity to the solution. The addition continues until a concentration of 25 wt% is reached, giving it a colloidal appearance. Finally, this solution is subjected to a dialysis process in deionized water to remove DMF [22].

3 Biomedical Applications of Polymersomes

Polymersomes are vesicles with structural similarity to liposomes that are capable of self-assembly but differ in that they are made up of amphiphilic copolymers made of covalently connected homopolymers. Polymersomes can be used for medical applications, and the following reasoning should be followed to obtain them: synthesis of the amphiphilic copolymer block, assembly of the copolymer blocks to form the vesicle, targeting of the vesicles by conjugation of specifically binding moieties, and strategies to control the release [24]. As they present a structure with properties similar to those of cell membranes of viruses and capsids, chemical versatility to adjust the properties and functionalities of the membrane, response to physical and biological stimuli, and drug delivery on demand with controlled doses, these structures proved to be extremely attractive for biomedical applications such as drug delivery and diagnostic imaging [25]. It is very common to find in the literature the application of polymersomes for cancer treatment. A great example is a study carried out by Pang et al. [26], where polymersomes have shown promise for DOX delivery across the blood-brain barrier for the treatment of brain tumors, especially after the incorporation of the transferrin antibody on the surface of the polysome, which enhances drug delivery to the brain. In another study also carried out by Pang et al., it was noted that the use of lactoferrin antibody on the surface of the polymersome achieves results similar to those of the use of transferrin antibody, with the outcome being the inhibition of glioma growth and prolonging the life of mice carrying the glioma [27]. Other applications can be used for the delivery of drugs made by polymersomes, such as the transport of antibiotics into infected cells, serving as an alternative to the usual treatment with antimicrobials that can result in resistance by bacteria, making the ineffective treatments. According to the study carried out by Fenaroli et al. [28], PMPC-PDPA polymersomes can be supplemented with small peptides (vancomycin), organic quinolones with low aqueous

solubility (rifampicin), glycols (gentamicin), and several other examples to treat intracellular pathogens causing infection with a lower dose of the drug and in a shorter treatment time to result in the death of the bacteria, being more effective than the drug used freely for *S. aureus*, BCG, *M. tuberculosis*, and *M. marinum* both in in vitro and in vivo tests.

The PMPC-PDPA-type polymersome was also often cited by Lomas et al. [29] in studies with DNA encapsulation. This polymersome is stable at neutral pH and can dissolve completely at cytosolic pH, and the study evaluates the delivery of DNA into cells by analyzing the concentration of plasmid DNA encoding the green fluorescent protein (GFP), as it is only expressed when a DNA sequence has been successfully incorporated into the cell nucleus. In the tests carried out, it was noted that among the various DNA delivery attempts, the ones that had the highest amount of GFP were those that used the polymersome as their carrier.

4 Characteristics of Responsive Polymers

For the construction of a drug carrier, the characteristics of the drug to be transported and the means through which it will pass must be taken into account. The carrier properties should be directed to increase its interaction with the environment, promoting a greater stimulus for the release of the active ingredient and, consequently, reducing adverse effects and increasing its bioavailability. This is how stimuli-sensitive polymersomes work. Because they are designed to respond to a specific type of stimulus, they can release the drug to the specific target, through the conformational modification caused in the nanostructure by the stimulus in the microenvironment [30]. The type of stimulus can be classified as physical, chemical, biological, internal, or external [31]. Internal stimuli are usually physiological differences that occur between healthy and diseased tissues. These differences can be pH, temperature, redox, enzymatic, and glucose concentration, among others. External factors can be light, ultrasound, magnetic field, or mechanical stimuli. Furthermore, polymersomes can also respond to two different stimuli [5]. Figure 3 exemplifies the main types of polymersomes, according to their responsiveness, which will be addressed in this chapter, showing the wide spectrum of possibilities to modulate the transport and release capacity of molecules upon specific stimuli.

4.1 pH-Responsive Polymersomes

Responsiveness to pH is one of the features incorporated into polymersomes by the insertion of specific bonds in their structure. In response, they acquire a chemical affinity for pathophysiological regions whose pH differs from that found in the human organism in ideal situations [26–28]. Therefore, this adaptation directs the polymersomes to selected sites, enabling a better efficiency in the release of

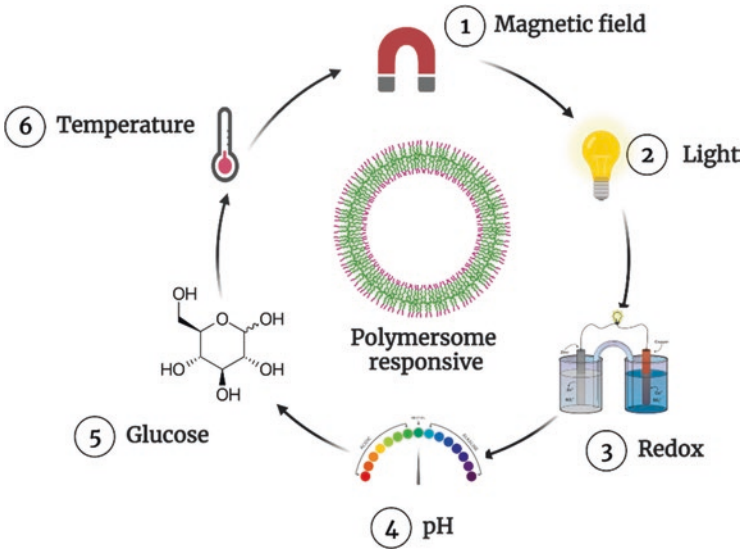

Fig. 3 Main types of responsive polymersomes and their triggers. (Created with BioRender.com)

molecules, as well as in pharmacological or genetic treatments [32, 36]. One of the main methodologies adopted in the synthesis of these polymeric vesicles is the incorporation of acid-cleavable bonds [33, 35, 36]. Historically, the first pH-responsive polymersomes were synthesized from polylactic acid (PLA) and polycaprolactone (PCL) as hydrophobic building blocks [37, 38] as the presence of ester bonds susceptible to hydrolysis at acidic pH in the copolymers allowed the vesicle to break down and release the material inside it [33, 39]. Chen et al. [40] synthesized polymersomes using polyethylene glycol (PEG) and polycarbonate of 2,4,6-trimeth oxybenzylidenepentaerythritol (PTMBPEC), which contained hydrolyzable bonds in acidic medium, allowing the disassembly of the vesicle at sensitive sites in this pH range. Therefore, the use of copolymers with pH-responsive bonds in the self-assembly of the polymersome proved to be a relevant alternative for the improvement of drug and gene delivery systems, as well as the insertion of ionizable groups in copolymer blocks [41, 42] and the formation of polyionic complexes [37, 38], described below. pH-sensitive polymersomes with ionizable groups generally contain weak acids and/or bases with primary, secondary, or tertiary amines. The sensitivity of these bonds comes from ionization, which promotes changes in solubility or conformation in response to environmental pH [43–45]. When there is a variation in the pH of the external environment to that in which the polymersome was programmed to respond, hydrogen bonds and hydrophobic interactions break, forming permeable channels in small and large molecules [40, 46]. Massignani et al. [47] showed that polymersomes with tertiary amines in their composition, such as those produced from poly(2-(diisopropylamino)-ethyl methacrylate) (PDPA), are stable at physiological pH. When these groups ionize, the vesicle is dissolved, which is normally hydrophobic and becomes hydrophilic in an acidic medium. This system

also proved to be effective for the delivery of the antineoplastic drug doxorubicin (DOX) proposed by Yassin et al. [48]. Koide et al. [49] developed polymersomes through the formation of polyionic complexes. Copolymer blocks were mixed with opposite charges containing PEG blockers, and an ionic block was prepared with aniomers and catiomers in an aqueous medium. These polymersomes proved to be more advantageous when compared to traditional ones, as they did not require an organic solvent and were able to easily encapsulate hydrophilic macromolecules [50]. Furthermore, according to Kokuryo et al. [51], when these polyionic complexes have encapsulated contrast agents inside, they can detect and diagnose small tumors in vivo.

4.2 Redox-Responsive Polymersomes

Redox-responsive polymersomes have been widely used to increase the efficiency of delivery systems for molecules at specific sites, mainly through the insertion of disulfide bonds in the structure of these vesicles [52]. Such bonds are sensitive to reducing agents, such as the tripeptide glutathione (GSH), which breaks them down into two thiols, allowing the disassembly of the polymersome and the release of the material trapped inside [32, 53–55]. Therefore, responsiveness to redox action constitutes an important driving mechanism for the delivery of drugs and genes in therapeutic systems. GSH is present at low concentrations in plasma and normal tissues, differing from the cytosol, nucleus, and tumor cells, where the concentration of this reducing agent is typically higher [25]. This enables the release of drugs, for example, in regions affected by tumors, whose GSH concentrations differ from those found in non-neoplastic tissues. Therefore, the disaggregation of the disulfide bonds contained in the polymeric vesicles enables greater efficiency and targeting of pharmacological treatments. Cerritelli et al. [53] developed polymersomes sensitive to reducing environments associating PEG, disulfide bonds, and propylene sulfide (PPS). The main results found reported the disassembly of the polymersomes through the cysteine concentrations inside the cells, resulting in the release of the encapsulated content in about 10 minutes after exposure to the reducing environment. Ren et al. [56] synthesized polymeric vesicles containing PEG and poly(ε-benzyloxycarbonyl-l-lysine) (PzLL) associated with disulfide bonds and loaded with DOX. In this case, the cleavage of the bonds was mediated by the high concentration of GSH in the tumor cells, providing the release of the drug inside the affected cells. In addition, the authors showed that DOX-carrying vesicles showed greater nuclear accumulation after incubation with cells affected by cervical cancer, being extremely useful to reverse drug resistance processes. The incorporation of disulfide bonds into the polymersome structure can also occur through crosslinking. In this perspective, Xu et al. [32] developed a polymersome from a triblock of copolymers associated with crosslinks using cysteine. The main findings of the experiment pointed to greater ease of dissociation in reducing environments, resulting, consequently, in the release of the inoculated content in the polymeric vesicle. As for

oxidizing agents, it is important to mention reactive oxygen species (ROS), which, although they arise from endogenous processes and are important for physiological roles, such as apoptosis, immune response, and cell proliferation [57], in excess can be indicative of oxidative stress present in various physiological conditions such as inflammation, diabetes, infection, cardiovascular disease, and cancer [58, 59].

In this perspective, Hubbell et al. (2004) [60] used, for the first time, oxidation to destabilize polymersomes, more specifically with the triblock synthesis of copolymers capable of vesicular self-assembly in the presence of aqueous solutions. This system was later used when evaluating the delivery of antigens inserted into a copolymer of PEG-b-PPS to analyze the immune response induced by the oxidation and release of these antigens. Boronic esters were also responsive to H_2O_2, leading to the self-assembly of copolymers into spherical nanoparticles and polymersomes in an aqueous environment [61]. Additionally, Yu et al. [62] reported the existence of hypoxia-responsive and hypoxia- and H_2O_2-sensitive vesicles. In the latter system, the synergistic action resulted in a better release of the contents of the interior of these polymersomes, thus favoring the transport of drugs and genes.

4.3 Temperature-Responsive Polymersomes

For the construction of temperature-responsive polymersomes, polymers capable of modifying their morphology as the temperature varies and thus releasing the encapsulated molecules are used. Upon reaching a certain temperature, these polymers become destabilized and a phase transition occurs, which leads to changes in the polymeric conformation, solubility, and hydrophilic-hydrophobic balance. They can be classified as higher solution critical temperature (UCST), which are those that become more soluble after heating; lower solution critical temperature (LCST), which are those that become less soluble with increasing temperature; or both [4, 24]. The polymers most commonly employed for this purpose include PNIPAAm (poly(N-isopropylacrylamide), poly(methyl vinyl ether), N,N-diethylacrylamide, poly(N-vinylcaprolactam), and poly(N-ethyloxazoline), [24] with PNIPAAm the most commonly used polymer to assemble temperature-sensitive polymersomes as it can vary between hydrophilic and hydrophobic when the temperature is changed around its LCST which is 32 °C and is just below body temperature. When it is applied as part hydrophobic of a system, it can self-assemble forming stable polymers at 37 °C (body temperature), as the structure dehydrates, which becomes insoluble, while at 32 °C it disassembles and releases the encapsulated molecules, as the polymer chains are hydrated and solubilize in an aqueous medium [30, 63]. The temperature stimulus, which causes the polymers to destabilize and release the encapsulated compound, can be internal or external stimulus [4]. As an internal release stimulus, solid tumors can be highlighted, where the local temperature is slightly (2–5 °C) higher than the body temperature, allowing the release system to accumulate in the tumor, thus providing a more targeted therapeutic alternative for the treatment of cancer. When the decrease in temperature applied to the tissue

comes from the external environment, it can come from focused ultrasound or hyperthermia, for example [64]. Kozlovskaya et al. developed thermosensitive polymersomes that self-assemble in water at temperatures around 20 °C, using copolymers composed of amphiphilic diblock of poly(3-methyl-N-vinylcaprolactam)-b-PVPONn (where *n* is the number of blocks, which were 20, 65, and 98). The copolymers aimed to store the drug promoting its release in situ, i.e., only in the tumor, when there was a change of temperature, to reduce the cardiotoxic effects of doxorubicin. After 30 days of dialysis at room temperature, the concentration of the drug in the solution was <5%. Quantity is considered low when compared to conventional liposomes, which present high leakage in vitro and blood at the same temperature. Structural stability was also observed in serum, and there was no change in size or aggregation in vitro. In the in vivo assay, a dose of 15 mg/kg of the free drug caused 100% mortality, while with the same dose of drug loaded in liposomes (Lipo-DOX) and polymersomes (Poly-DOX), all mice survived. For the Lipo-DOX system, however, gravimetric analyses showed atrophic toxicity, with a reduction in body weight in comparison with the group that received Poly-DOX and saline solution, in addition to a reduction in lung and splenic masses. The results demonstrate the efficacy of the polymeric system in storing the drug and releasing it at a specific site, reducing toxicity [65].

4.4 Light-Responsive Polymersomes

Light responsiveness is a particularly attractive feature to be added to polymersomes, as it is an external stimulus that does not require modification of the environment through the addition of reagents, such as acids or bases. Besides the responsiveness that can be induced in a specific location, the specific moment can also be elected by exposure to certain wavelengths, which can be in the visible, ultraviolet (UV), or near-infrared (NIR) light range, and is given by the addition of photosensitive groups incorporated to the polymers that suffer degradation under the action of light. The release profile of this type of vesicle may be adjusted by modifying the wavelength of light, exposure time, and intensity [31, 66]. A photolabile chromophore group must be added to the amphiphilic polymers for light responsiveness, such as azobenzene and spironane, which are groups that react by photoisomerization, and nitrobenzyl and coumarin derivatives, which react by cleavage [5]. Responsiveness can also be classified as reversible or irreversible, depending on the chromophore used [67]. Regarding the wavelength used, the light in the UV range has presented limitations because it has low penetration due to the large dispersion in tissues. The light in the near-infrared range (650–900 nm) has been of greater interest for biomedical applications because it presents low absorption by the skin and tissues, promoting better penetration [5, 68]. Zhou et al. [69] developed a light-responsive polymersome, named C12NB, with o-nitrobenzyl acting as the chromophore group and hydrophobic portion. The light sensitivity was confirmed by the UV-vis absorption spectrum, where changes in absorption were

observed after light irradiation. Hydrophobic and hydrophilic model drugs were used to test the release from the vesicles. In the assays, the substances showed little or no release when kept in the dark and significant release when exposed to radiation. The exposure time was directly proportional to the amount released. Doxorubicin hydrochloride (DOX-HCl) was also tested, and its effect on cancer cells and the effect was compared with the free drug. After incubation, the PC12NB + DOX complex decreased cell viability better than the free drug, probably due to targeting provided by the polymersome system. PC12NB alone showed no cytotoxic effect, showing that there was no influence of copolymers on the cytotoxic effect. The PC12NB + DOX + folic acid system was also tested, like folic acid (FA) which showed to increase cellular uptake of substances in cancer cells, which further reduced cell viability, as FA produced active targeting. Overall, polymersome PC12NB exerted satisfactory targeting, which in practice culminates in a less adverse reaction and increased the effect of the drug, showing to be a good candidate for the delivery of drugs to specific sites dependent on light stimulation.

4.5 Magnetic Field-Responsive Polymersomes

Magnetic field-responsive polymersomes are generally hydrophobic superparamagnetic nanoparticles included in hydrophobic polymersome membranes during their formation [24]. These polymersomes are attracted to specific sites due to their properties and can also be useful as contrast agents for imaging and used in diagnosis due to their noninvasive and easily controlled nature [70]. The polymersome can be deformed and transformed by the incorporation of these magnetic particles upon application of an external magnetic field, which results in the transient opening of the polymersome bilayer and release of the contents contained within the capsule [71]. Lecommandoux et al. [70] studied on magnetic field-triggered drug release and also the deformation that occurs in vesicle membranes by hyperthermia during magnetic field application, and they used self-assembled polymersomes of poly(trimethylene carbonate)-b-poly(L-glutamic acid) copolymer block (PTMC-b-PGA) with ultra-small superparamagnetic iron oxide nanoparticles and the antineoplastic drug Dox inside. In another work, they also carried out the use of amphiphilic polybutadiene-b-poly(glutamic acid) diblock copolymers (PBD-b PGA) for the preparation of micelles and vesicles. Sanson et al. [71], on the other hand, prepared multifunctional SPIO/Dox polymer vesicles for the investigation of their potential use in targeted therapy in cancer and also for imaging. Yang et al. [72] prepared polymers loaded with Fe_3O_4 nanoparticles present in the bilayer membrane at the hydrophobic-hydrophilic interface, and the formation of a bilayer bridge and oligo- and multilamellar vesicles occurs. Although most magnetic field-responsive systems are magnetic nanoparticles encapsulated inside polymersomes, some studies such as those by Rikken et al. [73] have demonstrated that amphiphilic copolymer blocks for magnetic manipulation can be effective by using PEG-b-PS amphiphilic copolymer blocks and regulating their shape in a restricted manner. These blocks

can have their aperture reversibly changed due to high anisotropic magnetic susceptibility, and these controlled apertures are under study for possible future applications.

4.6 Glucose-Responsive Polymersomes

Glucose-responsive systems have received great attention for their potential in insulin transport and delivery used to treat diabetes mellitus. Three glucose-responsive portions are widely used to structure the polymersomes and control the release rate of the encapsulated compound, glucose oxidase (GOx), glucose-binding proteins, and boronic acids. The encapsulation of insulin in polymeric vesicles for oral administration has been one of the focuses, as it can mimic the biodistribution of endogenous insulin and respond to the glucose concentration gradient, being released only when glucose levels are high, avoiding hypoglycemia pictures [68, 74]. Wang et al. [74] produced glucose-responsive polymersomes to transport insulin, using GOx as the glucose concentration-sensitive portion, which is orally administered and accumulates in the liver. Insulin release profiles were tested using a medium with varying glucose concentrations, and release was shown to be directly proportional to concentration. Size and morphology remained largely unchanged at low glucose concentrations, whereas at higher concentrations variation in size was observed. The vesicles were stable at pH 1.2 and 6.8, with insulin release of less than 10%, allowing the conclusion that they would remain stable in the stomach environment. In vivo studies with diabetic rats were performed, and the formulation was able to maintain postprandial glucose levels similar to those of healthy rats. Accumulation in the liver was desired so that the encapsulated insulin would make the same pathway as endogenous insulin and was estimated by fluorescence. The results showed high fluorescence intensity in the rat hepatocytes, indicating accumulation of polymeric vesicles. The rate of insulin release was measured and rapid response of the polymersomes was observed when the glucose concentration was increased. Yang et al. [75] manufactured glucose-responsive polymersomes from the complexation of the copolymers glucosamine (GA) PEG45-bP(Asp-co-AspGA) and PEG114-bP-(Asp-co-AspPBA), PBA being phenylboronic acid, using the α-inclusion complex cyclodextrin/PEG45 as a model structure, where the α-CD was later removed. The glucose response was evaluated employing dynamic light scattering (DLS) evaluating the light scattering intensity (LSI), which was stable for the vesicles in the absence of glucose. In the presence of glucose, the LSI increased rapidly, and this increase was faster as the amount of glucose increased. The hydrodynamic diameter (Dh) was also measured and increased according to the glucose concentration, probably because, in the presence of the trigger, the degree of cross-linking of the vesicular membrane decreases. Vancomycin was chosen as the model drug to be encapsulated, and it showed rapid release in the first 3 h in a glucose medium. In the medium with fructose, the release was even faster, probably because of its interaction with PBA, which is higher. In the medium without added sugar, there was little leakage of drug from inside the vesicles, which only occurred after

Table 1 List of polymersomes responsive to different stimuli and the preparation methods used for each one

Copolymer	Stimulus type	Preparation method	References
Poly(2-methacryloyloxyethyl phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA)	pH	Atom-transfer radical polymerization (ATRP)	[76]
Poly(ethylene oxide)-*b*-poly(2-((((5-methyl-2-(2,4,6-trimethoxyphenyl)-1,3-dioxan-5-yl) methoxy)carbonyl)amino)ethyl methacrylate) (PEO-*b*-PTTAMA)	pH	Reversible addition-fragmentation chain transfer (RAFT)	[33]
Poly(ethylene oxide)-*b*-poly(glycerol monomethacrylate)	Redox	Reversible addition-fragmentation chain transfer (RAFT)	[77]
Poly(ethylene glycol)-polylactic acid-poly(ethylene glycol)	Redox	Nanoprecipitation	[78]
Poly(ethylene oxide)-*b*-poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide) (PEO-PAA-PNIPAM)	Temperature	Reversible addition-fragmentation chain transfer (RAFT)	[32]
Poly(trimethylene carbonate)-*b*-poly(L-glutamic acid)	Temperature	Nanoprecipitation	[79]
Poly(pentafluorophenyl methacrylate)	Light	Reversible addition-fragmentation chain transfer (RAFT)	[80]
Poly(methyl caprolactone-*ONB*-poly(acrylic acid) (PMCL-*ONB*-PAA)	Light	Co-solvent	[81]
Poly(trimethylene carbonate)-*block*-poly(L-glutamic acid) (PTMC-*b*-PGA)	Magnetic field	Nanoprecipitation	[70]
Poly(trimethylene carbonate)-*b*-(L-glutamic acid)	Magnetic field	Nanoprecipitation	[71]
Benzoboroxolo (*o*-hydroxymethylphenylboronic acid)	Glucose	Reversible addition-fragmentation chain transfer (RAFT)	[82]
Poly(ethylene glycol)-*block*-poly(2-hydroxypropyl methacrylate)	Glucose	Reversible addition-fragmentation chain transfer (RAFT)	[83]

14 h of incubation. Vancomycin is a drug with a short half-life, and its encapsulation in controlled release systems can reduce the number of administrations required and reduce adverse effects (Table 1).

5 Polymersomes in Therapeutics Systems

Some drugs formed by macromolecules have shown to be very promising in the therapy for achieving excellent clinical results and becoming a viable alternative for the treatment of the most diverse diseases. Some examples of these macromolecules

are antibodies, peptides, proteins, and nucleic acids, which, compared to drugs composed of small molecules, have the advantage of having a greater specificity for the disease in the question of greater therapeutic activity due to their complex structural composition [84]. The major problem in the use of these macromolecules is precisely due to their structural complexity, which makes their delivery challenging. Studies have shown that macromolecules have poor in vitro stability due to the chemical and physical degradation they can undergo, in addition to activating the immune system and having a short blood half-life, as well as not being able to easily cross cell membranes [12]. A solution to these obstacles was the use of nanostructures for the delivery of these macromolecular drugs, since these structures offer greater protection, prolonged control, targeted delivery, and a longer blood half-life for these macromolecular drugs, thus being alternatives that promote greater efficacy and safety in therapy [85]. Polymersomes are structures composed of synthetic polymeric vesicles analogous to intracellular organelles that aim to mimic cellular structures and their functions [86, 87], whereas intracellular organelles are natural compartments that protect, isolate, and organize macromolecules within them [88]. The polymersomes are composed of amphiphilic copolymer blocks with an aqueous interior and a lipid bilayer that allows greater mechanical and physical stability when compared to other carrier nanostructures. Polymersomes can carry drugs both in their aqueous interior and in their lipid membranes, being an alternative with greater application potential when compared to other carriers [7, 89]. Polymersomes also have the advantage of colloidal stability and adjustable membrane permeability, thus making them an excellent alternative for macromolecular drug delivery [68]. These copolymer blocks can also be carriers for genes to enter the interior of cells, thus being an effective therapeutic alternative for genetic diseases. The biggest problem is that genes are structures sensitive to changes in pH and temperature, in addition to not being able to resist metabolism for a long time when administered without a carrier and being excreted or attacked by cells of the immune system before they can enter cells (which is also not possible without a carrier). Thus, it is of great importance to study the incorporation of polymersomes in therapy, always to improve available treatments by optimizing the incredible properties of macromolecules. Figure 4 shows the key macromolecules that can be incorporated into polymersomes for optimized cellular delivery. Note the location of hydrophobic drugs (stored in the lipid bilayer), while hydrophilic drugs are stored in the aqueous interior of the polymersome, as are peptides, genes, and proteins.

5.1 Polymersomes in Drug Delivery Systems

New technological perspectives for the creation and improvement of drug delivery systems have been described as important tools that contribute to the optimization of pharmacological treatments. Within this context, polymersomes stand out due to their morphological modulation capacity, which consequently reflects on the chemical properties and functionalities presented by these structures, whose

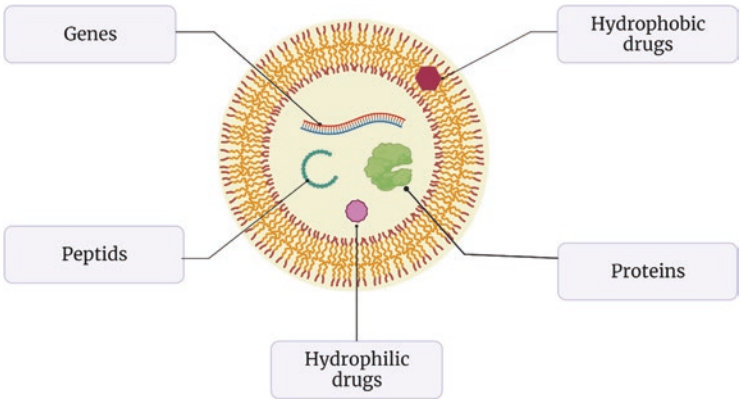

Fig. 4 Main types of macromolecules and their respective locations in polymersomes. (Created with BioRender.com)

characteristics allow the disaggregation of the polymeric vesicle and release of the drug encapsulated inside through internal or external stimuli [90]. In addition, when compared to liposomes, polymersomes have greater stability and the ability to protect sensitive materials inside, making them structures capable of carrying drugs [91]. Drug release by polymeric systems is triggered by changes in the balance of the hydrophobic and hydrophilic portions contained in the copolymer blocks used in the synthesis of polymersomes. Some examples are polyacrylic acid and polyethylene glycol (PEG), which are hydrophilic in nature, as well as polystyrene (PS) and polycaprolactone (PCL), which are hydrophobic in nature. Therefore, the presence of these copolymers allows the dissociation of the vesicle in specific environments through responsiveness to stimuli capable of promoting structural changes in the vesicles and their subsequent degradation [24]. Thus, the encapsulation of drugs within the polymersomes provides better targeting of these molecules to their release site, as well as the reduction of adverse effects in pharmacological treatments, enhancing the pharmacokinetics and biodistribution of drugs in the body. Figure 5 illustrates the endocytosis process undergone by a polymersome when in contact with the plasma membrane of living cells. In this example, we have a pH-responsive polymeric vesicle, which can be acidic or alkaline depending on the physiological characteristics of the site where the molecule (in this case, a hydrophilic drug) will be released. As illustrated in the Fig. 5, when the polymersome enters the cell and undergoes the action of a specific pH, its structure is dismantled and, consequently, the drug is released to exert its therapeutic function in the target compartment, conferring greater specificity in the drug delivery process.

However, for a polymeric drug release system to be efficient, some points need to be evaluated before its design, mainly in terms of solubility, both of the drug and the polymer. Hydrophilic drugs, for example, capture water to the system, promoting a greater degradation of the polymersome and, consequently, the release of the substance to a target site [92]. Other important aspects concern the molecular weight of the drug and the miscibility in the polymeric matrix because these parameters

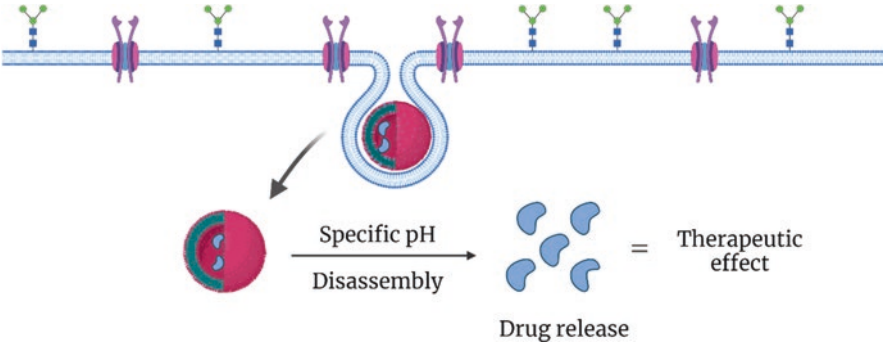

Fig. 5 Endocytosis, disassembly and release of a drug encapsulated in the hydrophilic core of a polymersome. (Created with BioRender.com)

directly influence the concentration profile of the drug and its distribution throughout the system, and the same drug may present different diffusivity and concentration rates depending on the composition and structure of the polymer used [4]. The molecular weight, for example, has great relevance in the kinetics of polymersome release. Generally, when the polymeric systems are structured from polymers with low molecular weight, they present a process of disaggregation and, consequently, drug release, faster, because this factor favors the structural disintegration of the polymers [93]. In addition, Massignani et al. reiterate that the structural organization of polymeric vesicles favors the carriage of hydrophobic, hydrophilic, or amphiphilic compounds because they can be incorporated either into the aqueous core (hydrophilic) or the lipid bilayer (hydrophobic) [94]. These attributes further favor the use of polymersomes in drug delivery systems, given that different molecules, in physicochemical terms, can be incorporated into polymeric vesicles using this mechanism. This occurs mainly by dissolving or dispersing the blocks of copolymers that make up the membrane in an organic solvent, followed by the addition of water or another aqueous solution to the solution or dispersion obtained [11, 40]. Thus, it is possible to visualize how the characteristics of the polymer and the drug used can interfere in the polymeric delivery system and, consequently, how the possibility of molding and organizing them, according to their physicochemical characteristics, can optimize the delivery of drugs in specific locations, corroborating the application of polymersomes in various therapeutic systems.

5.1.1 Hydrophobic Drug Carriers

The use of polymersomes as carriers for hydrophobic drugs has aided the targeted delivery of these molecules in various pathological situations. Paclitaxel (PTX), for example, is a drug used in the treatment of neoplasms, mainly breast and ovarian cancer [95]. However, it presents high lipophilicity, which hinders its solubilization in aqueous fluids, interfering with the absorption process by the organism and in the efficacy of the pharmacological treatment directed to cancer. Moreover,

adverse effects such as nephrotoxicity, neurotoxicity, and hypersensitivity have been reported for the commercial form of PTX, Taxol, which uses Cremophor EL as a solvent, responsible for most of these effects [96]. In this sense, the use of polymersomes as polymeric systems for the delivery of PTX presents itself as an important tool for optimization of its pharmacological activities and reduction of the adverse effects inherent to the administration of the drug for the treatment of cancer. Simón-Gracia et al. synthesized polymersomes using a copolymer diblock composed of poly(oligoethylene glycol methacrylate) (POEGMA) and poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) to evaluate PTX release in intraperitoneal cancer cases [97]. POEGMA is a hydrophilic block composed of polyethylene oxide, which presents the capacity of protecting polymersomes from immune system attack, prolonging the half-life of these structures inside the living organism [98]. The PDPA, on the other hand, does not present solubility in water but attributes to the polymersomes the capacity to respond to a slightly acid pH, allowing the disaggregation of the polymeric vesicle by the protonation of amines of the copolymer and the subsequent release of PTX in this specific environment [44]. The main results of this study showed that PTX-loaded polymersomes decreased about 50% of cell viability, besides presenting toxic activity directed to cancer cells, allowing a lower occurrence of adverse effects. Similarly, Bleul et al. [99] prepared polymersomes through a copolymer triblock (Pluronic L-121), carrying the antineoplastic drug camptothecin (CPT), cytotoxic and lipophilic, along with magnetic nanoparticles. The hydrophilic portion of Pluronic L-121, represented by the copolymer poly(ethylene oxide) (PEO), prevents the absorption of the polymeric vesicle by macrophages, having a protective function against the immune system [100]. On the other hand, the hydrophobic portion, constituted by the copolymer poly-(propylene oxide) (PPO), can be used to incorporate hydrophobic drugs [101], such as CPT. Moreover, the addition of magnetic nanoparticles in the structure of polymersomes enables the guided transport, through the magnetic field, of the drug to the cancer cells, avoiding adverse effects in adjacent tissues. The main findings of the study developed by the authors showed that the polymersomes loaded with CPT in its hydrophobic membrane reduced the viability of cells affected with prostate cancer from 40% to 20%, besides allowing a ten times higher dose of the drug, which is limited in free form by its low aqueous solubility. Therefore, the use of polymersomes as carriers of CPT, as well as PTX, discussed in this topic, has great relevance in the controlled release of these drugs and, especially, in reducing adverse effects caused by the targeting of drugs to undesired sites.

5.1.2 Hydrophilic Drug Carriers

Hydrophilic drugs, whose chemical structure is easily solubilized in aqueous fluids, can be carried in polymersomes through active or passive strategies. In the passive carriage, they are introduced inside the aqueous center of the vesicle, which presents similar polarity to the drug, allowing a better interaction in

physicochemical terms. The active carrier method, on the other hand, is generally used in ionizable substrates with ease of diffusion, based on a pH transmembrane gradient. Thus, the species devoid of electrical charge can permeate the membrane, by diffusion, and, from there, are directed to the core of the vesicle, which has a hydrophilic character [102]. In this sense, many hydrophilic drugs, with different therapeutic effects, can be carried in polymersomes as a way to enhance the biodistribution of these molecules in the body. An example, described by Broz et al. [103], is the pravastatin, a drug used in the treatment of atherosclerosis and in the prevention of cardiac effects, which was encapsulated in polymersomes produced from the copolymers poly(2-methyl-2-oxazoline) (PMOXA) and poly(dimethylsiloxane) (PDMS), constituting the PMOXA-PDMS-PMOXA triblock. The polymeric structures, associated with a sequence of oligonucleotides (polyG), were tested in muscular cells of rats and did not present toxic effects to the animals at the moment the drug was released in the target cells, showing to be useful, also, in the protection of the encapsulated molecule against macrophages. Usually, for these effects to be achieved with the administration of the free drug, a higher dose of the drug is required, increasing the possibility of adverse reactions, which, in this sense, can be avoided by directing pravastatin only to the diseased cells. Du et al. [44] encapsulated DOX, an equally hydrophilic drug, in pH-responsive polymersomes using the copolymers poly(2-(methacryloyloxy) ethyl-phosphorylcholine) (PMPC) and poly(2-(diisopropylamino)ethyl methacrylate) (PDPA), forming the diblock PMPC-PDPA. The vesicles were spontaneously structured employing adjustments in the pH of the solution, which went from 2 to a value higher than 6, and the hydrophobic chains, constituted by the PDPA copolymer, composed the walls of the polymersomes. From this, the authors evidenced high efficiency in the delivery of DOX to the target cells and, consequently, a decrease in the side effects caused by the drug. However, further studies regarding the biodegradability and cytotoxicity of polymersomes, in this case, are necessary to confirm the feasibility of using this type of carrier for the drug under analysis. Another study, developed by Qin et al. [104], reports the possibility of DOX carriage in temperature-responsive polymersomes, synthesized from poly(ethylene oxide) (PEO) and poly(N-isopropylacrylamide) (PNIPAm) copolymers. The synthesized vesicles showed stability when submitted to body temperature (37 °C), which allows the disintegration of the polymeric vesicle and the subsequent controlled release of the drug by the presence of a specific temperature gradient. Moreover, the vesicles produced allows the carriage of both hydrophilic drugs, such as DOX, and hydrophobic ones, expanding the spectrum of possibilities for the use of polymersomes in various pathological situations. Therefore, the results presented above for the transport of the drugs pravastatin and DOX collaborate with positive notes regarding the use of polymersomes and their therapeutic efficacy in the cases studied.

5.2 Polymersomes in Gene Delivery

Nucleic acids play vital roles in all living organisms. Diseases that occur at the genetic level are considered to be of relative importance and complexity and were previously considered untreatable. Gene therapy is now a new, powerful, and specific model of therapy for these diseases. Due to their structural complexity, genes need systems with specific characteristics to transport them, since they are molecules that are sensitive to environmental conditions, subject to physical and chemical degradation, with low absorption, and are rapidly metabolized by the body. Their free circulation can lead to the activation of immune cells, and they have low permeability in the cell membrane. Due to these characteristics, a carrier for this type of molecule should offer protection and efficient delivery, in specific locations, to avoid the genetic material suffering, among other factors, from enzymatic degradation by nuclease action, because the gene does not present directed intracellular traffic. It should ideally have low cellular toxicity, deliver the molecules in their active conformation, and be biocompatible. Polymersomes are carriers with modulatable characteristics and can adapt to the needs demanded by the genetic material [12, 105, 106].

Polymersomes have proven to be a good alternative for gene transport because they retain the content safely and have good stability [105]. Their membranes can be up to 10 times thicker than other vesicle carriers, such as liposomes, increasing, consequently, their robustness and stability. This last characteristic is directly linked to the molecular weight of the polymers used, that is, the higher the molecular weight, the greater the resistance to factors that may damage their structural integrity, such as erosion. As they can be constructed to be stimulus-responsive, they present, thus, greater specificity regarding the site of release of the material carried, preventing the degradation of the genetic material and taking it to the cell nucleus [4]. Because most of them are hydrophilic in nature, macromolecules such as proteins, peptides, immunoglobulins, and genes are favorably encapsulated in the aqueous nucleus of polymersomes. Laskar et al. [107] developed cationic, pH-responsive polymersomes by spontaneous formation using the copolymers poly-[(2-(dimethylamino)ethyl methacrylate)x-co-methoxy poly(ethylene glycol) methacrylate)y] and poly[DMAEMx-co-mPEGy], DMAEM being N,N-(dimethylamino) ethyl methacrylate. The mPEG monomers of DMAEM were joined in different proportions by random polymerization. Among several features, the self-assembly behavior of the cationic polymers and their potential for gene transfection by condensation and transfection of plasmid DNA (pDNA) inside the cell were evaluated. The cytotoxicity of the pure polymer and the polymer after complexation were evaluated and both systems showed low toxicity. The positively charged surface at pH 7 is a favorable factor for gene transport, and to evaluate the stability of the polymers in the presence of pDNA, high-resolution transmission electron microscopy (HRTEM) was used and sets of vesicles were observed, which proves the robustness and stability of the polymeric system. The transfection assay was performed on the breast cancer cell line MDAMA-231 and the results were output in

fluorescence. High fluorescence intensity in the cells indicates expression of the transfected gene and thus high transfection efficiency. This efficiency was higher for the complexes that had higher polymer to pDNA ratios and hence higher molecular weight. The results show the efficiency of polymersome vesicles in protecting and transporting genetic material.

5.3 Use of Polymersomes in Diseases

The self-assembly and modulation capacity of polymersomes enable the use of these structures under varied biomedical perspectives, especially through the controlled release of drugs at predetermined sites, optimizing their pharmacokinetics and biodistribution in the organism [108, 109]. The insertion of ligands on the surface of polymeric vesicles can improve the uptake of anticancer molecules from the targeted interaction with receptors expressed on tumor cells [110, 111]. In view of this, many studies reported the encapsulation of drugs in polymersomes in the treatment of cancers to improve the therapeutic effect of drug actions and prevent the spread of pharmacologically active substances to nontarget sites. In this perspective, Alibolandi et al. [112] developed folic acid (FA) nanopolymersomes conjugated with PEG and PGLA to carry DOX and *quantum dot* in therapy against breast cancer. The use of FA in this polymeric preparation was justified by the existence of receptors for folate in tumor cells, allowing the disaggregation of the polymersome and the release of the drug in the desired neoplastic site. Moreover, in vivo experiments showed that animals treated with these nanopolymersomes showed no histopathological and physiological changes in the long term, reiterating the effectiveness of polymersomes in therapy against breast cancer. Similarly, Fang et al. [113] synthesized polymersomes loaded with DOX and the peptide GE11 to target the encounter with epidermal growth factor (EGFR), whose overexpression is evidenced in mice affected with pancreatic cancer. Therefore, in this experiment, GE11 functioned as a signaling agent for DOX release by interacting with EGFR specifically in diseased cells, without compromising adjacent body sites, corroborating Alibolandi et al. [112] on the effectiveness of polymersomes to improve drug targeting in both breast and pancreatic cancer. Besides its relevance in the treatment of several types of cancer, there is evidence of the use of polymersomes in the treatment of bacterial infections. Fenaroli et al. [28] reported the efficacy and enhanced bactericidal and bacteriostatic power of using pH-responsive poly(2-(methacryloyloxy) ethyl-phosphorylcholine)-co-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) copolymer blocks for the delivery of antibiotics such as gentamicin, vancomycin, rifampicin, and isoniazid into macrophages (in vitro) and into zebrafish embryos (in vivo) against *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*. The ability of polymersomes to penetrate difficult-to-reach environments, such as granulomas tissues, to effectively eradicate and decrease the proliferation of intracellular bacteria was also verified. Another study, conducted by Porges et al. [114], used a copolymer block composed of

poly(ethylene oxide)-polycaprolactone for the delivery of antibiotics, in an intracellular manner, against *Burkholderia pseudomallei*, the etiological agent of melioidosis, obtaining the bacteriostatic result. Tuberculosis, whose etiological agent is *M. tuberculosis*, is difficult to treat with several antibiotics and has a high rate of bacterial resistance. Therefore, there is a need for the use of therapeutic alternatives, such as the PMPC-PDPA polymersomes, because the PMPC presents affinity for the scavenger B1 receptor, which results in specificity for nonprofessional phagocytic cells [115]. Furthermore, the pH-sensitive PDPA causes optimal drug delivery to the cytosol and endosome, as well as its internalization [26, 105, 116, 117]. In the in vivo study performed by Fenaroli et al. [28], infected zebrafish embryos were used, and it was found that the antibiotic activity was higher when compared to these drugs used freely both in vitro and in vivo, thus resulting in a faster eradication of microorganisms with lower doses and a lower risk of bacterial resistance.

5.4 Other Applications for Polymersomes

Polymersomes can be used for other therapeutic applications, as reported by Quer et al. [118] when using pH-sensitive polymersomes constructed with the polymer diblock poly[oligo(ethylene glycol) methyl methacrylate]-b-poly(2-(diisopropylamino)ethyl methacrylate) (POEGMA-b-PDPA) and Angiopep-2 (A-EP) on its surface to cross the CNS and deliver IgG macromolecules into its cells and parenchyma. It was observed that the polymersomes were able to cross the endothelium of the BHE in the in vitro studies by active transport, while in the in vivo studies it was reported that the polymersomes were able to prevent the systemic degradation of IgG and allow its proper penetration into the parenchyma and neurons and glial cells. In a study by Tai et al. [119], polymersomes were used for subcutaneous insulin delivery. The team used the PEG-based copolymer diblock and ketal-modified polyserine (PEG poly-Ser-Ketal) with insulin inside and reported that the polymersomes led to easier normalization of blood glucose levels to a normoglycemic state (levels less than 200 mg/dL) for up to 5 days. Kelly et al. [120] used the pH-responsive PEG-b-PLA polymersome with apolipoprotein E (Apo E) on its surface to perform β-galactosidase (βgal) delivery for lysosomal storage disease via enzyme-based therapy to the brain. These polymersomes demonstrated greater release at the pH of 4.8 characteristics of the lysosome and were able to restore βgal activity in GM1 gangliosidosis cells to normal levels. In vivo studies are being carried out in cats with GM1 gangliosidosis. Chen et al. [121] used polymeric vesicles based on amphiphilic graft copolymer for the delivery of bioactive vascular endothelial growth factor (VEGF) since angiogenic factors when released in a controlled manner can lead to tissue regeneration. Poly(L-lysine)-g-poly(lysine-(arachidic acid)) copolymer was used to properly encapsulate and condense the VEFG without damaging the size and surface charge of the polymers. In the same study, the use of polymers grafted with arachidonic acid was compared with those not grafted for the release of VEGF, and the results show that those grafted resulted

in greater release of VEGF (2.23 mg/mL), in addition to resulting in more efficient transfection and with fewer toxicity, thanks to the nature of the vesicular peptides. Finally, Liu et al. [122] made the use of theranostic vesicles for the application of magnetic resonance and also for the administration of antineoplastic drugs. In a study by Hao et al. [123], the amphiphilic diblock copolymer PEG-b-PLGA was used to encapsulate BSA-gadolinium (BSA-Gd) complexes. In vitro and in vivo results showed excellent T1-weighted magnetic resonance imaging, while the delivery of the antineoplastic DOX when administered together with BSA-Gd led to slower drug release when compared to BSA complexes, probably due to electrostatic and hydrophobic interactions between DOX and protein.

6 Conclusion

Polymersomes have emerged as a new tool to aid delivery systems for molecules, which, as elucidated in this chapter, include drugs, genes, proteins, and peptides. As described in this chapter, they are structures that bring the perspective of promising advances within nanobiotechnology, due to their intrinsic characteristics, applications, and abilities to potentiate pharmacological and genetic treatments through responsiveness to stimuli, such as luminosity, temperature, magnetic field, and pH variations, among others. With the notes presented, it was verified in several studies that polymersomes have the ability to minimize adverse effects in pharmacological treatments, which are very relevant events in contemporary society, as well as to enable the safe delivery of sensitive molecules, such as acids nucleic acids. As a result, these polymeric vesicles can be used in different functional systems, given their ability to morphologically modulate from the insertion of copolymers and other molecules capable of conferring distinct characteristics to the polymersomes, corroborating their broad spectrum of therapeutic targets.

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Quantum Dot-Based Nanomaterials for Diagnostic and Therapeutic Applications

Songul Ulag and Oguzhan Gunduz

1 Introduction

Nanotechnology covers understanding and managing materials in the 1–100 nm range. Over the past two decades, there have been many improved nanomaterials for diagnostic and therapeutic fields [1]. The area of nanomaterials for diagnostic/therapeutic purposes is vast. Using nanomaterials, drug delivery and imaging take advantage of anatomical changes combined with pathophysiological statements in the pathologic area. Nanomaterials usually accumulate in higher amounts than drugs used in the diseased area. This improved drug targeting causes reduced toxicity and successful delivery [2]. Nanomaterials can be named zero-dimensional such as nanoparticles (NPs), one-dimensional such as nanotubes and nanorods, and two-dimensional, such as nanofilms, graphene, or nanosheets [3]. Since nanomaterials have outstanding chemical, physical, and mechanical properties, new-generation computer chips, reliable insulators, low-cost flat-panel spectacles, solar cells, more complicated cutting devices, elimination of dyes and contaminants, high-power magnetic materials, high-energy batteries, high-sensitivity biosensors, long-lived satellites and biomedical materials [3].

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2 Role of Nanomaterials in Therapeutic and Diagnostic Applications

Diagnostic nanoparticles are used to visualize pathologies and understand different diseases and treatment methods for these diseases. Clinical nanodiagnostics is helpful in limited conditions due to their pharmacokinetic properties. For this reason, many of the nanoparticle formulations preferred in clinics are used for treatment. These particles used are therapeutic nanoparticles. Their task in the pathological area is to enhance the accumulation and release of active agents, increase therapeutic efficacy, and minimize damage to healthy tissue. Diagnostic and therapeutic agents that can be integrated into nanoparticle combinations provide significant advantages for therapeutic purposes such as target site monitoring, quantification, and visual inspection of drug release [4].

Recently, there are broad sorts of NPs under investigation and study for biomedical usability in cancer therapy, with particular notice being drawn to nano-drug agents and nanoparticle-based antioxidant agents for multidrug-resistant microbes (Fig. 1). In particular, in addition to highly preferred nanoparticles such as gold and silver, recently Fe_2O_3 nanoparticles, carbon nanotubes, metal oxides, quantum dots, and polymeric-based nanoparticles have received significant attention due to their diagnostic and therapeutic activities [5]. Bifunctional nanoparticles, which can be used for diagnosis and treatment, are preferred as a suitable carrier for different drugs. They are used to reduce the therapeutic drug's toxicity or control the pH-accelerated drug release [6, 7].

2.1 Types of Therapeutic Nanomaterials

One of the principal objects of nanomedicine is the advancement of safe and effective therapeutic nanoparticles. These nanoparticles are an approach to disease therapy based on the controlled release of targeted therapeutic agents over a long period. The diagnostic/therapeutic nanomaterial range is extensive. These nanoparticles provide the advantage of drug delivery and imaging by taking advantage of the anatomical changes in the damaged area. Nanomaterials generally accumulate in the diseased area in higher concentrations than conventional drugs [2].

Nanoparticles are divided into groups themselves. Nanogels, dendrimers, protein-based nanoparticles, and drug-containing nanoparticles belong to the polymer-based group. On the other hand, metallic nanoparticles, silica-based nanoparticles, nanodiamonds, and quantum dots constitute the non-polymer nanoparticle group. Solid lipid nanoparticles and liposomes belong to the group of lipid-based nanoparticles [8].

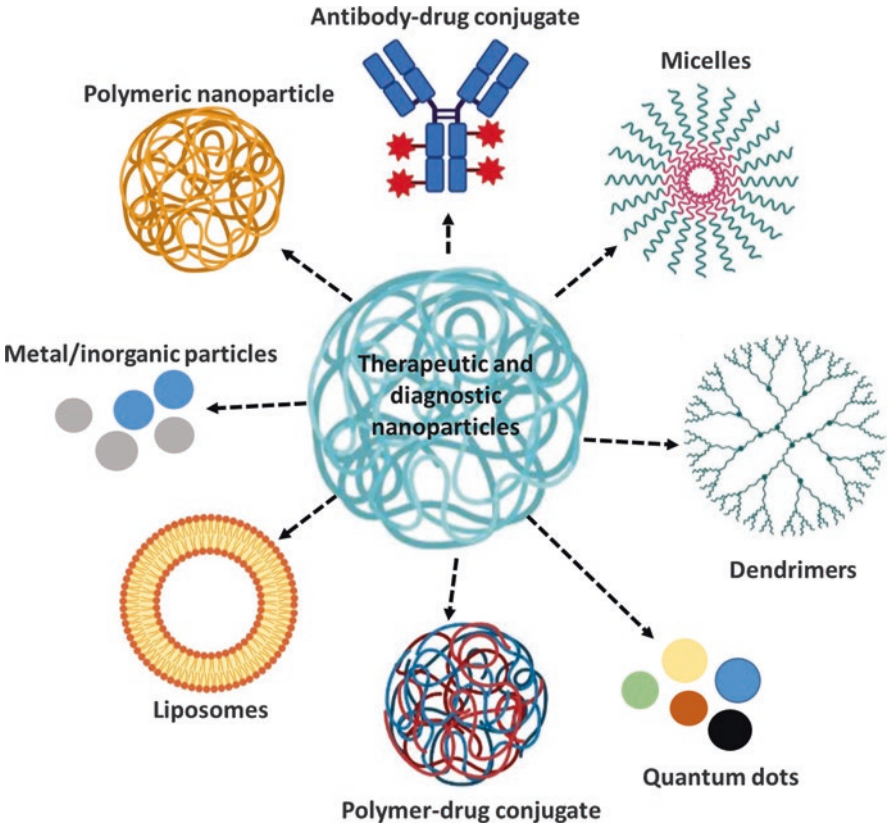

Fig. 1 The commonly used nanoparticles for therapeutic and diagnostic applications

2.1.1 Polymer-Based Particles

Dendrimers

Highly branched polymers primarily used in the clinical field are called dendrimers (Fig. 1). Since the number of branches in these polymers can be controlled, they can be produced in tiny sizes (1–5 nm). In creating these polymers, the creation of cavities inside the dendrimer molecule is provided through spherical polymerization. These high-generation dendrimers contain more than 64 surface groups that resemble small dendrimers and are utilized to deliver therapeutic agents. Another advantage of dendrimers is that they have free end groups that can be simply replaced or used to conjugate biocompatible compounds [9, 10].

Nanoparticles

Synthetic or natural polymer-based nanoparticles are frequently used in therapeutic applications. These nanoparticles are preferred because of their biocompatibility, nontoxicity, biodegradation over time, and non-immunogenicity (Fig. 1). Polycaprolactone (PCL) and polylactic acid (PLA) are examples of synthetic polymers. Polyester forms are utilized to reduce the toxicity of synthetic polymers. Gelatin, chitosan, and alginate, which are natural polymers, have high biocompatibility values and are preferred. Nanocapsule and nanosphere forms have been developed depending on the composition of the polymeric particles. The nanocapsules are surrounded by a membrane that covers the therapeutic agents. In nanospheres, agents are dispersed in spheres [11].

Micelles

Polymeric micelles are one of the polymers used in the controlled release of therapeutic agents which are not dissolved in water (Fig. 1). Micelles are smaller than 100 nm in dimension and form clusters in blends. The hydrophilic surfaces of the micelles help protect them from nonspecific intake by the reticuloendothelial system. The hydrophobic shell of polymeric micelles can hold hydrophobic therapeutics [12].

Nanogels

Gels, defined as polymeric or colloidal structures, enlarge when getting in touch with liquid. A nanogel is regarded as a gel particle less than 100 nm in diameter. Nanogels with high water content and swelling feature consist of a combination of natural or synthetic polymers that are physically or chemically crosslinked. The developed first nanogel is provided by physical crosslinking of amphiphilic polysaccharides, in which cholesterol-bearing pullulans self-assemble into nanogels in water. The most studied applications of nanogels are vaccines, nucleic acids, nasal vaccines, and cytokines [13, 14].

Protein Nanoparticles

Virus-like particles (VLP) are morphologically similar to viruses but are nanocarrier structures that do not comprise genetic material. On the other hand, caged proteins (CP) morphologically resemble viruses but are self-assembled proteins independent of viruses. In developing cancer vaccines, VLPs and CPs induce antigen-directed immune reactions against cancer cells. Therefore, their applications in cancer vaccines have innovative potential [15–17].

Drug Conjugates

Polymer-drug conjugate solutions, known as transmitters with good dissolubility and stability, support the effect of EPR in cancer cells (Fig. 1). By using pH-sensitive chemical bonds, pH-sensible polymeric drug conjugates are created between the polymer and the drug. This is the control of drug delivery in the tumor region due to the pH sensibility of the nanoparticle and its acidic environment. Polymeric drug conjugates are also known to enhance the bioavailability of the drug demonstrated by combination therapy, for example, paclitaxel and doxorubicin [18].

2.1.2 Nanoparticles Without Polymeric Structures

Carbon Nanotubes

Carbon nanotubes are carbon-based systems with a size of 1 nm and 1–100 nm. Carbon nanotubes are formed by wrapping a single sheet of graphite in a continuous cylinder. They are divided into three single-walled carbon nanotubes (SWNT), C60 fullerenes, and multiwalled carbon nanotubes (MWNT). Carbon nanotubes are attractive for therapeutic agents due to their steady geometric shape. Carbon nanotubes with an inner diameter of 1–2 nm, equal to nearly half of the mean DNA helix size, are C60 fullerenes and SWNT. The ability to penetrate the cell via endocytosis or cell membrane belongs to the SWCNT and MWCNTs. The properties that provide this advantage are their sizes [19].

Nanodiamonds

One of the carbon-based nanomaterials with a size small than 100 nm and two kinds of various faces with different properties is nanodiamonds (ND). The oldest and most common method of producing ND is to create a well-controlled displosion on carbon-carrying precursors in a closed reservoir. NDs are remarkable in biomedical applications, including their surface electrostatic properties, low toxicity, and low light bleaching with the combination of nitrogen deficiencies. Nanodiamonds can also be functionalized by immobilizing various types of biomolecules [20–22].

Metallic Nanoparticles

Metallic nanoparticles are nanoparticles with dimensions of 1–100 nm. They are generally composed of iron, cobalt, nickel, and gold elements and oxides such as magnetite, maghemite, cobalt ferrite, and chromium dioxide (Fig. 1). By adding biological molecules such as therapeutic agents, proteins, peptides, and DNA to metallic nanoparticles, they can be formed with versatile, functional chemical groups [23, 24].

Quantum Dots

Semiconductor crystals with 2–10 nm diameter are called quantum dots (QDs) (Fig. 2). The shell is composed of aqueous organic structures such as ZnS, and the core is semiconductor inorganic structures such as CdSe. Quantum dots are capable of producing distinctive fluorescent colors that are the result of high surface-to-volume ratios. The diffuse color is decided by the core configuration, while the outer aqueous shell is utilized to conjugate biomolecules like peptides, DNA, or protein [25, 26].

Silica-Based Nanoparticles

Silica-based nanoparticles have significant profits in nanotechnology due to the cost-effectiveness of designing complex systems. The attractive tools of quantum dots in therapeutic applications are due to their characteristic surface properties, porosity, and functional capability. Silica nanoparticles with a large surface area enclosed with polar silanol groups are suitable for water adsorption and increase the stability of therapeutic agents. Moreover, they can form an interaction between the nucleic acids and are an excellent tool for controlled release [27].

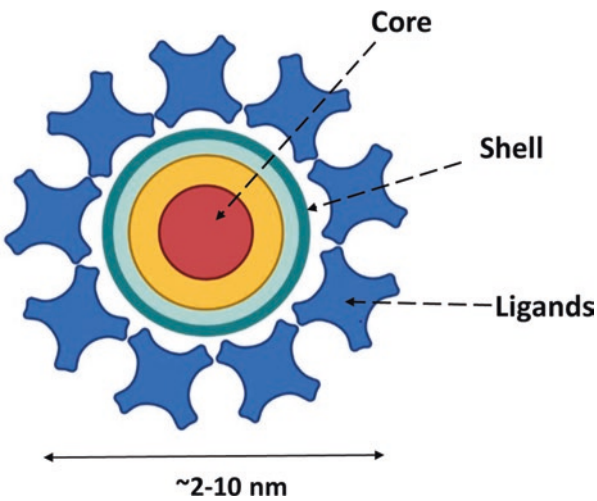

Fig. 2 The schematic illustration of the quantum dots

2.1.3 Lipid-Based Nanoparticles

Liposomes

Liposomes consist of a double lipid layer covering a hollow core with diameters of 50–1000 nm, and vesicles synthesized throughout the hydration of dry phospholipids are known as liposomes. Lipid nanoparticles are produced in different structures, sizes, compositions, and flexibility with surface modifications. The essential profits of liposomes are their skills to unite with the cell membrane and deliver their contents into the cytoplasm. This forms liposomes controlled release systems for targeted release [28].

Exosomes

Exosomes are endosome-derived extracellular vesicles, 30–150 nm in size, which happened in various body liquids like saliva, urine, blood, and breast milk. They are produced naturally and secreted by other cell types. They are cell membrane-like lipid bilayer vesicles containing different matters, including RNA, DNA, proteins, and glycolipids. These vesicles provide intracellular communication by carrying out numerous compounds in physiological systems like immune response, neural transmission, and antigen content in cancer, cardiovascular diseases, diabetes, and inflammation [29, 30].

Solid Lipid Nanoparticles (SLNs)

The solid lipid nanoparticles are aqueous colloidal distributions consisting of a solid lipid matrix at body and room temperatures. Surface-active agents increase their stability, while lipid selection affects drug release properties. The diameter of SLNs changes between 10 and 1000 nm, relying on the fabrication procedure. As a subcategory of lipid transporters, SLNs can involve very high quantities of lipophilic/hydrophilic drugs and nucleic acids, building them various drug delivery carriers. They are effective carriers for cancer, pulmonary, and oral administration aims [31, 32].

Nanocrystalline Particles

Nanocrystalline particles or known as nanocrystals are drug particles with a crystallite dimension of just a few nanometer scales. Nanocrystalline preparations are extensively fabricated for low-cost, water-soluble drugs with restricted active effects which are called bioavailability and absorption. In general, a bulk reduction is an appropriate choice to increase the bioavailability of substances. The crystal configuration leads to a raised total surface area, thereby increasing the dissolution rate.

This property rises solubility, mainly when the therapeutic index of the substance is restricted due to absorption difficulties. Nanocrystalline particles with fast dissolution features provide rapid absorption of therapeutic agents. The nanocrystalline surface can be modified to provide an extended or controlled release, permitting the use of small amounts of therapeutic agents and reducing side effects [33].

2.2 Types of Diagnostic Nanomaterials

Contrary to the therapeutic applications of nanoparticles, diagnostic fields are not yet sufficient. Although nanoparticles are often suggested as diagnostic agents, only one formulation of nanoparticles, called iron oxide nanoparticles, is used in clinical practice. However, recently even ferucarbotran has been withdrawn from the market. Instead, ferumoxytol, a therapeutic iron oxide nanoparticle formulation for the treatment of anemia, is used off-label by many radiologists [34, 35].

Despite progress in synthesizing diagnostic nanoparticle formulations, different limiting elements hinder the clinical translation of diagnostic nanoparticles. The significant difference between nanodiagnostics and nanotherapeutics is their specified pharmacological behavior. Nanotherapeutics should have pharmacological activity, while nanodiagnostics should not produce pathophysiological effects. Moreover, nanotherapeutics should be tested by long blood diffusion. Their primary aim is to discriminate drug accumulation in tissues tested by enhanced permeability and retention (EPR), such as tumors. In this respect, therapeutic nanoparticle preparations are advantageous over standard low molecular weight drugs, as their renal excretion is reduced, resulting in prolonged circulation times and reduced volume of distribution [36, 37]. Because of their short circulation times and rapid biodegradation in nanodiagnostics, they are preferred to be eliminated without pharmacological and toxicological activity. Diagnostic nanoparticles should have a good and efficient release at the target site and display high specific binding and internalization abilities [37].

3 Novel Properties of the QDs

Zero-dimensional nanostructures, one of the semiconductor nanocrystals, are called quantum dots. These nanostructures are of enormous attraction because these materials have characteristic optical possessions like high absorption amplitude, strong fluorescence, and high resistance to photobleaching. Due to these properties, it is widely employed as a fluorescent agent in vitro and in vivo bioimaging fields. They are also used in the production of multifunctional theranostic nanomaterials. Especially in recent years, organic dyes and quantum dots produced with heavy metals are preferred in cell imaging due to their environmental friendliness, less toxicity, and more stable fluorescence properties (Fig. 3) [38]. Quantum dots have

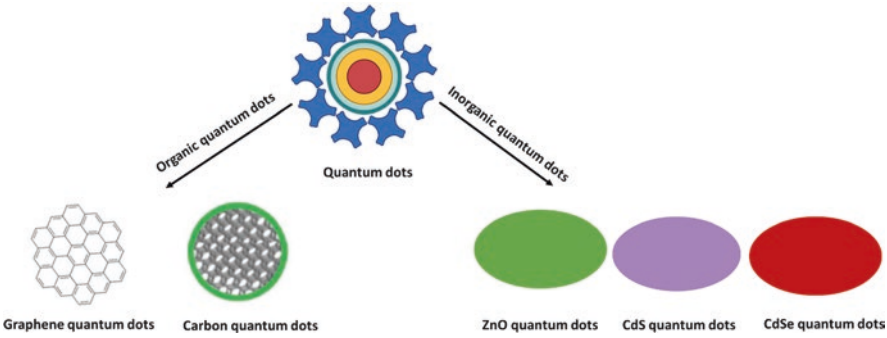

Fig. 3 Types of the quantum dots

many advantages over currently used fluorescent dyes and proteins. The first is that they are brighter and thousands of times more photostable.

Other advantages are wide excitation spectra, narrow symmetric emission spectra, and significant stock shifts. Quantum dots are the most suitable probes for multicolor imaging due to their unique spectral properties. In these materials, nanocrystals of different colors can be excited from a single source at once, and the emission peaks can be separated with high resolution. Due to their high absorption coefficients and quantum efficiencies, quantum dots do not require intense photon beams for excitation. This feature also prevents light damage to the biological material during analysis [39]. Depending on their composition and core size, the spectral properties of quantum dots can be tuned, providing a vast optical fluorescence range from near-ultraviolet to near-infrared. Quantum dots emitted in the infrared region are preferred for in vivo and in vitro deep tissue imaging [40, 41]. The functionalization of quantum dots with biomolecules such as peptides, proteins, antibodies, and drugs requires their use in biomedical applications. Many solutions have been presented for modifying the surface properties of quantum dots. One of them is to obtain stable bioactive tags by binding quantum dots to specific targets. Although quantum dots have unique photophysical properties in biomedical applications, they have some difficulties in use due to their relatively large size and complex surface properties. However, the main obstacles to clinical applications are their toxicity and biodegradability [42, 43].

3.1 Semiconductor QDs

One of the quantum dot types that has attracted attention in recent years is semiconductor quantum dots. Semiconductor quantum dots are also nanoscale clusters of 102–105 atoms. Semiconductor quantum dots are unique structures since the energies, and wave functions of quantum bounded states can be adapted by controlling the size, composition, and shape [44]. Semiconductor quantum dots are produced

by chemical synthesis or epitaxial growth. Of these synthesis methods, chemical production is cheaper, but the epitaxial growth method provides high optical quality. This feature is also valuable for manufacturing electrical and optical devices using epitaxial heterostructure technology. In addition, the semiconductor quantum dots produced by this method provide the possibility of direct integration into the high-quality crystal matrix [45]. Epitaxial InAs quantum dots synthesized by Stranski-Krastanov growth on GaAs surfaces are one of the most widely investigated epithelial semiconductor quantum dot types [46]. InAs/GaAs quantum dots have proven helpful model systems for broad-spectrum experimental quantum mechanics applications [47]. Semiconductor quantum dots can be synthesized in both aqueous and organic hydrophobic solvents. Fabrication in nonpolar solvents and hydrophobic ligands reveals semiconductor QDs with the best optoelectronic properties. Although the quantum dots produced by these methods have extraordinary photoluminescent properties, their tendency to aggregate and precipitate in aqueous solutions is one of their negative aspects. Therefore, surface modification of QDs needs to be performed to protect their surface with molecules that provide good colloidal stability to QDs in an aqueous medium, presenting hydrophilic groups to the medium [48].

3.2 Metal-Doped QDs

The improvement of hybrid nanoparticles that integrate the exciting dimension and shape-related possessions of semiconductor quantum dots with longtime phosphorescence-type emission has been enhanced by advances in the logical pattern of nanomaterials. New types of quantum dots promising in bioanalytical applications are formed by incorporating appropriate atoms or ions into host cages. Main cages containing transition metals and lanthanide ions (mostly ZnS, ZnSe, ZnO, CdS, CdSe quantum dots and elements such as Mn^{+2}, Co^{+2}, Cu^{+2}, Pb^{+2}, Eu^{+3}) are used as agents. The presence of these elements and lanthanide ions forms luminescent quantum dots with new properties. Among these, ZnO and ZnS host cages are the most widely used. The features that make them attractive can be listed as follows. They do not comprise toxic metals and they are tested by a more significant energy bandgap as they are suitable for incorporating more doping agents [49]. The added dopant agents extend the lifetime of quantum dots. In addition, these dopants remove the fluorescence background that commonly occurs in bioimaging and biosensing applications. The result is a phosphorescence-like emission that gets over the restrictions of fluorescent nanoparticles or dyes [50].

3.3 Carbon QDs

Carbon-based nanoparticles such as carbon quantum dots (C-QDs) and graphene quantum dots (G-QDs) are inorganic semiconductor quantum dots that have been mainly studied as luminescent nanomaterials in bioanalytical applications. The reasons for these materials to become alternative materials are their high photoluminescence quantum yields, low light-bleaching impacts, low toxicity, high biocompatibility, and not containing commonly available heavy metals [51]. Due to the small size of these materials, they offer colloidal stability in aqueous environments, because the aggregation that may occur between them with the Brownian motion is prevented [52]. Moreover, the excitation wavelength-dependent fluorescent emissions make C-dots the most exciting materials in optical imaging. Currently, investigations into the origin of the fluorescent emission of C-dots are ongoing, so the principle of such characteristic emission has not been fully clarified. C-dots are obtained by synthetic means or different precursors and exhibit other optical behaviors. This shows that carbon-based quantum dots are highly complex [53].

Graphite is used as the carbon matrix to synthesize dots, and surface passivation of carbon dots is required to provide better fluorescent properties. On the other hand, when natural precursors are used to synthesize carbon dots and with cost-effective, environmentally friendly syntheses, there is no need for any surface modification of the produced C-dots. This provides high photoluminescence in aqueous media [54]. The diagnostic application areas of carbon dots have been extended by the metal doping of nanoparticles with N and lanthanide. As a result, they are co-doped nanomaterials that show both strong fluorescence and high magnetic resonance and computed tomography contrast capabilities [55]. There are two types of techniques for the synthesis of carbon-based quantum dots. These are known as bottom-up and top-down techniques. These techniques are done through chemical, electrochemical, or physical systems. The top-down process provides a decomposition of carbon material into carbon nanotubes. Examples of bottom-up processes are pyrolytic treatment, template strategy, hydrothermal and aqueous methods, microwave-assisted strategy, material oxidation, and assisted synthetic technique [56].

4 Application Areas of the QDs

They have emerged as alternative materials to conventional organic dyes in diagnostic analyses with their nano-sized properties, extraordinary photochemical and photophysical properties, bright and fluorescent crystal semiconductor structures, invariant photostability, unique materials, and multiplexing abilities in conjunction with their fundamental narrow and symmetric emission bands. With the new methods developed, quantum dots can be dissolved in solution and labeled with

biological molecules. These features have led to the opening and growth of quantum dots in biomedical application areas [57]. Figure 4 illustrates the common application areas of the QDs. The studies on the use of QDs show that they can be used as a suitable probe in DNA hybridization, receptor-mediated endocytosis, control of parasite metabolism, and real-time visualization of tissue and cellular forms. They have been utilized as fluorophores in an in vivo study, but there aren't certain results about their safety, yet. Another study has proven that in the early stages of the embryonic development of *Xenopus* embryos, quantum dots are stable, nontoxic, and resistant to photobleaching when injected. This result paved the way for investigating the effect of cellular distinction that occurs during the period of the formation and development of an embryo. QDs used as fluorophores are an essential agent in real-time tumor recognition. Research using quantum dots have demonstrated that these particles are promising due to their sensitivity in detecting cancerous tumors and in vivo models. Zinc-based QDs, which are heavy metal-free QDs, are more promising in terms of use. To interpret the disease process and develop new treatment modalities, it is essential to introduce cells tagged with QDs into little animals and trace a specific path through their fluorescent properties [58, 59].

4.1 Role of QDs in Disorder Diagnosis

QDs, when labeled with particular antibodies against particular tumor markers, have an important place in cancer detection because of their size resemblance to special biomolecules when used with tissue, human serum, and other body liquids. Many methods are used to diagnose cancer, including chemotherapy, surgery,

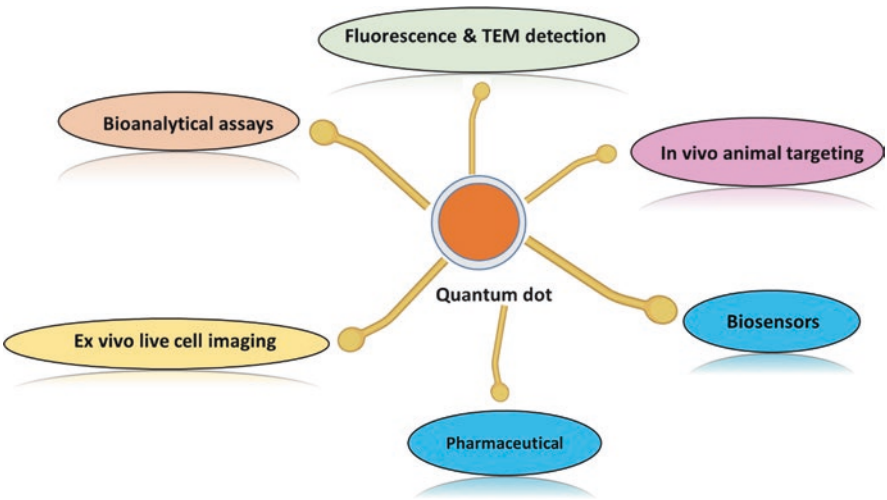

Fig. 4 The common application areas of the quantum dots

immunotherapy, medical visualization, enzyme-linked immunosorbent test which uses the absorption of antibodies by insoluble preparations of antigens, and tissue biopsy. These techniques can only be employed for less sensitive spotting of cancer at the early stage. QDs are preferred over conventional fluorophores such as fluorescent proteins and organic dyes due to their broad emission spectra, limited absorption range, and short fluorescent lifespans. The emission and absorption wavelengths of QDs can be adjusted according to the particle dimension. The QDs are preferred for both in vivo and in vitro fluorophores in numerous medical and biological fields like tissue visualization, detection, and treatment of multiple disorders, involving cancer [60].

4.2 The Role of Quantum Dots in Immuno-Based Searches

Although toxicity concerns about QDs have been addressed by using suitable surface coatings, they are still not considered a standard fluorophore for diagnostic fields [61]. In another study, they performed a fluoroimmunoassay to mark prostate-specific antigen (PSA). This test uses 107 nm streptavidin-covered QDs with B-diketones that sequester N30,000 europium molecules. Detection limits for biotinylated PSA were set at 0.38 ng/L. PSA spot was carried out at both solid and liquid stages. Specific PSA was imaged with a fluorescent microscope [62].

4.3 Potential of QDs in Biomarking and Bioimaging

Modification of QDs with biomolecules is an important parameter that enables QDs to identify and adhere to cognate biomolecular objects. Several methods have been developed to map biomolecules to the surface of QD that are defined as covalent and noncovalent conjugation. Covalent conjugation includes amine, carboxyl, thiol, etc. The bonding method, which includes electrostatic interaction, biotin-avidin binding, and metal chelation, is called noncovalent conjugation. By electrostatic interaction, protein particles or nucleic acids which are charged negatively can adhere to positively charged QDs. Those that bind directly to the QD surface are proteins/peptides that have powerful metal ion chelating parts. Due to the strong binding property of histidine which consists of most protein remainders with zinc and nickel ions when combined with polyhistidine protein molecules, it acts as a good binder to combine proteins to the surface of QDs. In addition, these DNA and protein molecules act as ligands in synthesizing biofunctionalized QDs. Biotin-avidin conjugation is a different solution for binding biomolecules to the surface of QDs [63, 64].

Once functionalized with appropriate biomolecules, QDs are suitable for cell labeling and imaging. Thanks to their strong photoluminescence and outstanding photostability possessions, QDs give concentrated and persistent signs. This

QD-based strategy also contributes to the single-particle trailing of cells and improves multicolor visualization.

QDs now have great use in cell surface receptor labeling, intracellular spotting, sensing of biomolecules, and organelle marking. Various sorts of biomolecule-derived ligands have been used for special identification in the functionalization of QDs. Proteins, antibodies, peptides, aptamers, and other nonessential biomolecules are the most used ligands. These ligands are often used to label proteins on cellular surfaces and overproduced receptors such as PSA and epidermal growth factor (EGFR). They are overproduced in numerous cancer cell lines and can be labeled with biofunctionalized QDs, thus enabling diagnoses for different types of cancer.

Highly specific nucleic acid aptamers are collected from an excellent RNA/DNA memory, either by in vitro selection with surface cellular proteins or with cells as pure targets. Compared to antibodies, these aptamers are steady and simple to control and manufacture [65].

The potential usability of organic fluorophores like indocyanine green in NIR fluorescence visualization is limited due to the small quantum yield and destructive photostability. In the case of highly luminescent NIR QDs, in vivo visualization of blood vessels, mapping of lymph nodes, tumor marking, etc., application areas have been significantly increased. NIR light is chosen over visible light in deep tissue, thanks to superior tissue attachment and detection down to 1 cm. QDs are becoming current in vivo cancer-sensing markers utilizing xeno-implanted tumor models of little animals. Some other in vivo usage of QDs is to assist in real-time visualizing of lymph nodes of SLNs located at a depth of 1 cm due to the tissue permeability properties of NIR QDs. This supplies surgeons with uninterrupted graphical supervision during the SLN mapping process, reducing unneeded incisions [66].

4.4 QDs in the Field of Biodetection and Biosensors

The potential of QDs to be versatile biosensors has been demonstrated to detect a large number of molecules like metal ions, nucleic acids, large proteins, and other tiny molecules. QDs can oversee changes in pH, temperature, and oxygen parameters. QDs were used as fluoro-marks for the spotting of proteins. The basic procedure which contained protein detection with the support of QDs resembles conventional immunoassay procedures. Recently, several new detection procedures for proteins have been found. These advances comprise DNA-linked protein detection, double-aptamer protein detection, protein separation QD-based multidimensional detection, ratiometric QD protein sensor, and electrochemically luminescent immunosensor [67].

Protease, a vital biomarker employed in many disorders, is upregulated in various conditions. It can be noticed with the help of QD-contained biosensors. The FRET acceptor binds to QDs via a peptide linker. The peptide binder is hit when treated with a particular protease, and the FRET signal is blocked. FRET-contained QD biosensors are preferred because of their protease activity. A set of FRET

acceptors is used to map FRET to QDs. The organic fluorophores, fluorescent proteins, gold nanoparticles, and quenching dyes are examples of these FRET acceptors. Multicolor QD-based biosensors have also been used for the multiplex spotting of various proteases. For example, FRET and BRET have also been employed to detect protease activity, wherein bioluminescent proteins acted as BRET donors, while QDs were used as BRET acceptors [68].

Based on biosensors, QD has been frequently used to detect various tiny biomolecules like amino acids, nucleotides, ascorbic acid, glucose, and dopamine. Electrochemistry and FRET-based fluorescence microscopy are the widely utilized techniques for detecting biomolecules with the support of QD biosensors [61]. With its multicolor properties, photoluminescence makes QDs an essential agent for the multiple detections of several DNA targets. One of the best-familiar techniques for DNA detection is the FRET strategy. Enzymes are used to enlarge the target to increase detection sensibility. Detection outside of FRET can be performed with a DNA-focused QD-tagged DNA probe and a magnetic nanoparticle-labeled DNA probe throughout hybridization [69].

4.5 Q-Dots in the Pharmaceutical Field

Theranostics refers to types of formulations that assist both diagnostic and therapeutic purposes. Recently, nanotherapeutic systems combined with targeted therapeutic moiety delivery have attracted interest in clinical oncology. This concern has caused manipulations in nanotechnology to use multiple functions. The use of QDs as image contrast agents is of interest in improving the in vitro and in vivo evaluation of tumors compared with the conventional fluorophores [70]. To be suitable for theranostic use, QDs require functionality either by drug ligand conjugate or by crosslinking with both drug and ligand. Biomolecules such as antibodies, aptamers, or chemical ligands specific to receptors at the targeting site are ligands for targeting [71, 72].

With recent advances in surface functionalization techniques, complex nanomedicine with QDs as drug carriers has advanced. QDs coated with hydrophilic stabilizers such as mercaptoacetic acid, mercaptoethylamine, and polyethylene glycol have chemical interactions, covalent bonds, electrostatic interaction, and amide bonds between stabilizers. It readily forms conjugates with active drugs [73].

Recently, many inorganic nanoparticles with antimicrobial efficiency have been examined. These are various metals and their combinations of silver, titanium dioxide, and zinc oxide nanoparticles. QDs also have advantages such as supported optical properties and are identified to create free radicals found by the core of the semiconductor on irradiation. It is known that these radicals are toxic to microbes and free heavy metal ions released during the irradiation process are also toxic to bacteria [74].

5 QDs' Role in Diagnostic and Therapeutic Applications

5.1 QD Aqueous Strategies

Traditional superior-quality fluorescent QDs are usually fabricated in organic solvents at elevated temperatures. On the other hand, QDs used in bioanalytical applications must be made compatible with water while maintaining their optoelectronic properties. For this cause, surface adjustment of QDs after synthesis is essential. In addition, QDs should have practicable classes on their surface for further association with biomolecules (Fig. 5) [75].

With proper QD surface passivation, crystalline NPs can simply make surface failures that satisfy the fluorescent possessions of bare QDs. Furthermore, bare QDs can be damaged by surface oxidization, photochemical degeneration, and leaching of metal ions from the NP core after exposure to ionic or biological environments that affect the optoelectronic properties of QDs and produce undesirable cytotoxicity. Therefore, alteration of the QD surface with suitable ligands is important to stabilize NPs in physiological environments and reduce nanocrystalline surface defects by minimizing the reactivity and toxicity of QDs. Significant advances have been made in the synthesis of QDs. Despite these advances, the biological uses of QDs necessitate the conversion of such nanoparticles into biocompatible probes. Hydrophilization of QDs is achieved by three fundamental strategies [76].

The first strategy is based upon ligand exchange of the original surface hydrophobic coating of the QD, which is taken off and modified by bifunctional

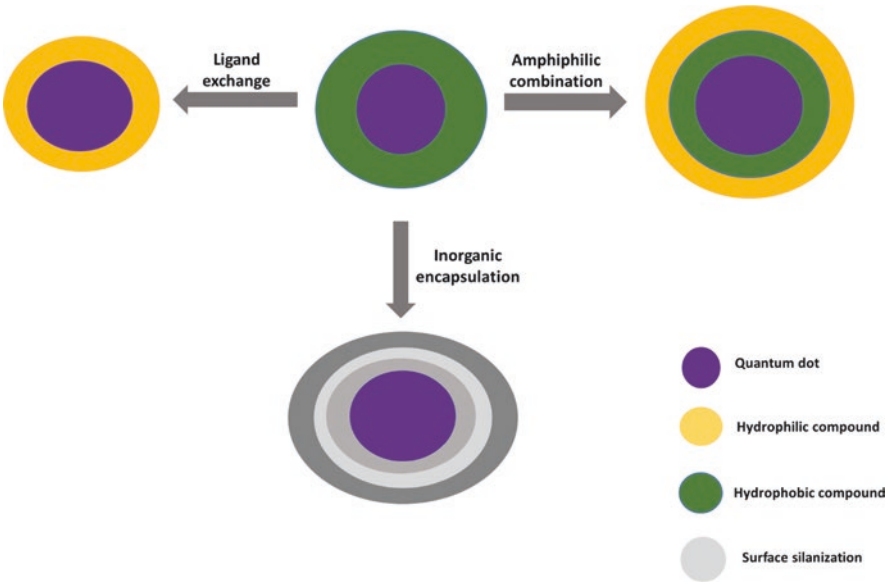

Fig. 5 Quantum dots' stabilization strategies

molecules that bind to the surface of QDs. Hydrophilic functional groups like carboxyl or sulfonic acids provide adequate NP solubility in polar and aqueous environments and can be used for further bioconjugation. Dithiothreitol (DTT) or dihydrolipoic acid (DHLA) are bidentate ligands such as oligomeric phosphines, crosslinked dendrons, and peptides. These ligands are preferred to obtain stable and aqueous QDs [77].

The second strategy involves silica used for inorganic encapsulation in the hydrophilic structures of QDs by forming a silica shell surrounding the NP surface. It is an interesting strategy for water stabilization of QDs since the silica surface is nontoxic, transparent enough in terms of optical properties, and chemically inert. In this strategy, a precursor molecule such as mercaptopropyltrimethoxysilane (MPTMOS) is added. The thiol groups of MPTMOS react with the inorganic surface of the QDs. Methoxysilane groups, which form a strongly crosslinked protective shell around the QDs, polymerize by forming siloxane bonds. As a result of this approach, it was observed that the biocompatibility rate of silica was high. It is easy to give function to its surface with suitable (bio)analyte recognition biomolecules. This type of approach makes QDs encapsulated in the silica layer, making it ideal for other bioanalytical areas [78].

Similar to liposomes, encapsulation of hydrophobic QDs in hydrophilic agents belongs to the third stabilization procedure. One of the suitable carriers for hydrophobic QDs is liposomes. Liposomes can be used as suitable carriers, thanks to their core-shell structure and high loading capacity. In addition to these properties, liposomes minimize the nonspecific interactions between the QDs, which have both water-soluble and water-insoluble properties, and the surface material. In addition, the surfaces of liposomes can be simply adjusted with functional groups to permit simple conjugation with protein [79].

5.2 QD Bioconjugation Strategies

The main challenge for using QDs in biomedical applications is the robust binding of the suitable target recognition molecule on the NP surface. This vital parameter directly affects their application in biological habitats.

The property of having a surface-to-volume ratio applies to all NPs. These properties are determined by their chemical structure and the layer of cap molecules called ligands. These ligands can bind to QD surface metals. Thus, ligands can prevent aggregation of particles, resist nonspecific adsorption of bordering molecules, and supply a conjugation point to useful biomolecules. The synthetical process used to organize QDs settles their shape, size, and chemical makeup and the ligands that seal the surface. These ligands are preferred because they manage the shape, size, and polydispersity of the QDs in the fabrication step, and their uniform mixture in the solvent is required after preparation [80].

The main points to be considered during the bioconjugation process are as follows: (1) There can be control over the desired ratio variable, the biomolecule/QD

ratio, with the sort of practice. QDs are generally larger than BMs, except perhaps for some large proteins. (2) Control of BM over its orientation on the QD while maintaining the optimal activity of both QD and BM has (3) an impact on the separation between QD and BM and (4) an impact on the strength of the QD-BM bond. The simplest form of attaching a biomolecule to a QD interface is adsorption in which the binding of BM is relatively weak and protected by hydrogen bonding, London dispersion, and weak interactions such as Coulomb forces and lone pair electrons. Proteins found in the human body have shown a tendency to bind nonspecifically to the surface of QDs, something to be avoided. The electrostatic connections between BM and QD are closely related. Although this functionalization procedure is usually fast and straightforward, it suffers from serious disadvantages such as simple adsorption, electrostatic interaction instability, and lack of orientation control on biomolecule and BM/QD ratio [81].

Bioconjugation occurs in four different ways. These are the direct union of BM, BM bonded to a ligand, BM bonded to an encapsulating shell, and BM bonded through biotin/Streptavidin. In the direct association of BM, peptides, proteins, and nucleic acids can bind to the surface metal particles of QDs via (cysteine) thiols and (histidine) imidazole groups. Histidine and thiol patterns can be combined with "native" biomolecules, creating thiol groups that reduce peripheral S-S bonds. In some cases, biomolecules bind directly to QD in their synthesis process.

The second strategy involves covalently binding the biomolecule to a ligand previously connected to the QD surface. Graphene QDs (GQDs) usually contain hydroxyl, carboxyl, carbonyl, and outer deposit groups. It is known that CQDs can selectively have amine, carboxyl, or other groups on their surface. With some exceptions that can be prepared in water, natural semiconductor QDs do not have suitable ligands that can bind to the BM in a steady state. Bidentate or monodentate molecules such as dihydrolipoic acid (DHLA) and 3-mercaptopropionic acid (MPA) form new ligands. Free terminal carboxylic groups can be found to bind to BM, and they can bind to the QD surface, thanks to their thiol groups [77]. Problems with ligand exchange, such as poor bonding between the thiol group and the metal surface of the QDs, and diminished photoluminescent quantum yield are the problems that can be seen in BM connected to an encapsulating shell. Silanization (production of the silica outer sphere) raises the stability and solubility of QDs. In addition, silanization retains many of its emission possessions. Encapsulation of the semiconductor QD with the amphiphilic copolymer is achieved due to the capacity of its big hydrophobic tails to intertwine and interact with the robust QD ligands. In addition, the hydrophilic and functionalized parts get in touch with the water molecules of the dissolving agent [82]. The powerful interaction of biotin-avidin with a decomposition constant of 10–15 M is a commonly used strategy to assemble biomolecules onto the QD surface. The protein found in egg white is avidin. Each avidin is a single biotin-binding site. Vitamin H is known as biotin. Thanks to the different biotin derivatives, the biotinylation of QDs and biomolecules is facilitated. In addition, BM and QDs can be functionalized with the avidin protein. On the other hand, it should be noted that one or more of the biotin-binding sites present will likely be hidden by the binding of the avidin protein to the QD during the initial modification.

QDs functionalized with avidin can then bind to biotinylated biomolecules. On the other hand, QDs functionalized with biotin are useful in assembling biomolecules functionalized with avidin [83].

6 Diagnostic and Therapeutic Applications Involving QDs

6.1 Therapeutic and Diagnostic Applications Involving QDs

With the increase in the incidence of deadly diseases such as cancer, the need for the efficacy of therapeutic agents and definitive diagnostic methods is increasing. Therapeutic nanoparticles are of great importance in protecting healthy tissues, controlling the delivery of active agents in the pathological region, increasing therapeutic efficaciousness, and reducing the effectiveness of side outcomes.

In a study by Sriparna De et al., graphene oxide quantum dot (GOQD) was combined with folic acid-functionalized chitosan (FA-CH) to improve a drug-release nanocarrier (FA-CHGOQD) to treat cancer. The drug doxorubicin (DOX) was chosen for encapsulation to confirm the efficacy of FA-CH GOQD as nanocarriers. The in vitro release model of DOX has been tested at different pH ranges. According to the results, the drug release rate in the tumor cell microenvironment with a pH value of 5.5 was higher than in physiological environments with pH values of 7.4 and 6.5. It was carried out to determine the cytotoxic properties of GOQD and FA-CH-GOQD/DOX by MTT analysis. Cytomorphological micrographs of A549 cells showed different morphological rearrangements that were subject to cell apoptosis. The results of cellular uptake studies showed that FA-CH-GOQD could deliver DOX within a cancerous cell [84].

In a study by Ghorai, oxidative functionalization following acid-free and oxon oxidant-assisted solvothermal synthesis of graphene quantum dots from coal was performed first. This method of oxidative functionalization of coal was accomplished through heat treatment of nitric acid followed by acid recovery. Due to the synergistic synthesis technique, the developed GQDs exhibited solid blue photoluminescence (PL) property with a significantly high quantum yield (~14.42%). High-resolution transmission electron microscopy (HRTEM) has proven that nanocrystalline GQDs have smooth and narrow particle sizes in the 2.5–5 nm range. RITC-labeled GQDs demonstrated cellular incorporation in breast cancer cells [85].

In a study by Shivaji et al., a green, biogenic synthesis route was used to make CdS QDs with a particle size of 2–5 nm which were developed utilizing tea leaf extract (*Camellia sinensis*) as a nontoxic stabilizing agent. CdS proved that QDs efficaciously inhibited bacterial growth and exhibited cytotoxicity against A549 cancer cells [86].

In a study by Kholikov et al., GQDs were magnified by focusing nanosecond laser pulses on benzene and combined with methylene blue (MB). These GQDs have been used to act on *Escherichia coli* and *Micrococcus luteus*. MB-GQD singlet

oxygen production was observed by calculating the photobleaching rate of 9,10-anthracenediyl-bis(methylene)dimalonic acid. The formation of singlet oxygen has occurred with GQDs and MB. The MB-GQD combination successfully destroyed the bacteria. MTT test was used to examine whether GQDs cause human cellular side impacts in dark conditions and affect cancer-noncancer cellular viability. As a result of this analysis, viability was not changed under dark conditions, even at high GQD concentrations. This indicates that the MB-GQD combination is a good form of photodynamic therapy [87].

In another study performed by Justin et al., biodegradable chitosan-magnetic graphene quantum dot (MGQD) nanocomposites were used to observe release mechanisms between the large and small molecular weight (MWt) therapeutics from biodegradable microneedles. The existence of MGQDs in chitosan raised the degradation rate and electrical conductivity of chitosan, also preserving its mechanical properties. Poly(ethylene glycol) (PEG) was chosen as the microneedle material. The PEG ring did not block microneedle capacity with its mechanical qualifications and low MWt lidocaine hydrochloride drug release behavior nearly the same as ringless microneedle arrays. The chitosan-MGQD microneedles were electrically conductive and permitted electrically stimulated delivery of big MWt therapeutics without the PEG ring [88].

Daou et al. used commercial ITK705 amino QDs coated with methoxy-terminated poly(ethylene glycol) (PEG) of different chain lengths. After that, long-term stable homogeneous QD solutions were fabricated. The effect of particle coating on in vivo behavior after tail IV injection was visualized with a fluorescent microscope. The rate of first-pass extraction of coated QDs toward the liver reduced with PEG length. On the other hand, it increased with the hydrodynamic diameter of the particles [89].

In a study by Erogbogbo et al., steady dilute suspensions of Si QDs were produced utilizing phospholipid micelles. Si QDs used in the study are luminescent labels for pancreatic cancer cells. As a result of the study, it has been proven that silicon quantum dots can be applied as potential probes in diagnosis [90].

In a study by Roy, a new method was developed using plant leaf-derived graphene quantum dots (GQDsremodeleded with annexin V antibody (AbA5) to generate (AbA5)-remodeled GQDs (AbA5-GQDs). GQDs were used to screen the human cervical cancer cell line (HeLa cells), human breast adenocarcinoma cell line (MCF-7 cells), and normal human breast epithelial cell line (MCF-10A). In addition, the biotoxicity of GQDs was evaluated and shown to have little toxicity at concentrations <2.0 mg/mL. According to the result, GQDs were low toxicity [91].

7 Conclusions

QDs have a significant role in cell labeling imaging, diagnosis, and drug release. Many studies have shown that QD is an excellent imaging agent and can assist in the structure of theranostic devices. According to current studies, QDs could be modified to be more benign biologically and environmentally. QDs can be successfully

incorporated into release systems. The properties of these hybrid substances are determined based on the respective release system for in vitro and in vivo practices. Although QDs offer significant advantages, more systems should be developed, and in vivo studies should be increased to evaluate the practicability of QD-based devices for clinical uses.

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Carbon-Based Nanomaterials for Targeted Drug and Gene Delivery Systems

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1 Introduction

Nanomaterials have several medical applications, including novel applications, for instance, vectors for vaccine development, genetic material and drug distribution of curative substances to the immune system, photothermal therapy, and tissue engineering, as illustrated in Fig. 1 [1]. Furthermore, various diseases, including cancer, can now be detected and treated simultaneously with carbon nanomaterials. Current treatment methodologies have many shortcomings, including the incapability to eradicate all cancerous cells; with that said, chemotherapy and radiotherapy possess severe poisonous adverse reactions. Furthermore, the use of carbon nanomaterials (CNs) within the medical field centers around the controlled delivery of anticancer treatments and the careful demolition of cancerous cells [2–4]. The size of carbon nanomaterials ranging between approximately 0.5 and 100 is ideal for use in both in vitro and in vivo natural structures. This is due to their size analogous to

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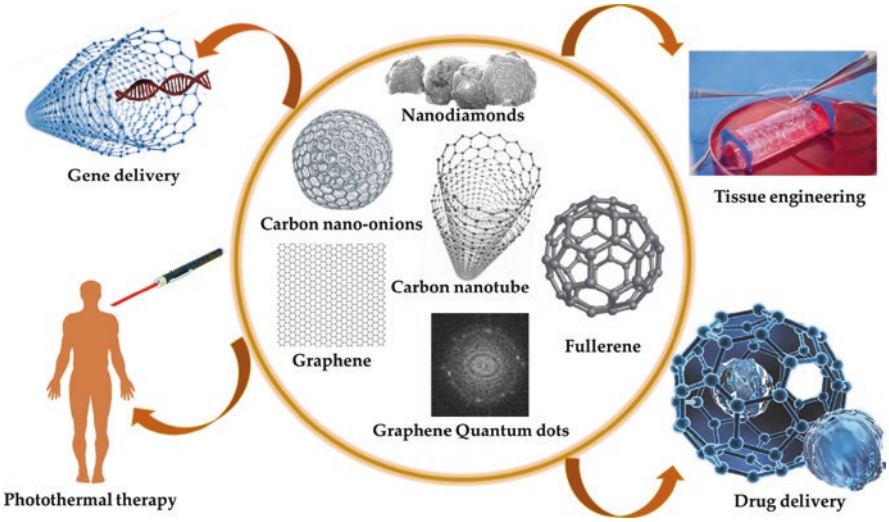

Fig. 1 Graphical illustration of the applications of carbon-based nanoparticles in medicine. (Reproduced with copyrights permission from [1])

biological molecules like DNA plasmids, enzymes, proteins, etc. [5, 6]. They are also easily transported across a cell membrane, most likely via endocytosis [7].

Additionally, the ability of carbon-based nanomaterials, namely, graphene, fullerenes, carbon nanotubes (CNTs), and nanodiamonds, to easily cross cell membranes makes them excellent vehicles for small molecules and biopharmaceuticals [8]. Carbon nanomaterials benefit morbid regions like tumors because they are small in size. In addition, nanomaterials accumulate selectively inside tumorous material at considerably greater concentrations than in the surrounding and nearby healthy materials. The preceding fact is abnormal leaky blood vessels and reduced lymphatic drainage related to tumorous material. The above dynamics cause the improved permeation and retention effect (EPR) that permits nanomaterials to accumulate carefully in carcinogenic tissue [9, 10]. By using carbon nanomaterials specifically created for tumor uptake, photothermal therapy (PTT) can be utilized to selectively destroy cancerous bodies through the use of near-infrared light ablation and the activation of photosensitizing agents that yield singlet oxygen and responsive oxygen molecules that destroy the neighboring tumor cells. The applications mentioned above are a few of the possible utilization of carbon-based nanomaterials (CBNs). This chapter addresses targeted drug and gene delivery systems that are carbon-based.

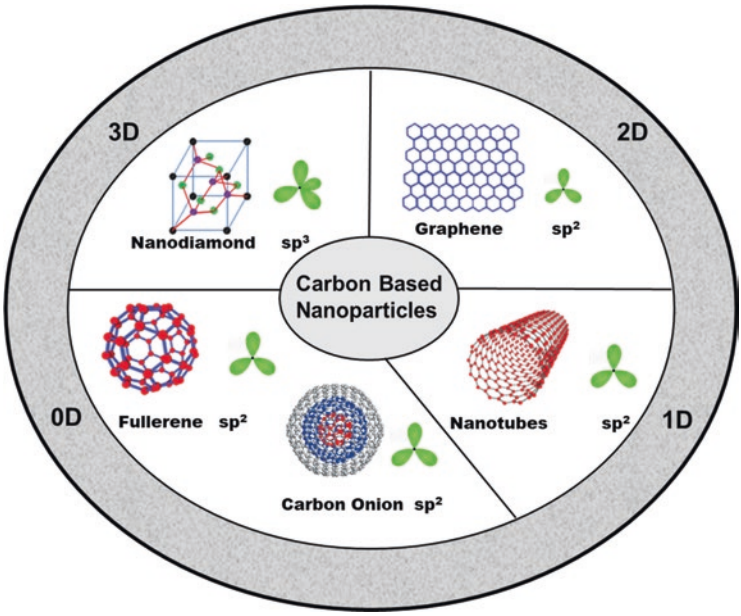

Fig. 2 Illustration of the different CBNs and their hybridization states

2 Classification of Carbon-Based Nanomaterials (CBNs)

Initially discovered in 1985, carbon-based nanomaterials (CBNs) are, as the name implies, fundamentally composed of carbon atoms [11, 12]. Moreover, the existence of CBNs such as fullerenes (C60, C70, and C84) was predicted in 1970 [13], and as already mentioned, they were discovered only in 1985. Besides, fullerenes (i.e., C60) characteristically have a sphere-like shape, even though oblong shapes (i.e., C70) are also possible. In 1991, the discovery of carbon nanotubes accelerated the research into carbon-related nanomaterials [14]. According to Fig. 2, CBNs are classified according to their geometrical structure and are found in various forms (tubes, spheres, ellipsoids) with two crystalline allotropes (graphite and diamonds). The carbon element is therefore distinct and diverse, and CBNs can be three-dimensional (diamond and graphite), two-dimensional (graphite sheets), one-dimensional (nanotubes), or zero-dimensional (fullerene) [15, 16]. Furthermore, its structural configuration and hybridization state greatly influences CBNs' chemical behavior and physical properties. The carbon element with its six electrons has a ground state configuration of $1s^2 2s^2 2p^2$.

A small energy gap in the 2s and 2p orbitals facilitates electron transition, allowing hybridization into sp, sp^2, or sp^3. For sp^2 and sp^3 hybridization, the covalent bonds with neighboring atoms at higher energy levels provide energy to accommodate this configuration, as for sp, sp^2, or sp^3 hybridization. In unhybrid orbitals, the concept of π-bonding is considered. The unhybrid p-orbitals contemplate π-bonding

among themselves [17]. Therefore, if one studies organic compounds, they mainly differ in their hybridization states. Depending on its configuration, a significant variation in carbon's bulk can be observed. For example, when high temperatures and pressures are applied to diamonds, a trigonometric sp^3 arrangement is apparent. In the absence of heat, the planar form interlayer sp^2 configuration supplements to provide a layered sheet that is singular in the structure, having a single π-bond and three sigma covalent bonds. Conversely, weak interplanar forces caused by shear, chemical, and physical separation cause the graphene sheets to slip [18].

Furthermore, research into CBNs in several fields has been done: computerized devices and semiconductors, data-storing applications, biosensors and regular sensors, energy storage research, and nano-based biomedicine. The subsequent section describes the physical properties of the following CBNs in this order: graphene, fullerenes, nanotubes, and nanodiamonds.

2.1 Graphene

Graphene, including graphite sheets, is a CBN with higher-ranking properties. CBNs of this type are two-dimensional nanomaterials, with honeycomb crystal lattices made from carbon atoms with sp^2 hybridization [19]. In addition, graphene molecules are the building blocks of other graphitic structures, namely, zero-dimensional (0D) fullerenes, one-dimensional (1D) carbon nanotubes, and three-dimensional (3D) graphites. In addition to being wrapped up in buckyballs (0D), graphene can also be rolled up into nanotubes (1D) and stacked into graphite (3D), as illustrated in Fig. 3 [20]. It is due to this fact that graphene is the mother of all graphene-like structures.

Furthermore, graphite sheets are more steady than 3D structured molecules at the nanoscale [21]. Graphene's strange electronic behavior allows for several relativistic phenomena of quantum electrodynamics. It also possesses extraordinary conductivity properties that enable the relativistic effects in quantum electrodynamics [22]. This makes it highly useful in various fields, including optical research, electronic investigations, spintronic studies, composites, and hydrogen storage [15]. In addition, the unique planar structure of graphene, its outstanding electronic, optical, magnetism, thermal, and electrochemical properties, has its advantages. Moreover, its easy biofunctionalization makes graphene a versatile material that has been applied in various biological applications. These applications include drug delivery, diagnostic uses for terminal illnesses, tissue altering, fluorescence quenched by graphene, graphene-improved differentiation, and graphene-supported laser desorption [23, 24]. In addition to its excellent surface area (2630 $m^2.g^{-1}$), exceptional Young's modulus (1.0TPa), outstanding thermal conductivity (5000$Wm^{-1}.K^{-1}$), high optical transmittance (97.7%), and excellent electrical conductivity, graphene has many other distinctive properties, i.e., physical, chemical, and mechanical [25–27].

There are four leading derivatives of graphene, namely, (i) graphene oxide (GO), (ii) reduced graphene oxide (RGO), (iii) graphene quantum dots (GQDs), and (iv)

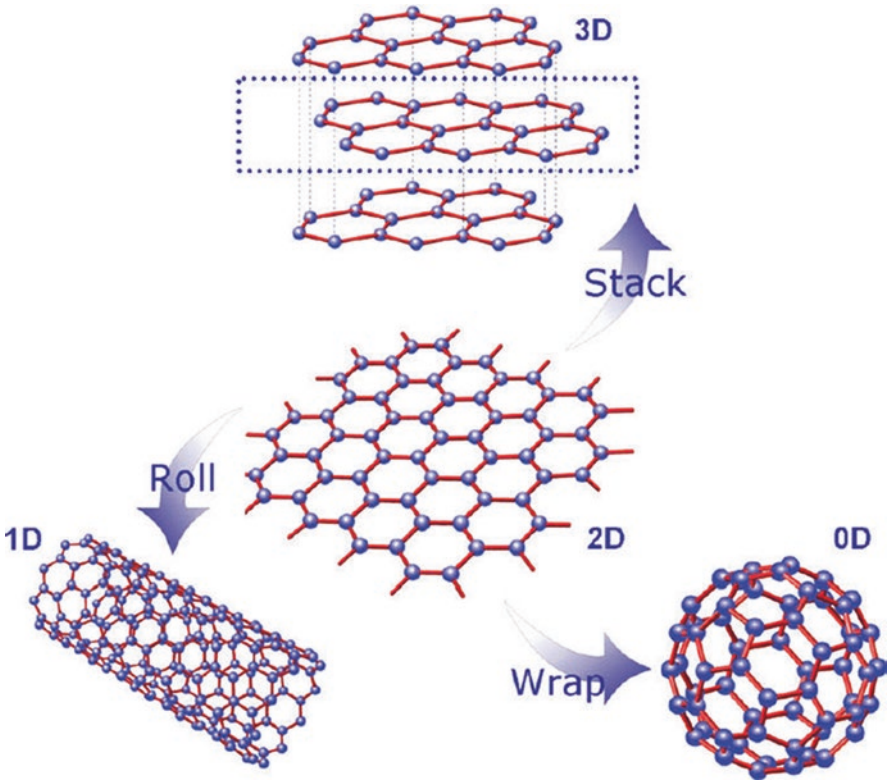

Fig. 3 Illustration of the various types of graphene: 0D is fullerene, 1D is carbon nanotubes, 2D is graphene, and 3D is graphite. (The picture is reproduced with permission from [20])

graphene nanosheets (GNs). Additional derivates are graphene nano-onions (GNOs), graphene nano-ribbons (GNRs), graphene nanoplatelets (GNPs), ultrathin graphite, monolayer graphene, and few-layer graphene. However, graphene's most important derivative is graphene oxide (GO).

2.2 Fullerenes

Carbon nanomaterials that take the form of ellipsoids or spheres are known as fullerene (0D), buckminsterfullerenes, or buckyballs, and these types of CBNs retain unique chemical and physical properties [28]. Fullerenes have a hexagonal ground structure because of the sp^2 bonding. This makes fullerenes stand out from other materials. In theory, a micro-sized graphene sheet is rolled cylindrically in the nanometer range, and then, it is topped up with a fullerene sphere. There are 120 symmetry operations in the molecular structure of fullerenes, including rotation

about an axis and reflection, making fullerenes the most symmetrical cage-like molecule of all CBNs [29]. A large amount of fullerene in a synthesized sample is C60 (creating a round shape with 60 vertices). It contains 60 carbon atoms involving C5–C5 single bonds, which equate to 12 pentagonal rings, and C5 = C6 double bonds equating to 20 hexagonal rings [30, 31]. Fullerene comprising $2n + 20$ carbon atoms has "n" hexagons; it is solid and can withstand pressures as excellent as 3 000 atm (at which it still holds its original shape). Concentrations of C60 and C70 are highest at 1000 °C and increase with pulsation interval [31]. The fullerene structure has nonlinear optical responses when π-electrons are delocalized. Fullerene molecules may contain 30–980 carbon atoms to formulate altered forms with various properties suitable for multiple uses. When the hexagons in the C60 molecule are altered (i.e., added or removed), the particle can lose its soccer ball-like appearance. When having 70 carbon atoms and 25 hexagonal units, C70 forms a rugby ball-like structure. A pentagonal shape is more giant fullerenes, while asteroids are smaller fullerenes. There are 1.65 grams of fullerene in a cubic centimeter, which is less than the 3.51 grams of diamond in a cubic centimeter [18]. Because of the possible twofold performance of C60 among reactive oxygen species (ROS), this nanoparticle can behave contrarily within altered conditions. As C60 produces oxygen species when exposed to visible light, it is a contender for photodynamic therapy (PDT) and biological applications [32–34].

2.3 Nanotubes

The spherical or ellipsoidal form of nanomaterials is called fullerene, while the cylindrical shape is called carbon nanotubes (CNTs). These materials are hexagonal 1D sp^2 hybridized carbon sheets that possess unique properties, and the nanostructure of CNTs is illustrated in Fig. 3. Due to the CNTs' graphene sidewalls, which have sp^3 bonding properties, CNTs are less susceptible to chemical alterations than fullerenes. In carbon nanotubes, the nano-diameter is coupled with a length in cm, creating an unusual structural aspect ratio. As a result of noncovalent interactions, CNTs interact with organic molecules via electrostatic, van der Waals [35, 36], hydrogen bonding, or hydrophobic forces. Because of their higher strength, rigidity, conductivity, and elasticity, they are potentially functional materials in a range of applications.

Furthermore, to synthesize CNTs, various methods may be used (e.g., laser ablation, arc discharge, and chemical vapor deposition (CVD)), and three types of CNT structures exist, namely, single-walled carbon nanotubes (SWCNTs), double-walled (DWCNT), and multiwalled carbon nanotubes (MWCNTs). The classification of CNTs is based on how many graphene layers there are in the cylindrical tube [15]. An SWCNT is typically 0.4 to 2.5 nm, whereas an MWCNT is usually a few nanometers to 100 nm. By combining 2D crystals with different electrical, optical, and mechanical properties, multilayer CNTs can provide other physical phenomena and device functionalities [37]. Depending on the orientation of the hexagonal lattice,

SWNTs exhibit metallic or semimetallic properties and are semiconducting. DWNTs and MWNTs can be considered "carbon onions" in one dimension. Both MWNTs and bulk SWNTs have similar characteristics, and the coupling between layers is feeble in these two NTs. As opposed to SWNTs, MWNTs have a semiconducting behavior rather than a metallic character [18].

2.4 Nanodiamond

In the 1960s, diamond nanoparticles were first created by the explosion in the Soviet Union and were largely unknown to the rest of the world for many years [38, 39]. Scientists have been fascinated for decades by nanodiamonds (NDs); these are diamonds with a size range between 4 and 10 nm. Particles of nanodiamonds containing small monocrystalline-sized particles of around 4 nm are termed detonated nanodiamonds. In 1933, adamantane was first discovered in crude oil. It has the chemical formula C10H16, and then tetra-C22H28 becomes adamantine. Nanocrystalline carbon atoms are connected tetrahedrally in nanodiamonds, forming a three-dimensional cubic lattice. This makes NDs look like diamonds, and they possess the physical properties of the onion-like-shaped carbonaceous shell with coverings of functional groups [40–43]. Because NDs have extremely flexible sp^2/sp^3 ratio bonds, their stretched faces may function as graphene planes, while puckered surfaces act as diamond surfaces. NDs are fascinating because of their intrinsic properties, and the smaller they are, the better their properties [44–46]. NDs vary in size and properties depending on synthesized [47, 48]. In addition to functionalizing NDs, they are nontoxic and functionalized with various ligand molecules. Medicinal compounds, chemical compounds, and biological molecules can be conjugated using these different ligands [49, 50]. Moreover, despite their high surface areas and tunable surface arrangements, nanodiamonds devise excellent mechanical and optical properties.

3 Biomedical Application of Carbon-Based Nanomaterials

Carbon-based nanomaterials (CNs) with exceptional characteristics are progressively being used in numerous scientific and industrial suits. The diverse carbon-derived nanomaterial family has been widely used in biomedical applications, namely, bioimaging, biosensing, diagnostics, drug and gene delivery, regenerative medicine, tissue engineering, and phototherapy due to its unique properties. We hope to provide an overview of CNs in this chapter, focusing on intrinsic structural, electrical, and chemical features. The properties and characteristics of CNs and their derivatives, such as carbon nanotubes (CNTs), graphene, carbon dots, nanodiamonds, fullerene, and mesoporous carbon nanomaterials, are summarized in depth in this section.

3.1 Bioimaging

Carbon nanomaterials are attracting immense interest for their unique optical properties and potential applications in imaging due to their small size and low cost. In many imaging applications, carbon-based materials have been studied for numerous years. Fluorescence imaging, Raman imaging, multimodal imaging, magnetic resonance imaging, computerized tomography, photoacoustic imaging, and computed positron emission tomography are only a few examples [51]. Nanomaterials based on carbon quantum dots have gained much attention in bioimaging. Carbon quantum dots outperform traditional organic fluorophores and contemporary inorganic semiconductor quantum dots in photobleaching resistance, chemical inertness, and ease of surface functionalization, among other things [52]. Carbon quantum dots, for example, have minimal cytotoxicity, excellent aqueous solubility, and a high emission quantum yield, making them ideal for use in biomedical research, particularly in in vivo and in vitro bioimaging [53]. Karakoçak et al. demonstrated the usage of hyaluronan-conjugated nitrogen-doped carbon quantum dots (HA-nCQDs) for bioimaging of tumor cells. In addition, they showed how they might be used as drug carriers. Their findings suggest that HA-nCQDs could be employed to image CD44-specific tumors in preclinical human cancer models and could also be used as carriers for directed medication distribution into CD44-rich cells [54].

3.2 Biosensors

Carbon nanotubes, grapheme, grapheme oxide, carbon nanowires, and graphene quantum dots have excellent physical, chemical, and electrical properties. They have a high surface-to-volume ratio, so most surfaces are exposed, allowing them to bind many sensors [55]. Furthermore, the exceptional surface-to-volume ratio and the sp^2 hybridization structure guarantee surface alteration by detecting elements and signal transformation [56]. In transistor-type sensors, carbon nanotubes and graphene, for example, function as electrical channels, while chemiresistor-type sensors use them as sensitive elements with specialized detecting probes to detect molecules with high sensitivity and selectivity. Since glucose is an essential molecule in diagnosing diabetes and fermentation technology, using enzyme-carbon nanotube electrodes for glucose detection is the most widely used analyte. Immobilization of enzymes for glucose determination employs various techniques [57]. Gokoglan et al., for example, developed a new flexible glucose biosensor that measures the glucose content of multiple beverages utilizing vertically allied carbon nanotubes and conjugated polymers. They claimed that the novel biosensor has more significant kinetic limits, including excellent detecting capability and sensitivity [58]. In another study, Maity et al. produced a screen-printed carbon electrode modified using amine terminated multiwalled carbon nanotubes/polyaniline/reduced graphene oxide/gold nanoparticles with glucose oxidase (GOx)

immobilized on SPCE. They reported that the fabricated glucose biosensor shows extraordinary sensitivity, a wide linearity range, and the lowest micromole detection range. The researchers also emphasized that the biosensor is cost-effective, reproducible, repeatable, and stable [59].

3.3 Drug Delivery

Carbon nanomaterials have attracted researchers with their unique properties, enormous surface area, significant cellular internalization, superior tumor accrual, and ease of surface functionalization, allowing nanomaterials to carry chemotherapeutic agents, especially to tumor locations and decreasing drug toxic side effects [60]. Carbon nanomaterials can be modified to deliver bioactive peptides, nucleic acids, proteins, and medicines to cells and organs. Because functionalized carbon nanomaterials are non-immunogenic and have minimal toxicity [61]. Garriga and his co-researchers tested the in vitro toxicity of carbon nanomaterials such as carbon nanotubes, carbon nanohorns, carbon nanoplatelets, nanodiamonds, graphene oxide, and reduced graphene oxide in human epithelial colorectal adenocarcinoma (Caco-2) cells and human breast adenocarcinoma (MCF-7) cells. They reported that nanodiamonds have the most negligible toxicity, followed by grapheme oxide and carbon nanotubes. In contrast, carbon nanoplatelets have high toxicity, producing an elevated level of reactive oxygen species. Further, they emphasized that the anticancer drug-loaded carbon nanomaterials show improved anticancer activity compared with the free drug. This efficiency largely depends on carbon nanomaterial hydrophobicity and surface chemistry [62]. Similarly, many researchers have reported that surface functionalized carbon nanotubes are target-specific, non-immunogenic, and low toxic, have controlled drug release, and are efficient in killing cancer cells [62–64].

3.4 Gene Delivery

Zhao and his co-researcher prepared gene delivery systems using fluorine-doped cationic carbon dots. Their study showed that fluorine-doped cationic carbon dots are highly efficient in delivering low DNA doses to high serum concentrations. However, despite having high gene delivery efficiency and biocompatibility in vitro, its in vivo application is limited because it lacks a degradable chemical structure [65]. However, surface functionalization and chemical modification of carbon nanomaterial will reduce the cytotoxicity in in vivo conditions. Because of carbon nanotubes' unique properties, including high surface-to-volume ratios, optical properties, increased conductivity and strength, ease of surface functionalization, and biocompatibility, they have been considered gene carriers [66]. MicroRNAs (miRNAs) regulate gene expression post-transcriptionally, making them a promising

therapeutic target for various disorders. A study proved that functionalizing CNTs with two different polymers like polyethyleneimine and polyamidoamine, followed by miR-503 oligonucleotides, regulated the target genes, cell proliferation, and angiogenic budding [67]. Golshadi and his co-researchers developed an array of aligned hollow carbon nanotubes for rapid, high-efficiency transfer of DNA. They reported that plasmid DNA delivery through the developed nanofabrication was 84% with low cytotoxicity. Further, they emphasized that carbon nanotube utilization for gene delivery will overcome the molecular weight limits, and it could be modified to transport genes with added drugs or proteins [68].

3.5 Regenerative Medicine

Bones are connective matter that provides the structure of an entity and act by way of a hinge for the movement of muscles by providing a position of attachment. Bone tissues can self-heal, but multifaceted fractures and bone defects can lead to non-unions. The scaffolds are essential for bone regeneration, and they provide a site for new bone tissue formation and proliferation. CNTs are a great alternative to titanium or ceramic bone scaffolds since they are robust, rigid, and flexible [69]. In addition to the in vitro studies on ectopic bone formation using human mesenchymal stem cells derived from adipose tissue, Li's research paper evaluates the use of carbon nanotubes to induce osteogenic differentiation in mice models. Their results indicated that the CNTs cause osteogenic gene expression and ectopic bone construction in the dorsal musculature of the mice model. According to their report, CNTs induce inductive bone by concentrating more proteins in cells located in bone tissues [70]. Similarly, Das et al. tested canine mesenchymal stem cell proliferation and differentiation characteristics using functionalized carbon nanotube scaffolds. They reported that the COOH-functionalized CNTs promoted chondrogenesis and neural differentiation. As CNTs have electrical conductivity properties, Kunisaki et al. reported that the CNT yarns promote peripheral nerve regeneration in peripheral neural defeats. These reports suggest that the CNTs are a promising scaffold for treating bone and neuron regeneration [71].

3.6 Tissue Engineering

The treatment of human bone abnormalities, such as those caused by tumor excision, trauma, and aberrant bone formation, has significant limits. There is little evidence that current therapies like autografts, allographs, and metal prostheses promote bone regeneration. Instead, they use an artificial material to replace the missing bone [72]. It is preferred that CNTs be used for bone tissue engineering due to their biocompatibility, rigidity, ability to mimic natural tissue nanofibers, ability to stimulate the adhesion and proliferation of cells, and ability to form solid 3D

structures [73]. Osteocytes and bone cells have been demonstrated to be completely biocompatible with CNTs [74]. Uzui et al. found that multiwalled CNTs adjacent to bone cause slight local inflammation, show high bone-tissue similarity, play a role in bone repair, integrate into the new-fangled bone, and accelerate bone development when stimulated by transgenic human bone morphogenetic protein-2. This research establishes a preliminary exploratory foundation for CNTs in biomaterials utilized next to the bone, such as those used to promote bone regeneration [75]. Through a dual in situ method of producing CNTs in hydroxyapatite powders by chemical vapor deposition, Li and his co-researchers manufactured CNT-reinforced hydroxyapatite compounds. They reported that the composites are suitable biocompatible and drastically stimulated the propagation of fibroblasts and osteoblast in in vitro conditions. Similarly, Mukerji et al. observed soft callous growth on the implant surface after the CNTs-hydroxyapatite composites were placed in an in vivo study. They reported that the CNTs and hydroxyapatite composites improved the host-graft and bone-implant interfaces [76].

3.7 Phototherapy

Carbon nanotubes and graphene, which have properties like colossal surface areas, thermal conductivity, and electrical properties, have attracted consideration in phototherapy due to their unique properties. Phototherapy is a hopeful next-generation healing approach for many current medical diseases, including cancer diagnoses, targeting, and therapy [77]. Commonly used phototherapy agents are photosensitizers, which absorb light sources and produce reactive oxygen species in the cells [78]. There are different types of phototherapy, such as (i) photobiomodulation (PBM), (ii) photodynamic therapy (PDT), and (iii) photothermal therapy (PTT). In PBM, a light-emitting diode or laser is used at a specific wavelength level to cure various illnesses. The PDT therapeutic method uses light at a particular wavelength to activate photosensitizers to kill tumors. In contrast, photothermal therapy (PTT) involves exposing the photosensitizer to a light source and activating the PS molecule to generate heat energy. These phototherapy methods treat acute and chronic pain, rheumatoid arthritis, scleroderma, dental surgery, neurological disorders, colon cancer, wound healing, Parkinson's disease, and musculoskeletal syndrome [79]. Nanoparticles of graphene quantum dots (GQDs) possess unique electronic properties, such as solid and tunable photoluminescence, which can be used for bioimaging and biosensing [80].

4 The Impact of Physical and Chemical Properties of CBNs in the Delivery of Drugs and Genes

CBNs are becoming promising and attractive nanomaterials. CBNs have a wide range of applications, including biomedical applications. The wide range of applications is due to organic π-π stacking characteristics and semiconducting properties. CBNs are rapidly becoming more popular and more and more mass-produced. They exist in diverse allotropes of carbon. Intensive research has been done on the nature of CBNs. CBNs have been the most used type of nanoparticles over the past two decades. CBNs for biomedical uses have captivated much focus owing to their inherent properties. They are extensively used in various products, including drug and gene transporters. The unique physicochemical properties of CBNs include optical, electrical, mechanical, thermal, electrochemical, structural diversity, and electrical properties. The elucidation of the structures of CBNs and the characterization of their physicochemical properties is vital to ensure product excellence (i.e., increased purity, determination of shortcomings, chemical species on surfaces, etc.) and to understand their structure. These properties provide flexibility, electrical conductivity, and superior strength toward drugs and genes. In addition, CBNs can effectively interact with biomolecules. The widespread application of CBNs increases the possibility of them entering the environment. Spectroscopic methods are used to elucidate the chemical and physical properties of the CBNs. The most used methods are atomic force microscopy, electron microscopy, and Raman spectroscopy. The physicochemical property nature of the CBNs makes them inert, stable, and difficult to degrade. The CBNs have demonstrated distinct biological interaction due to their diversified physical properties and nanosized nature. The different physicochemical properties have been discussed in detail in this subsection.

4.1 Degradation by Enzymes and Microbes

In the therapeutic use of CBNs, the enzyme degradation of these compounds is critical to immune-competent cells since they assist in a superior instance of biological interactions. Various CBNs are associated with a wide assortment of enzymes, leading to the mechanism by which enzymes degrade. Degradation by enzymes is defined as a heterogeneous process involving adsorption and hydrolysis. Furthermore, biodegradation reduces the number of atoms in a molecule through a specific type of biological transformation. Therefore, the biodegradation of CBNs by enzymes from microbes is of utmost significance. This reduces their harmfulness to existing organisms and removes them from the environment. However, the number of microbes and enzymes involved in the biodegradation of CBNs remains limited.

Further studies are needed to characterize the enzymes and microbes involved in biodegradation. CBNs may be degraded more effectively if microbes and enzymes

are enhanced. Microorganisms excrete enzymes that decompose the CBNs found in plant litter—the degradation of CBNs by microbes important for immune-competent cells. Single-walled carbon-based nanotubes (SWCNTs) degrade relatively quickly, and their biodegradation occurs naturally via microbes and enzymes catalysis [81]. This was revealed from previous studies. The degradation of SWCNTs was increased in a low concentration of hydrogen peroxide and horseradish peroxidase. MWCNTs degrade differently than SWCNTs.

The degradation of MWCNTs is unlike SWCNTs since MWCNTs have multiple layers, so they may affect HRP secretion. Multiwalled carbon nanotubes (MWCNTs) are degraded layer-by-layer [82, 83]. The functionalization of MWCNTs changed the rate of degradation. The time of oxidative acid action was dependent on the degree of carboxylation. The hydrophilic interaction among horseradish peroxidases and oxygen concentration caused the degradation and oxidation of CNTs. SWCNTs degrade in a shorter time than MWCNTs. This is because MWCNTs are more resilient to degradation by horseradish peroxidase. The degradation of SWCNTs was influenced by myeloperoxidase in a separate study. The degradation was enhanced by the addition of sodium bromide and hydrogen peroxide. Myeloperoxidase (MPO)-mediated degradation and cellular-mediated degradation of SWCNTs work the same way the body's immune systems offend nanomaterials and microorganisms. SWCNTs are also completely degraded when MPO, hypochlorite, and H_2O_2 are present in in vitro cultures of human neutrophils. Human neutrophils can degrade SWCNTs with MPO, but macrophages cannot entirely degrade SWCNTs. CNTs are susceptible to degradation under acidic conditions since hypochlorite and free radical intermediates are present in MPO [84].

Furthermore, the free radicals will also facilitate the breakdown of the CNTs. SWCNTs are also susceptible to degradation under certain in vitro conditions, in addition to in vivo. Also, like other enzymes, peroxidases such as eosinophil peroxidase (EPO) usually catalyze redox reactions involving two electrons, using H_2O_2 to oxidize halides into their hypohalous acids producing reactive radical intermediates.

4.2 Biocompatibility and Biological Interactions

CBNs have various physical and chemical properties of interest. These properties increase their demand in various fields of biomedical science. CBNs have drawn remarkable attention, including CNTs, graphene, fullerenes, and nanodiamond [85, 86]. However, the CBNs are not compatible with biological systems, causing potential toxicity. This incompatibility of limits their wider spectrum acceptance. The assessment of the biocompatibility of CBNs is therefore highly ideal [87]. Designing nanoparticles for biomedical applications requires surface modification. The surface modification of nanoparticles can influence biological reactions, particle uptake, and biodistribution. Surface modification has made the CBNs more compatible and susceptible to degradation by enzymes and microbes [88].

Furthermore, CBNs (like superparamagnetic iron oxide nanoparticles, SPION) can be used to regulate the immune system's behavior differently, as illustrated in Fig. 4 [89]. For instance, the SPIONs used in magnetic resonance imaging (MRI) are not labeled, resulting in macrophages taking up the particles [90]. Whereas, if SPION is PEGylated or sized less than 20 nanometers, macrophages are less likely to take them up, and the circulation time is longer [91]. CBNs can associate with a diversity of mammalian cells and enter mammalian cells. For instance, the existence of CNTs in the blood allows the interaction of CNTs with leukocytes in the blood. The exchange is through antigen-presenting cells.

The activation of leukocyte markers allows understanding of the direct and indirect interaction between blood and the immune reaction. The CDs have minimum impact on the serum albumin and gamma-globulins. CDs possess good tolerance and higher cell viability with serum compared with polyethyleneimine. Different CBN subclasses have other physical and chemical properties [83]. A thorough

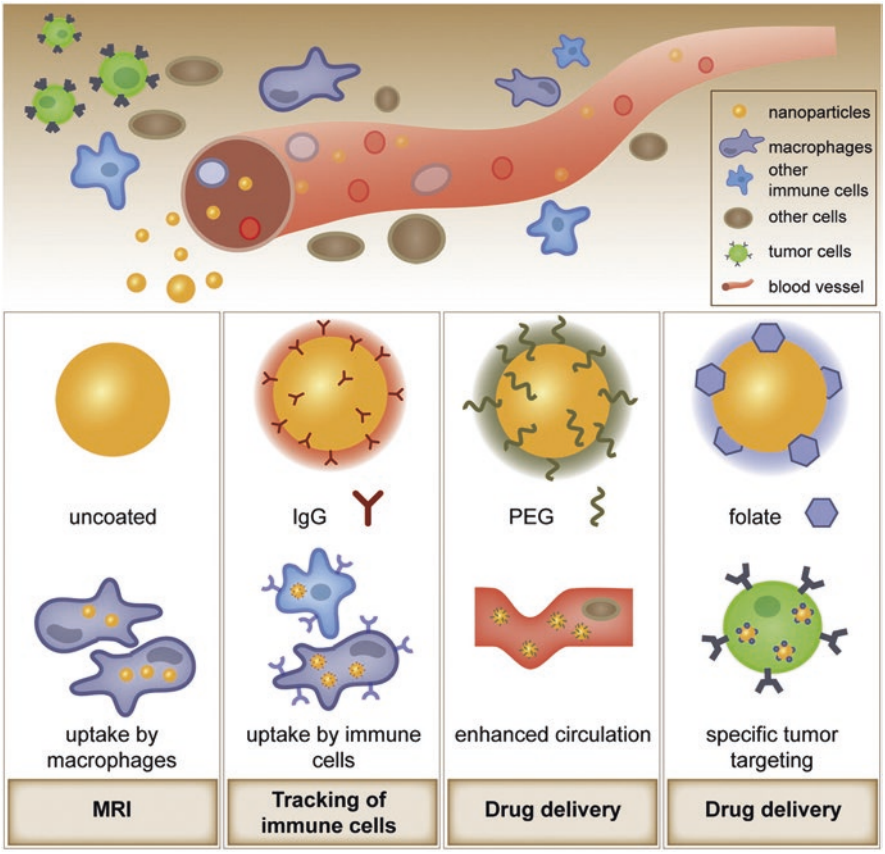

Fig. 4 Graphical illustration of effect of surface coating on nano-immuno-interactions. (Reprinted with copyrights permission from [89])

assessment of biological interaction is a prerequisite to exploring the biomedical application of the CBNs. The innate immune system is comprised of soluble mediators like cytokines and chemokines, as well as inflammatory cells like all phagocytic cells and macrophages. Inflammatory cells and monocytes are two different sets of cells. Monocytes are solely responsible for producing cytokines, presentation of antigens, and phagocytosis. The soluble mediators include complement factors, chemokines, and cytokines [92]. The metabolic activity of macrophages is affected by interference with materials from graphene. The interaction damages the mitochondrial membrane, thereby increasing the levels of reactive oxygen species. CBNs may lead to damage to DNA and lipids. This damage is followed by cell death via the excitation of reactive oxygen species when uptake by cancer cells [13]. The mechanism of entry into cells by CBNs is still unclear. Passive diffusion takes place on the cell membrane via the phospholipid bilayer. Active uptake occurs through the attachment of CBNs to the external the cell membrane. The addition of the CBNs to the exterior surface results in absorption. Diffusion is used to internalize small CBNs of up to 400 nm in length. In addition, endocytosis is responsible for the internalization of some small CBNs. CBNs with enhanced biofunction enhance drug and gene delivery efficacy by reducing clearance and increasing retention.

4.3 Bio-corona

Biomedical researchers face a significant challenge in nanoparticle investigations, such as developing safe and active bio-corona formation on nanoparticle surfaces. Much of the research on nanoparticles and bio-coronas has been qualitative, posing a critical drawback. In part, this is due to the emerging nature of the field and the need to develop more quantitative study designs and technologies [93]. An environment containing bio-corona molecules refers to an adsorbate layer of molecules that resides on the surface of a nanoparticle. Nanoparticles can form complex coatings when biomolecules absorb them in a physiological environment [94, 95]. The coating changes the nanomaterial's physical properties, cellular viability, and immune responses.

In a bio-corona particle, the CBNs are coated with biomolecules like lipids and proteins. The CBNs present themselves with new biological identities soon after the coating. The new form that is acquired is called bio-corona. Bio-corona is valid for various toxicological and biological interactions with the CBNs in biological systems. Bio-corona coated with albumin is effectively adsorbed on the SWCNT surface. Albumin-coated bio-coronas helped macrophages take up nutrients from the environment. The scavenger receptors interacted with the damaged albumin. The albumin is then eliminated from systemic circulation [96]. Studies of the interaction of SWCNTs with major serum proteins were analyzed [97]. The uncoated SWCNTs produce high toxicity than serum protein-coated SWCNTs in leukemia cells and human umbilical vein endothelial cells. As demonstrated by a cell line study,

SWCNTs bind to serum, changing cellular interaction pathways. The π-π interaction between the aromatic residue and SWCNTs plays a critical role in tissue binding. The cell membrane was damaged due to the cytotoxicity of graphene oxide (GO). GO was coated with bovine serum albumin (BSA) to reduce cytotoxicity toward A549 cell lines. Bio-corona is dominant in the gastrointestinal tract, lung, and other organs. The environment determined the formation of different types of bio-corona.

The pharyngeal aspiration of SWCNTs in mice models led to the absorption of proteins, lipids, and lung surfactants. Phosphatidylcholines and phosphatidylglycerol are highly absorbed on the surface of SWCNTs with varying binding affinities. Alkyl chains of hydrophobic phospholipids adsorbed on the surface of coated SWCNTs. The uptake of SWCNTs by cells was increased by surface coating. Conformational changes and orientation may be changed by bio-corona. This alteration could result in an immune response via the cell surface receptors. The activity of enzymes may be inhibited by the adsorption of proteins on the CBNs. For example, CYP3A4 isoenzymes may be inhibited by SWCNTs and GO. When CBNs are coated with BSA, the efficiency of inhibition decreases significantly.

4.4 Toxicity

CBNs have unwanted toxic effects on biological systems, which has become a significant concern. CBN exposure poses potential hazards, but information on these hazards is rare and still under investigation [98, 99]. Fiber-like materials are biopersistent because of the toxicity of CBNs. Because carbon nanotubes are structurally similar to asbestos, they may cause asbestos-like toxic effects. Furthermore, a person's lungs may develop asbestosis (a progressive fibrotic disease), lung cancer, or malignant mesothelioma from asbestos inhalation [100]. Several factors contribute to the toxicity observed in CBNs [97]. Remarkable toxicity on cells is due to metal impurities during synthesis. Substantial influence on toxicity is due to the length of the CBNs through cellular internalization. Fibrosis is caused by CNTs which initiate inflammation [101]. A higher diameter of CNTs elicits substantial toxicity. Altered toxicity on cells is shown by chemical surface variation in size and structure between SWCNT and MWCNT [102]. The introduction of functionalization has managed the problem of toxicity. The biocompatibility of CNTs is increased by surface modification.

MWCNTs treated with acid and MWCNTs functionalized by taurine detected cellular apoptosis and cellular phagocytosis. Altered macrophages respond differently to MWCNTs. The different types of macrophages are dependent on the concentration of MWCNTs. In an investigation by Tabei et al. (2019), they reported the toxicity of MWCNTs. The research group found that the MWCNTs have elevated phagocytic activity toward undifferentiated HL-60 cells [103].

Furthermore, they showed that the MWCNTs have some cytotoxicity for already differentiated HL-60 cells. Moreover, as well as being genotoxic, MWCNTs will affect DNA repair mechanisms. MWCNTs induce apoptosis of murine bone marrow at a fixed concentration range [96]. MWCNTs produced no cytotoxic effects on mammalian cell lines at a specific concentration. A lower cytotoxic effect of fullerenes against macrophages was observed. The cytotoxic effect has been shown in alveolar macrophages in an in vitro analysis [104]. CBNs' influence on cell death was investigated in a separate study. The exposure of alveolar macrophages and RAW264.7 was characterized by the cell membrane rupture, enlargement of the cell, leakage of lactate dehydrogenase, and activation of caspase-1 [105]. In vitro toxicity on standard mouse fibroblasts was conducted. The results showed no abnormality in the morphology of the cell; intracellular trafficking and cell cycle were detected. CDs are in high demand as promising materials for biomedical applications. Pristine-CDs produced higher oxidative stress without entering the nucleus. Severe toxicity at low concentrations can be caused by polyethyleneimine-coated dots entering the nucleus [106]. Photo-induced toxicity and the effect of CDs' degree of carbonization on cytotoxicity were observed in cells. A significantly higher quantum yield was shown in negatively charged CDs smaller than 5 nm. Enzymes and microbes degrade most of the materials under the CBNs. Long-term toxicity studies are critical in analyzing the potentially harmful effects of CBNs.

5 Considerations for Target-Specific Delivery of Drugs and Genes

The specific delivery of therapeutics (i.e., drugs and genes) to their target sites has certain factors to be considered. Targeted delivery of drugs and genes is a system of specifying the therapeutic moiety directly into its targeted body area. The targeted body areas include organs and cellular or subcellular levels of a specific tissue. The system is there to overcome the specific toxic effect of conventional therapeutic delivery, reducing the dug or gene required for therapeutic efficacy. The targeted delivery of nanoparticles is currently widely studied in cancer treatment. The therapeutics must successfully move and accumulate within a desirable site in targeted delivery. For the process of targeted drug delivery to be efficient, the agent-loaded system should be in the physiological system for the preferable time. The agent-loaded system must target specific cells or tissues. Furthermore, the agent-loaded system must evade the immune system and release the drug or gene to the site. Conditions of diseases are managed by improving the novel formulation of targeted therapeutics. The section will explore the disease targets, effective therapeutics, and cargo carriers to transport therapeutics. Before stepping forward to novel CBNs for targeted drug and gene delivery, the following points need to be considered.

5.1 Mechanism of Targeting Drugs and Genes

Targeted therapeutic delivery enables the delivery of a therapeutic that enhances the concentration of that medicine in particular parts of the body relative to others. Therapeutic targeting refers to the ability of the therapeutic agent to accumulate in the target site selectively, independent of the sites and methods of administration. The mechanism by which a drug or gene is targeted can be broadly divided into two types. The two types of targeting are passive targeting and active targeting. These types operate by two different mechanisms. The importance of therapeutic targeting approaches has been understood since the early clinical trials. Targeted therapeutic delivery is also called smart therapeutic delivery. The nanoparticles would be loaded with therapeutics and targeted to specific body parts where there is solely diseased tissue. During the process, interaction with healthy tissue is avoided. Targeted therapeutic delivery systems have been developed to optimize regenerative techniques. The efficacy of a therapeutic can be impacted significantly by how the therapeutic is delivered. It is possible to maximize the performance of a therapeutic inside the body. Therapeutics can be introduced into the body via several different routes. Targeted therapeutic delivery approaches are of great importance. These enable concentrated delivery of a therapeutic to its desired target—increasing efficacy and reducing off-target effects. The unique feature of CNTs is the ability to interact with a wide variety of therapeutics, higher stability, and large expanded surface area. The therapeutics include drugs, therapeutic antibodies, DNA, and enzymes. CNTs can release the loaded therapeutics to their target sites. The CBNs carry the therapeutics by two mechanisms, namely, the CNTs can load them into the matrix or attach them to their surface. The method of internalization is generally recommended for effective attachment to the surface. The microenvironment of the cell matrix destructs the binding after the entry of therapeutics into cells. Therefore, the therapeutics can be released before entering the cell. However, there are different types of attachment to the surface. In one method, the drug is released before entering the cells. Several ways are used to move the CBNs into cells. The methods involved include insertion, diffusion, and endocytosis [107].

5.2 Fabrication of Drug and Gene-Laden Nanocarriers

There are many different methods of fabrication of nanomaterials. Some of the methods are coprecipitation, hydrothermal synthesis, inert gas condensation, etc. Various synthesis methods prepare therapeutic-laden nanocarriers. The best suitable method is nano-encapsulation. Other synthetic methods include emulsion polymerization, the continuous phase method, and the continuous aqueous phase method. Numerous factors affect therapeutic loading capacity apart from synthesis methods and reaction conditions. A nanocarrier can be designed by fabricating the nanosystem with optimum clearance characteristics. Computational modeling is often

used to develop new nanoparticles and better understand the existing ones. The three delivery methods are passive targeting, active targeting, and physical targeting. CNTs have several challenges before being considered effective methods for delivering genes and drugs. CNTs are restricted in their vast application in the delivery of drugs and genes by rapid release and low loading profile. The therapeutic-loading capacity must be improved. Therapeutics can be loaded by solvent evaporation, melting, and adsorption equilibrium. The therapeutics used depends on the efficiency of loading. Melting serves as an approach for loading therapeutics. Therapeutics are unable to enter into pores. However, therapeutics are distributed throughout the carrier by melting technique. Therapeutics are unstable in this form. The drugs and genes do not bind with carbon materials to crystalline states. CBNs are dipped into a solution of therapeutics. The therapeutics enter and arrange themselves until they reach equilibrium. Centrifugation is used to separate the solution from therapeutic-laden CBNs. The solvent has to evaporate after adsorption. The method has higher efficiency of loading than the melting method. An inhibition effect was observed when cisplatin was loaded into MWCNTs. Furthermore, a significant increase in loading efficiency was observed. The CNT-based hydrogel was fabricated using biodegradable hydrogel and DWCNTs [108], increasing electrical conductivity and mechanical properties. Several drugs reported exceptional therapeutic loading efficiency of CNOs. Examples of these drugs include 5-fluorouracil, ibuprofen, and paracetamol. Therapeutic loading efficiency can be increased by porosity, drug-CBN interaction, surface area, and attachment with polymer matrix. Carbon dots can improve drug loading capacity. For example, the loading capacity of doxorubicin is increased by the hollow structure of the CBNs [109].

5.3 Uptake of Carbon-Based Nanotubes by Cells

CNTs have long been regarded as promising carriers in biomedicine. CNTs are uniquely equipped to carry therapeutic molecules across biological membranes due to their high surface area and needle-like structure. This results in CNTs widely researched for use in theranostic applications. CNTs can carry many molecules; hence, they have been used to design nanotube-based delivery systems. To tailor nanotube function, therapeutic molecules can be added to functionalized CNT (F-CNT) side walls. F-CNTs can be filled to keep the surface available for further modifications by taking advantage of their inner cavity. The F-CNTs are supposed to have a high propensity to cross cell membranes. F-CNTs are taken up by the cells through passive diffusion and endocytosis. Cells do not show any impact on the permeability of the cell in the presence of inhibitors of endocytosis. This impact was observed after incubation at low temperatures. Labeled CNTs would be helpful to internalize and track within the cytoplasm of fibroblasts. The tracking can be done by fluorescence or microscopy. Cells detected oxidized CNTs that were conjugated with fluorescent and streptavidin. The acid resulted in the oxidation of the sidewalls of SWCNTs. Noncovalent binding was observed. Moreover, nonspecific

conjugation of protein and nanotube was observed. The protein migrated inside the mammalian cells. Nanotubes acted through the endocytic pathway as transporters.

The conjugated materials successfully entered the cytoplasm and initiated biological activity within the cells. A wild-type functionalized nanotube was able to enter the membrane. The insertion required hydrophobic-hydrophilic matching. Nanotube fluorescence spectra were used to analyze pristine SWCNTs' uptake into macrophages [110]. Fluorescence intensity was directly proportional to incubation time and concentration of the CBNs. The uptake of the nanotube occurred via phagocytosis. The cytotoxicity and apoptosis of cells increased by functionalized carbon spheres that were loaded with verapamil and doxorubicin. The study highlighted a relationship between high anticancer activity and low systemic toxicity [111]. CDs have been observed to be present in plasma. The metabolism of food is believed to be the primary source of high anticancer activity associated with low systemic toxicity. The activities and health conditions are regulated by the shape, size, and concentration of CBNs [112].

5.4 Ways to Augment the Solubility of Therapeutics

Solubility is essential for achieving the desired therapeutic concentration in systemic circulation for the desired pharmacological response. The solubility of the therapeutic depends on the solvent used, temperature, and pressure. Solubility is a significant challenge for formulation scientists. Any therapeutic to be absorbed must be present in the solution at the absorption site. Various methods are used for the enhancement of poorly soluble therapeutics. The selection of solubility improving approach depends on a variety of factors. The solubility by covalent or noncovalent bonding is enhanced by surface modification. Surface modification is possible between functionalized CNTs and therapeutics. Covalent and noncovalent modifications have advantages and limitations. Stable CNTs are produced by the drug gene-loaded CNTs bonded using covalent bonding. The bonding takes place in both intracellular and extracellular compartments. The covalently bonded CNT retards the release of therapeutics within a cell. Therefore, CBNs must be purified and solubilized in biological fluid. This activity must be done before use as therapeutics in nanomedicine. The most commonly used method for purification is oxidative acid treatment. The method includes refluxing or sonicating the CBNs with concentrated nitric acid or sulfuric acid. Covalent and noncovalent methods may improve biocompatibility and solubility [113]. Functionalization by covalent bonding is superior as it forms a strong bond between the CBNs and the biomolecules. Covalent functionalization grafts chemically reactive molecules onto the surface carbon. Materials have been lost during the acid-oxidation process, but the issue is less concerned with therapeutics delivery. The performance of the material is improved by surface functionalization. Doxorubicin was incorporated to evaluate it biomedically. The decoration of sidewalls of CNTs with oxygenated functional groups has been introduced. The oxygenated functional groups include carbonyl, carboxyl, and

hydroxyl groups. The carboxyl group is commonly used in functionalization. The amine site of biomolecules binds with CNTs using the carboxyl group. Several agents activate carboxylic acids. These cross-linking agents include esters, oxalyl chloride, or carbo-di-imides. Several other functionalization approaches are available for the functionalization of the sidewalls of CNTs. These approaches include ozonolysis, radical additions, electrophilic reactions, and hydrogenation. Covalent functionalization ruptures the sp^2 bonding, whereas noncovalent functionalization does not damage the sp^2 bonding.

5.5 Penetration of Blood-Brain Barrier by CBNs

Permeation facilitates the efficient delivery of therapeutics to target sites. The blood-brain barrier (BBB) is concrete for drug molecules. Endothelial cells are dominant cells in the blood-brain barrier. It is quite hard to penetrate the blood-brain barrier for different sizes of therapeutics. This barrier is a highly selective semipermeable border. The blood-brain barrier prevents circulating solutes from freely crossing into the central nervous system. The central nervous system is highly protected as it contains neurons. Different invasive methods (i.e., injections and even surgery) administer therapeutics to the brain cells. However, infection and edema may result from these invasive techniques [114]. Therefore, there is a need to precisely transport therapeutics to the brain, using vehicles that can cross the BBB [115]. Intracranial drug delivery might be a promising approach to overcome this difficulty, which shows promise to overcome the blood-brain barrier crossing, as indicated in Fig. 5. A transcytosis is an approach that can be used to cross the BBB, and it can be achieved in three different ways: via adsorptive-mediated transcytosis, transporter-mediated transcytosis, and receptor-mediated transcytosis [116, 117]. When the nanoparticles are inside the brain, they should be able to reach the target, i.e., tumor cells, neurons, or even fibrils, associated with many neurological diseases.

Additionally, researchers in the biomedical and pharmaceutical fields have been interested in functionalized multiwalled carbon nanotubes (fMWCNTs) for various applications [118]. These include neural prostheses and the delivery of therapeutic complexes to the brain [119, 120]. For example, MWCNTs with angiopep-2 PEGylated have been shown in vivo to target and decrease the proliferation of brain cancer cells [121, 122]. In addition, functionalized single- and multiwalled carbon nanotubes have value-added results in murine strokes and excitotoxic situations [123]. In addition, fullerenes possess additional activities like anti-aggregation and antioxidants. Therefore, fullerenes may be used to generate novel therapeutics for neurodegenerative disease.

Furthermore, graphene quantum dots and fullerene demonstrated anti-amyloid action. This could open a new avenue in the management of neurodegenerative disorders. The carbon nanostructures include CNTs, carbon dots, and nanodiamonds. The biological molecules include proteins and amyloid molecules. The biological

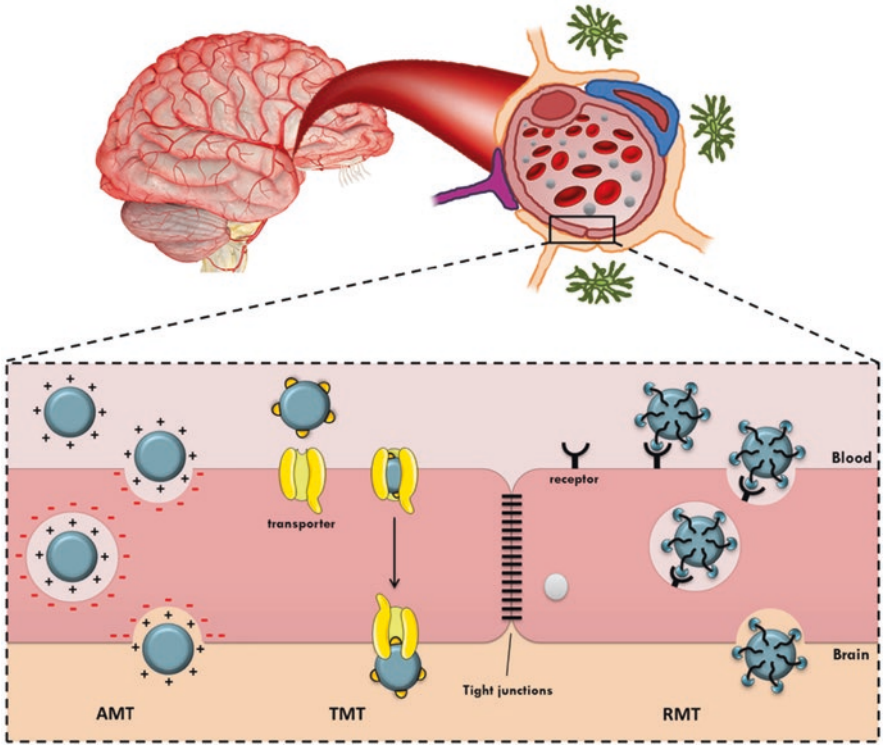

Fig. 5 An illustration of how nanoparticles cross the blood-brain barrier (BBB). (Reproduced with copyrights permission from Ref. [155])

molecules can cross the blood-brain barrier. Moreover, these biological molecules are tiny and can repair worn-out neurons [124].

6 Functionalization of Carbon-Based Nanomaterials for Drug and Gene Delivery

6.1 Functionalization of Carbon Dots

Surface functional groups can modify carbon dots' characteristics. The most common groups found in carbon dots are amino, carboxy, and hydroxy, which can be added via covalent and noncovalent modification [125]. The capacity to adjust carbon dots' size, shape, and physical properties through covalent modification is desirable. A covalent bond enables the covalent bonding of functional groups to carbon dots, increasing electron cloud density, improving conjugate arrangement, and changing the fluorescence spectrum of carbon dots. In addition, it acts as a link

between nanoparticles and biological systems, or it can be further conjugated with other molecules to give perfect carbon dots new capabilities [126].

Moreover, carbon dots and modified molecules interact electrostatically to form the noncovalent modification. As a result, the carbon dots will lose their structure. New functional groups will target the molecules or metal nanoparticles on the surface, allowing a more efficient bonding between nanoparticles and biological surfaces. The most apparent benefit of noncovalent modification is its structural integrity, created by the carbon dots' diverse functional groups and structure properties [127]. Havrdova et al. studied the cytotoxicity consequence of carbon dot surface functionalized with carboxylic groups, polyethylene glycol, and polyethyleneimine. Their result showed that the polyethylene glycol didn't induce any abnormalities in the cell morphology at the 300 $\mu g ml^{-1}$ concentration, whereas the carboxyl and polyethyleneimine-modified carbon dots showed toxicity at 100 $\mu g ml^{-1}$ concentration [107]. Park and his co-researchers synthesized multifunctional carbon-based nanodots using atmospheric plasma treatments involving O_2 and N_2. They used polyethylene glycol as a precursor. They tested the synthesized multifunctional carbon-based nanodots in different cell lines and reported that the materials are biocompatible. Further, an antibacterial study showed that it inhibits the growth of *Escherichia coli* and *Acinetobacter baumannii* [128].

6.2 Carbon Nanotubes

Carbon nanotubes' vast surface area, high aspect ratio, and unique material properties like mechanical strength, drug-loading capability, pH-dependent healing delivery ability, and thermal and electrical conductivity make carbon nanotubes ideal for next-generation fabrication composite materials. Despite their appealing characteristics, they clump together due to their chemical nature, which confines their application. Surface functionalization is necessary to overcome agglomeration and promote dispersibility, resulting in improved functionalized carbon nanotube interactions with polymer materials. Carbon nanotubes can be classified into single-walled (SWCNT) and multiwalled (MWCNT) based on their wall structure. Carbon atoms in SWCNTs are arranged in three ways based on their basic arrangements: armchair arrangement, zigzag arrangement, and chiral or helical arrangement. An MWNT is made up of a series of concentric graphene cylinders. Three to five sheets of SWCNTs are rolled on top of one another to create a multilayered structure. MWCNTs come in various shapes and sizes, depending on how the graphitic sheets are arranged [124]. Haddon et al. used chemically modified CNTs as a substrate for the progression of neurons. They reported that neural processes' extension and branching pattern could be modulated by altering the charge carried by different functional groups of functionalized CNTs [129]. Kaur et al. developed and evaluated the 5-FU-loaded FA-PEG bis amine/MWCNTs formulation in vitro, ex vivo, and in vivo. Their results showed that surface-functionalized CNT has potential tumor targeting. The pharmacokinetics study result showed that the drug was

released in a controlled manner at the targeted site. CNT advancements have resulted in "smart bio-nanotubes," and these next-generation nanomaterials have supramolecular characteristics compared to regular nanotubes. A microtubular protein covered with a lipid bilayer, namely, tubulin, creates these tri-layered structures. The thicknesses of protein-lipid and protein coatings are crucial formulation variables that control the drug release rate from such bio-nanotubes. Several pharmaceutical formulations of carbon nanotubes have recently been created for various controlled drug delivery uses [130].

6.3 Graphene

Graphene usage in the delivery of pharmaceuticals is based on its unique features. This results from the high surface-to-volume ratio, polyaromatic arrangement, and flexibility of different functionalization options. This structure is capable of loading, transporting, and targeting cargo. In addition, the combination of hydrophobic and hydrophilic regions of the graphene family of nanomaterial flakes ensures their solubility in aqueous solutions and their consequent interaction with lipids in cell membranes [131]. Zhi and his team studied the immune toxicity of graphene oxides and the effects of PVP coating. The group reported good immunological biocompatibility and immune-enhancement effect in in vitro conditions. They emphasized using surface-functionalized graphene oxides as immune-adjuvant in the future [132]. The graphene oxide nanocarrier demonstrated by Zhang et al. helped deliver two anticancer drugs with controlled loading and targeting. In this study, graphene oxide was functionalized with sulfonic acid groups followed by covalent binding of folic acid and then targeted with folic acid receptors, leading to the growth of human breast cancer cells. In addition, a controlled loading of two anticancer drugs (doxorubicin and camptothecin) was performed onto the surface-functionalized graphene oxides via noncovalent bonding. Their result showed that the combined usage of the two drugs' therapeutic efficacy was more efficient [133]. Chen and his co-researchers conjugated polyethylenimine-graphene oxide (PEI-GO) through covalent bonding. They found that PEI-GO could successfully transfect cells with plasmid DNA when evaluated for transfection efficiency [134].

6.4 Mesoporous Carbon Nanoparticles

Mesoporous carbon nanoparticles' (MCNs) carbonaceous framework is usually generated by high-temperature calcination or hydrothermal treatment [135]. MCNs were designed to be highly hydrophobic in the initial stage. The oxidation of MCNs with a strong concentrated acid improves their hydrophilicity. It produces functional groups, a regularly used approach to attain a hydrophilic surface and make surface modification conceivable [136]. Alternatively, in a diluted H2SO4 solution

containing ammonium persulfate, MCNs can also be oxidized. On the surface of MCNs, this acid oxidization procedure can produce a lot of functional groups (mostly carboxyl groups). The oxidized MCNs could then be modified for PEGylation, stimuli-responsive grafting, polymer coating, targeting, and diagnostic imaging, among other things [137]. Miguel and his colleagues investigated two types of MCNs with varying shape, size, and pore structures as drug nanocarriers after functionalizing them with a self-simulative polymer sensitive to pH variations. They reported that a small amount of drug was released at the physiological pH of 7.0 and acidic pH of 5.0 significantly fewer drugs. Further cytotoxicity tests revealed that when loaded with the anticancer drug doxorubicin, the hybrid nanocarriers are less cytotoxic and impede cell growth [138].

6.5 Fullerenes

Fullerene is proving to be promising in widening the horizons in nanomedicine owing to its inertness surface tailoring ability to be used for biomedical applications [139]. Several attempts are made to exploit fullerenes' controlled and targeted drug delivery potential. Non-immunological properties, high efficacy, and low production cost have lifted fullerenes to be a promising agent for nucleic acid delivery [140]. The major disadvantage of fullerene is its hydrophobicity, and therefore several approaches are employed to promote its hydrophilicity [141]. Hydrophilic, cationic tetra-amino fullerene, and siRNA formed nanocomplexes suitable for lung tumor targeting. The complexes agglutinate with plasma proteins, thereby clogging lung capillaries and rapidly releasing siRNA into cells silencing cancer gene expression [29]. In another study, modified (functionalized) fullerene C60-Dex-NH2 formed aggregates resembling micelles in water and guarded siRNA against ROS destruction. This system also enhanced gene silencing efficiency to 53% MDA-MB-231-EGFP in vitro and 69% in tumor-bearing mice by promoting lysosomal escape through controlled ROS generation and lysosomal membrane destruction upon exposure to visible light [142]. For instance, an amine-functionalized C60-PEI-FA/DTX drug delivery system enhanced DTX uptake in tumor cells and was safe for normal cells in vivo [143]. Molecular docking studies of fullerenes functionalized with sulfasalazine, naproxen, and curcumin indicate that functionalized Ca-decorated C20 fullerenes could effectively inhibit pro-inflammatory cytokines compared to naïve drugs [144]. Apart from nucleic acid delivery, its structure enables it to be explored for drug delivery applications. Fullerenes can be explored for drug delivery by assisting as noncovalent carrier of various drugs and facilitate covalent attached of drugs to its derivatized structure [145].

6.6 Nanodiamonds

Nanodiamonds (NDs) can carry high payload capacity and release cargos sustainably, depicting its promising nature in targeted drug delivery-controlled release [146]. NDs' size influences cellular uptake of drug-loaded ND. For instance, DOX conjugated ND of 45.8 nm displayed an increased cellular uptake than larger ones [147]. Employing ND for targeting cancer cells was achieved by folic acid ligand conjugation to generate the ND-PEG-FA complex [49]. Selective targeting of tumor cells and minimizing off-targeted effects on normal cells were achieved by developing PEGylated NDs loaded with DOX and pH-responsive release behavior [148]. Alendronate conjugated with NDs was a promising bone disease due to its selective uptake by osteoblast cells in vitro and in vivo [64]. Amine modified ND poly(1-O-methacryloyl-2,3:4,5 di-O-isopropylidene-β-fructopyranose) with carboxyl groups conjugated to the polymer chains were found to exhibit quick and pH-dependent release of DOX [149]. In addition to this, it was found to exhibit less toxicity over free DOX. pH-sensitive drug release behavior was also observed in NDs conjugated with periodic mesoporous organosilica nanoparticles [150]. PEGylated NDs controlled the low premature release of DOX in the tumor microenvironment [151]. Apart from anticancer therapies, PEI-modified NDs loaded with amoxicillin were effective against urinary tract infections [152]. Amine and carboxy groups modified NDs exhibited controlled release of loaded drugs vancomycin and tetracycline [153]. NDs can also co-deliver drugs such as DOX and malaridine under physiological conditions. Peptide vector and PEGylated polyglutamic acid grafted on NDs enhanced melittin (peptide drug) binding and effectiveness against breast cancer cells [154]. The studies mentioned above are few among several kinds of literature on the drug delivery property of NDs. They possess a wide range of drug release applications in various tumors. One may appreciate that multiple surface modifications are adopted to enhance the biocompatibility, dispersibility, stability, and drug release properties of ND. It is expected that NDs-based nano-drug carriers will revolutionize the drug delivery approaches in the near future.

7 Conclusion

Nanotechnology continues to push technological boundaries as nanoscience and materials make tremendous advances at an incredible rate. In nanoscience and nanotechnology, nanomaterials are crucial. Among the many accomplishments of nanomaterials in the modern era are applications in anticancer studies, neurodegenerative illnesses, antimycobacterial investigations, anti-inflammatory uses, topical agents, biomolecules, etc. Moreover, CBNs are viewed as a groundbreaking prototype for drug distribution as they allow for enhanced transfer of drugs in both systemic and topical applications. Additionally, they allow for control over stability, solubility, and drug loading capabilities. The scope of this chapter included an

outline of the classification, biomedical applications, the impact of the physical and chemical properties in drug and gene delivery, and, finally, the functionalization of carbon-based nanomaterials for drug and gene delivery. Nanomaterials derived from organic molecules, namely, carbon nanotubes, fullerenes, graphene, and nanotubes, were discussed. The use of organic nanomaterials in drug delivery systems is stable and controlled. Concerning the toxicity of CNMs, it is still causing concern, which can impact the in vivo application thereof. In addition to decreasing the biological toxicity effects of CNMs, the degradation investigations (which primarily depend on enzymes and microbes) aim to enable their application in biomedicine. Even though toxic effects in biological systems are still a concern, numerous chemical modification strategies have successfully addressed their toxic effects. These strategies include altering the effects on the delivery of drugs, tissue engineering, recognition of biomolecules, and cancer treatment. This chapter describes some accomplishments in the request of CBNs in biomedicine. In conclusion, the advent of nanotherapeutics and nanoformulations in due course offers pharmacological experts with means that, if technologically advanced, can modernize drug distribution studies, and CBNs are anticipated to be a fundamental component of it.

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Hybrid Multifunctional Nanomaterials for Diagnostic and Therapeutic Applications

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1 Introduction

The development of nanoscience and molecular biology, in the last decades, inevitably led to spectacular progress in the bio-nano interface. This scientific evolution is also obvious in material science. Thus, great effort has been devoted to the exploration of the possible clinical applications of the innovative nanotechnology achievements [1, 2], particularly in the detection, diagnosis, and treatment of various diseases [3].

A variety of nanomaterials with interesting properties is now available, and a continuously increasing number of new nanomaterials is reported, with sizes that range from a few to several hundred nanometers [4]. Some of the nanoscale materials have been proven promising in biomedical applications, for a wide range of purposes, such as for drug delivery, cancer treatment, and diagnosis [5], since they allow high pharmaceutical loadings, controlled size, charge, crystallinity [6–8], release manipulation [9], biocompatibility improvement, and precise targeting and accumulation in the area of interest [10]. Thus, these materials present numerous opportunities, improving particularly the current anticancer approach and minimizing the undesirable side effects of the conventional diagnosis and treatment.

A hybridism in nanomaterials synthesis is consequently observed. Hence, the previous well-known categorization of two classes of nanoparticles, the organic and the inorganic ones, has enriched with a third category, the so-called hybrid nanomaterials [11]. Hybrid nanomaterials are designed with both inorganic and organic components. Various strategies can be applied to prepare hybrid nanomaterials. Moreover, many combinations of different materials can be chosen to develop hybrid complexes [12]. For instance, gold nanoparticles can be mixed with natural polysaccharides, or metal oxides with polymers, leading to advanced composites with specific properties. Actually, researchers often choose to mimic complex natural materials, while sometimes they create artificial materials with hybrid composition and structure [13].

Recently, hybrid materials have attracted significant attention, since not only they retain the beneficial properties of all their ingredients, but also, they show additional synergistic performance, such as target specificity or biodegradability, improving the outcome of many biomedical procedures [14]. Therefore, it is reasonable that these nanomaterials are exploited in various applications, particularly in nanomedicine.

Hybrid nanomaterials can be utilized in diagnostics, as imaging and contrast agents, enabling precise visualization at a molecular level, exploiting the potential of a various novel or multimodal imaging techniques, and reducing any adverse effect in healthy tissues. Active targeting enhancements have been incorporated into the nanoparticles to create multifunctional formulations [5]. These materials allow the improvement of the implementation of well-known effective imaging techniques, such as MRI (magnetic resonance imaging), PET (positron emission tomography), CT (computed tomography), etc. [15, 16].

Hybrid nanomaterials can also be used in therapeutic applications and modalities, particularly in anticancer therapy, such as photodynamic and photothermal therapy, hyperthermia and radiotherapy, neutron capture therapy, magnetic therapeutic approaches, drug delivery, and gene therapies [17].

Furthermore, hybrid multifunctional nanomaterials can be incorporated with several components that can be used for simultaneous diagnostics and therapeutics (theranostics), allowing real-time monitoring of an efficient treatment process, leading to more personalized healthcare systems [18]. The new field of theranostics is referred to as a treatment strategy that monitors the response to treatment, increasing the efficacy of a drug and permitting the concurrent detection of the target area throughout the treatment process [19]. Limitations and safety issues are still under consideration before the clinical routine finally incorporates nanotheranostics.

Thus, the synthesis of advanced hybrid nanomaterials for targeted and on-demand theranostics can be considered a state-of-the-art topic in nanomedicine, and for this reason, these applications will be discussed in this chapter.

2 Nanomaterials

Engineered nanostructured materials are among the most impressive materials and unveil distinctive chemical, physical, and/or biological characteristics. Various nanostructured materials (NSMs) have been developed, in the last decades, so the need for their classification was early presented [4]. Several attempts of categorization have been reported. The most systematic classification was achieved by Pokropivny and Skorokhod [20] that was based mainly on the number of dimensions of a nanomaterial that are not considered on nanoscale. Thus, according to this scheme, there are zero-dimensional (0-D) (e.g., nanoparticles, nanocapsules nanospheres, composite nanoparticles, etc.), one-dimensional (1-D) (e.g., nanotubes, nanowires, etc.), two-dimensional (2-D) (e.g., nanoplates, nanobelts, graphene, etc.), and three-dimensional (3-D) nanomaterials (3-D electrodes, fullerene, etc.) (Fig. 1).

The group of nanoparticles is a very important class of nanomaterials, possessing specific properties that differentiate them from their bulk materials. Among the most characteristic properties, it is worth mentioning their controlled particle size, stability, bioactivity, bioavailability, and the fact that they include large active surfaces and easily controllable surface chemistry that allows binding to small molecular drugs, imaging labels, and several ligands [21, 22] (e.g., antibodies, peptides, and nucleic acids). Their interesting characteristics make them the materials of choice for various applications, particularly in biomedicine. Their small size allows for exclusive intracellular and extracellular interactions, such as extravasation via endothelial cells and increased permeability and retention in tumor tissues. Due to their capacity to enter cells, tissues, and organs than macro particles so, conquer the poor bio-accessibility and high toxicity of present pharmaceutics, their potential for medication delivery can be considered as incredible [23].

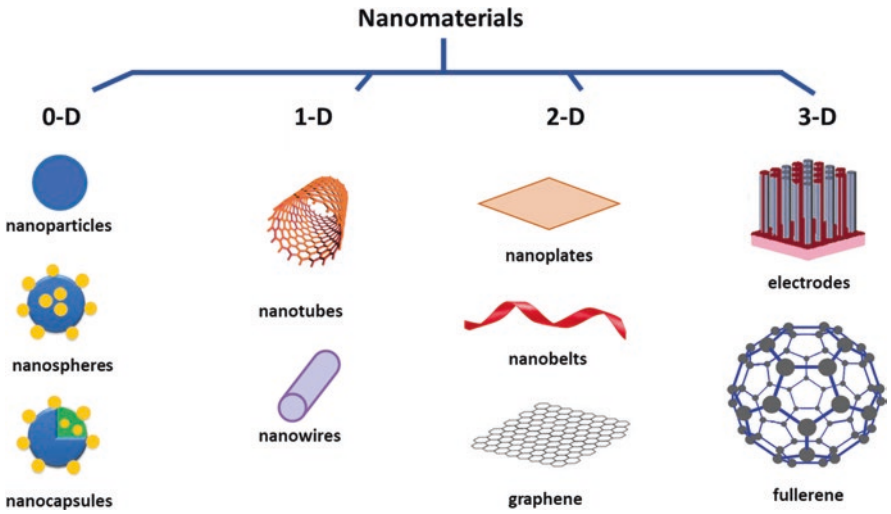

Fig. 1 The common classification of the nanomaterials is mainly based on the number of dimensions of a nanomaterial that are not considered in nanoscale

Thus, focusing on the category of 0-D nanomaterials and particularly on nanoparticles, two main classes can be traditionally detected: the inorganic (e.g., metallic and semiconductor nanoparticles, quantum dots, magnetic nanoparticles, etc.) and the organic nanoparticles (e.g., liposomes, dendrimers, micelles, etc.) [24]. The inorganic nanoparticles typically are biocompatible and less cytotoxic than organic ones. They provide novel electrical and optical characteristics which can be adjusted during assembling [4]. Gold, silver, iron oxide, silica, and titanium dioxide are among the characteristic nanoparticles of this group. The organic nanoparticles are commonly used as nanocarriers, loading several types of pharmaceuticals. There is also one more class, that of hybrid nanoparticles which is a mixture of nanoparticles from the two previously mentioned categories (Fig. 2).

2.1 Hybrid Nanomaterials

The progressing demand for innovative materials with tailored physicochemical properties has led to the development of hybrid materials with remarkable properties and multifunctional nature [25]. Hybrid nanostructured materials (HNMs) are formed by two or more components of nanoscale dimensions. HNMs are also defined as a mixture of organic and inorganic compounds. These materials can be either homogeneous or heterogeneous systems including domains of each organic or inorganic component that range from a few angstroms to a few tens of nanometers [26]. The nature, structure, and contents of the organic and inorganic components of a hybrid nanomaterial affect its properties and determine its behavior.

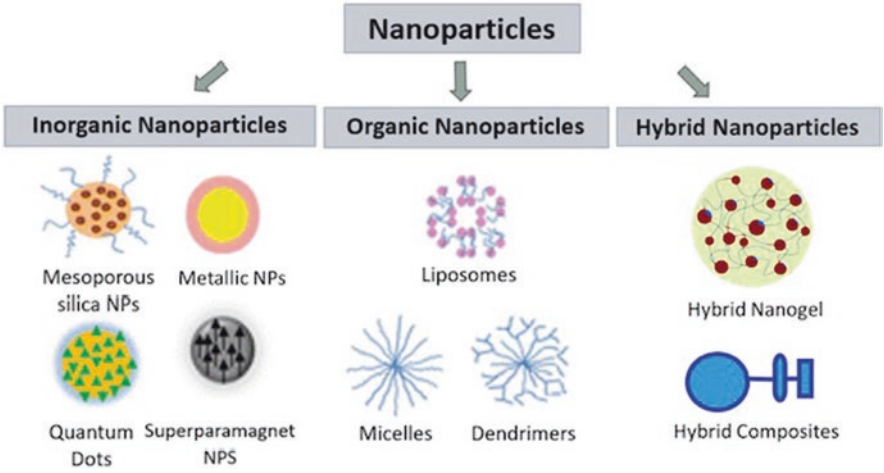

Fig. 2 The main categories of nanoparticles are three, the inorganic (e.g., metallic nanoparticles, quantum dots, magnetic nanoparticles, etc.), the organic nanoparticles (e.g., liposomes, dendrimers, micelles, etc.), and the hybrid nanoparticles (a mixture of organic and inorganic components)

Various studies suggest several strategies for the preparation of hybrid nanomaterials. Moreover, many combinations of different materials can be chosen to develop hybrid ones [12]. Metal oxides are commonly mixed with natural polysaccharides or polymers. Also, liposomes can encapsulate many types of nanoparticles. The combination of different nanomaterials leads to the development of advanced composites with specific properties [4, 21]. Recently, based on the physicochemical properties of hybrid materials, they have attracted significant attention, since not only they retain the beneficial intrinsic properties of all their individual ingredients, but also, they show additional synergistic performance [25]. Hence, the properties of the HNMs can be tuned by modifying their structure and morphology leading to advanced materials with improved performance characteristics, such as target specificity or biodegradability, electron conductivity, high thermal stability, mechanical strength, etc. improving the outcome of many biomedical procedures [14, 27]. The wide spectrum of their accessible properties allows the exploitation in significantly diverse fields (microelectronics, optics, energy, environment, catalysis, etc.) [28]. It is reasonable that hybridism in material science is considered as a burgeoning and highly interdisciplinary research field. Consequently, it is logical that these nanomaterials can be exploited in various biomedical applications, in the frame of nanomedicine.

2.2 Hybrid Multifunctional Nanomaterials

Mono-functional nanoparticles typically provide a single function, while multifunctional nanoparticles can participate in a series of different processes. The functionality of a nanoparticle can be modulated by several strategies, such as surface modification, to fit some specific and targeted applications [29]. Stabilization of the nanoparticles in a solution for the development of a controlled growth system; incorporation of functional groups at the surface for enhanced performance or additional capabilities; improvement of the solubilization in various solvents to extend the application spectrum; changes in optical, spectroscopic, mechanical, chemical, optical, and magnetic properties; and modification of the toxicity are among the main goals of a functionality modification [24, 29]. Thus, multifunctional nanomaterials have gradually attracted scientific interest due to their ability to combine numerous properties [29]. Multifunctional nanomaterials are smart and highly functional materials which gather all the desirable characteristics of their components and sometimes possess some modified properties, quite different from those of the individual conventional materials [30]. Thus, multifunctionality gives them the ability to perform various goals such as co-delivery of contrast agents, targeted treatment, and surface modification in order to attach different ligands.

Great achievements are reported in biomedicine, regarding diagnosis, treatment, and prevention. However, worldwide, the highly prevalent diseases (cardiovascular diseases, cancer disease, neurodegenerative diseases, etc.) are still leading death causes or quality of life reduction factors [31]. Therefore, the development of new nanotechnological tools for relevant application in biomedicine is still an ongoing research goal addressed by different methodological approaches. In this context, the specific targeting of disease-related pathways and molecules, mostly proteins, is a promising strategy [32].

Hybrid nanomaterials can be utilized in diagnostics, as imaging and contrast agents, enabling accurate visualization at a molecular level, and exploiting the potential of several innovative or multimodal imaging techniques. In parallel, these materials can reduce any adverse effects on healthy tissues [33]. The main advantage is that they can allow the improvement of the implementation of well-known effective imaging techniques. Hybrid nanomaterials can also be used in therapeutic approaches, or palliative treatment, particularly in anticancer therapy [17]. Hybrid nanomaterials can also be used for the prevention of various diseases through their use in nanovaccines or point-of-care applications, such as rapid diagnostic tests and lab-on-chip approaches [34]. Furthermore, hybrid nanomaterials are used in tissue engineering for the development of scaffolds. Hybrid multifunctional nanomaterials can be used for simultaneous diagnostics and therapeutics (theranostics), allowing real-time monitoring of an efficient treatment process, leading to more personalized healthcare systems [18]. Hence, there is a great variety of applications of hybrid multifunctional nanomaterials in biomedicine (Fig. 3).

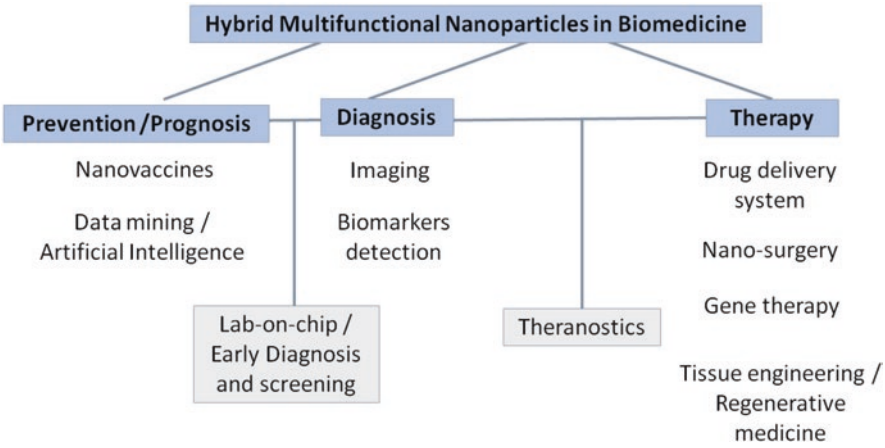

Fig. 3 The applications of hybrid multifunctional nanomaterials in biomedicine

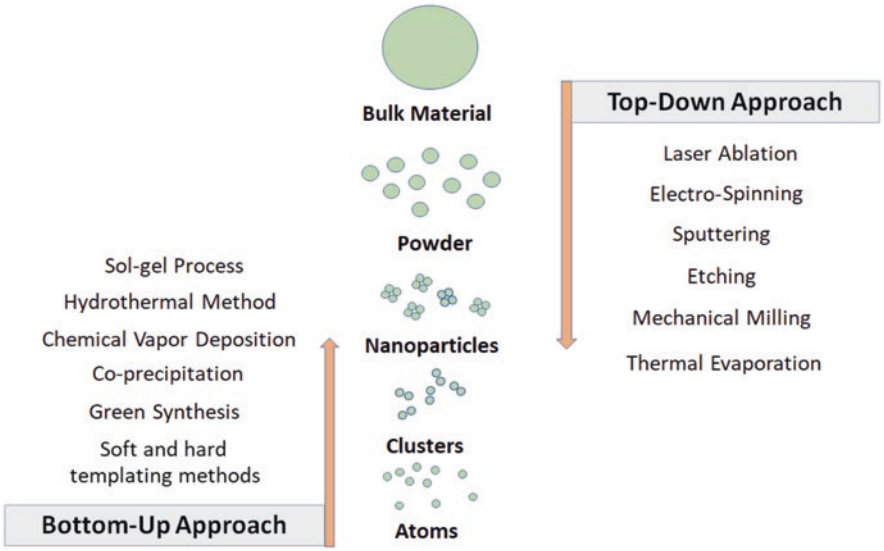

Fig. 4 Top-down and bottom-up synthesis methods for nanomaterials synthesis

3 Synthesis of Nanomaterials

Top-down and bottom-up approaches are used for the synthesis process of nanomaterials [35]. Figure 4 shows the most common methods of synthesis.

3.1 Top-Down Methods

Applying top-down methods, bulk materials can be transformed into small nanoparticles. These techniques are quite simple to use, but are not efficient for applications with advanced requirements, regarding difficult geometric shapes and very small sizes of nanoparticles [4]. Laser ablation, electrospinning, sputtering, etching, mechanical milling, and thermal evaporation are among the most common top-down methods of synthesis [4, 35].

3.1.1 Laser Ablation

Laser ablation is a straightforward process of nanoparticle synthesis from various solvents [36]. A pulsed laser is used to remove molecules from a surface, creating micro- and nanostructures. Ablation occurs when the surface area absorbs enough energy to melt or evaporate [37]. This method has numerous applications in ceramics, glasses, metals, as well as polymers.

3.1.2 Electrospinning

This is a very simple method, generally used for nanofiber production. Coaxial electrospinning is among the most famous techniques. The required equipment includes two coaxial capillaries including viscous liquids. It is also common to use a viscous liquid as the shell and a non-viscous liquid as the core, if the producing material is core-shell structured [38].

3.1.3 Sputtering

During sputtering, high-energy particles, such as plasma or gas, target solid surfaces. The sputtering gas is added to an evacuated chamber, and a high voltage is applied, and free electrons-gas collisions lead to the production of gas ions. The positively charged ions continuously hit, due to the acceleration in the electric field of the cathode. This fact results in the ejection of atoms from the target area. Thin films are usually produced through this method [39].

3.1.4 Etching (Lithography)

Two main types of lithography are distinguished, masked and maskless lithography. Masked lithography applies a mask or template, and nanopatterns are transferred over a surface area. Photolithography, nanoimprint lithography, and soft lithography are among the most famous processes. Maskless lithography-free nanopatterns are

created without the use of a template. Scanning probe lithography, electron beam lithography, and focused ion beam lithography are the most common methods of maskless lithography [40].

3.1.5 Mechanical Milling

Mechanical milling allows the production of nanoparticles by attrition. Kinetic energy from the grinding medium is transferred to the material that is reduced. Metal alloys and various nanoparticles can be produced via this method [41].

3.1.6 Thermal Evaporation

Thermal evaporation is an endothermic method. During this process, heat causes chemical breakdown. Stable monodisperse suspensions, several inorganic nanoparticles, and thin films are often produced through this method [42].

3.2 Bottom-Up Methods

The bottom-up approach is based on the exact opposite fundamentals, compared to the top-down approach. Atoms and molecules are used as structural units, and through the growth and self-assembly of them, chemical development of nanomaterials is achievable. Hence, it is considered as a constructive approach [43]. Sol-gel process, hydrothermal method, chemical vapor deposition, co-precipitation, and green synthesis soft and hard templating methods are among the most famous bottom-up approaches.

3.2.1 Sol-Gel Process

Sol-gel method is a simple wet chemical technique and, for this reason, is widely applied. The term sol-gel is derived from the combination of sol and gel. Sol is a colloidal solution that is produced when solid particles are suspended in a liquid medium. Gel is a solid macromolecule which dissolves in a liquid. During this method, the liquid precursor is transformed into a sol [44]. The sol is finally converted into a jelly network structure that is called gel. Actually, sol-gel method is a five-step procedure. Hydrolysis, polycondensation, aging, drying, and calcination are the aforementioned steps. A great variety of nanomaterials, including thin films, nanoparticles, glasses, and ceramics, can be produced through this method.

3.2.2 Hydrothermal Method

The hydrothermal process is a well-known and extensively used method to produce nanomaterials. Nanostructured materials are attained through a heterogeneous reaction carried out in an aqueous medium at high pressure and temperature around the critical point in a sealed vessel. Nanowires, nanorods, nanosheets, and nanospheres are usually produced hydrothermally [38].

3.2.3 Chemical Vapor Deposition

Carbon-based nanomaterials are commonly developed via chemical vapor deposition (CVD). A thin film is created on the heated substrate surface as a result of a chemical reaction of vapor-phase precursor compounds. At high temperatures, the decomposition of the gas leads to the release of carbon atoms. These carbon atoms can recombine, forming carbon nanotubes on the substrate. Thus, the production of one- or two-dimensional nanomaterials is achievable through CVD [45].

3.2.4 Co-precipitation

Co-precipitation is a wet chemical technique, which is considered as basic and is widely used. The method refers to the use of a precipitation reaction to consistently compose two or more cations in a homogeneous solution. Then, this solution is combined directly or dropwise with another solution which contains dissolved precipitation agents. To obtain nanostructured material with desired crystal structures and morphologies, post-treatment such as annealing, sintering, or calcination is also applied [46].

3.2.5 Green Synthesis

The biogenic synthesis (green synthesis) of nanomaterials requires the use of non-toxic chemical substances (solvents, reducing and stabilizing agents, etc.) and generally utilizes plants and microorganisms. It is an eco-friendly approach with several applications [47].

3.2.6 Soft and Hard Templating Methods

Nanoporous materials are typically produced via soft and hard template methods. These methods are generally easy and allow a great range of morphologies and sizes. Soft templates like block copolymers, flexible organic molecules, and anionic, cationic, and non-ionic surfactants are used in soft templating methods. Interactions between the precursors and the templates are based on electrostatic forces,

hydrogen bonding, and van der Waals forces. Nano-casting or hard templating method uses solid templates, whose pores are filled with precursor molecules [38].

3.3 Synthesis of Hybrid Multifunctional Nanomaterials

The design and development of advanced multifunctional hybrid nanomaterials is a challenging procedure. The synthesis methods that are commonly applied are already mentioned. Additional methods to produce liposomes and dendrimers and generally several nanocarriers and drug vehicles are also needed to be mentioned as well as some methods improving the functionality, such as chemical surface modification and doping and standard methods for conjugation between the separate parts of the hybrid nanomaterials [24].

3.3.1 Liposomes Preparation and Drug Loading

The common methods that are used to prepare liposomes involve the following steps: drying lipids obtained from organic solvents, dispersing the lipids in aquatic solutions, purifying, and analyzing the produced material. Furthermore, as far as drug loading to liposomes, two approaches are distinguished, the active and the passive one [48].

3.3.2 Dendrimers Preparation and Drug Loading

Dendrimers lie at the interface between the polymer and molecular chemistry. The controlled synthesis based on specific steps is related to molecular chemistry procedures, and the structure which is consisted of monomers is associated with polymer chemistry techniques. Dendrimers are commonly prepared through a divergent or a convergent method. A multifunctional core molecule reacts with monomers including reactive groups and gradually leads to the first-generation dendrimer which is enriched with more monomers. The interactions between dendrimers and drugs can be categorized into two categories, physical and chemical bonding [49].

3.3.3 Functionalization of Nanoparticles and Conjugation Strategies

To modify the nanoparticles to achieve different properties, a variety of ligands can be incorporated into them, allowing them to be used in drug delivery systems, diagnosis, treatment, and sensoring [50].

Green fluorescent protein (GFP) has been electrostatically complexed with nanoparticles in many studies, for the accurate detection of healthy and cancer cells [51]. Other studies give an effort to functionalize nanoparticles with ligands

exhibiting differential affinity toward proteins. Thus, various cell surface molecules are employed for their identification [52]. Also, several studies focus on the modification of the surface charge, the hydrophobicity to optimize their performance in sensors.

Moreover, coating with polymers (e.g., polyethylene glycol (PEG)) can facilitate passive targeting of tumor areas exploiting the enhanced permeability and retention effect (EPR effect). Furthermore, coating with biomolecules leads to specific attributes, such as improved efficacy of drug delivery and minimal toxicity [50].

Various conjugation techniques are applied to produce hybrid multifunctional materials. Arginine-glycine-aspartate (RGD) and folate (FA) can be used as the functional targeted ligands for the conjugation of polydopamine (PDA) functionalized nanoparticles [53]. Biotin-streptavidin binding is also a widely used technique to conjugate two or more parts of a hybrid material [54].

3.3.4 Surface Modification

The modification of the surface of nanoparticles, to change physical, chemical, or biological characteristics, is called surface modification. It is usually applied to solid materials. Reactivity, biocompatibility, surface charge, hydrophilicity, roughness, and photocatalytic properties are among the characteristic that is commonly improved through surface modification [55].

3.3.5 Doping, Dye Sensitization, Coupling

The electrical, optical, and biological properties of the nanoparticles can be modified through the doping process. Light-sensitive dyes are used in dye sensitization methods to increase the light absorption of the nano-photocatalysts. Coupling with metals leads to the improvement of the semiconductivity, due to the enhanced charge separation and the increased light absorption [56].

3.4 Characterization of Hybrid Multifunctional Nanomaterials

Various characterization techniques are used to study the physical and chemical properties as well as the morphology of the synthesized nanomaterials. Hybrid multifunctional nanomaterials are typically characterized through the same techniques that are going to be presented concisely.

Dynamic Light Scattering (DLS)
Dynamic light scattering is utilized to estimate the size (hydrodynamic radius) and the zeta potential of the produced nanoparticles. Particles' size can influence the

medication discharge. Particles with a small size typically provide a bigger surface area. Zeta potential can give a sense of the surface charge [57].

X-Ray Diffraction (XRD) Analysis
The crystallinity and the crystallite size can be estimated through the XRD method [58].

Raman Spectroscopy
Raman spectroscopy can clarify the complexity of the structure of the nanomaterials, detecting different crystal forms [59].

Fourier-Transform Infrared (FT-IR) Spectroscopy
FT-IR spectroscopy is used to obtain an infrared spectrum of absorption or emission of a solid, liquid, or gas identifying the composition of the produced material, compared to standard databases [60].

Nuclear Magnetic Resonance Spectroscopy
NMR solid-state spectra can confirm the data obtained by FTIR analysis, providing further information on the structure of the material network and its condensation rate [44].

Ultraviolet-to-Visible (UV-Vis) Spectroscopy
UV-Vis spectrometer allows diffuse reflectance measurements for the determination of the band gap (Eg). It is also used for the quantitative determination of different analytes in a compound [61].

X-Ray Photoelectron (XPS) Spectroscopy
XPS can identify the elements existing within a material (elemental composition) or are covering its surface, as well as their chemical state, and the electronic structure and density of the electronic states in the material [62].

Scanning Electron Microscopy (SEM)
SEM allows morphological observation with direct perception. This technique is tedious, costly, and frequently needs reciprocal insights about estimating dispersion, but it is considered among the most reliable methods of characterization [63].

Transmission Electron Microscopy (TEM)
The transmission electron microscope is mainly utilized for recognizable proof of the morphology of the produced nanoparticles [64].

Atomic Force Microscopy (AFM)
AFM images are used to determine the surface topography and roughness profiles of nanoparticles [65].

Surface Hydrophobicity
Surface hydrophobicity is determined by numerous techniques along with hydrophobic interplay chromatography, biphasic partitioning, and adsorption of probes [66].

Drug Release
Drug release assays are used to investigate the drug release mechanism. Drug loading assays are widely used [67].

Stability
The conventional methods to check the stability of the produced nanomaterials include TEM, DLS, UV-Vis spectroscopy, and zeta potentiometer [68].

4 Hybrid Nanomaterials for Diagnostic Applications and Population Screening

Nanoscale materials are designated to allow the implementation of highly effective imaging techniques. The majority of the imaging applications include both inorganic materials, such as quantum dots, gold nanoparticles, iron oxides, etc., and organic ones, like dendrimers, liposomes, micelles, and polymeric nanohydrogels. A significant increase in the use of nanomaterials in bioassays and biosensors has been seen in the last decades [24]. New strategies for surface functionalization to create versatile recognition agents allow the development of innovative biodiagnostic assays and sensors, allowing precise and robust detection of a wide range of compounds. Hybrid stimuli-responsive nanomaterials can be used to transduce molecular interactions into biomedical signals [69]. These signals are recorded by standard equipment or optical observation. Several biosensors can provide reliable and fast monitoring with high sensitivity and specificity. Sometimes it is crucial to detect even incredibly low levels of a marker for a particular disease. Thus, the optimization of the materials that are used is of crucial importance for early diagnosis and population screening [70, 71].

Gold nanoparticles have been thoroughly studied for bioassay applications and particularly for biosensors and molecular diagnostics. Their optical properties as well as the well-established synthesis methods allow their wide use. Gold nanoparticles can form stable suspensions in aquatic solutions. Hence, they allow simple conjunction with biomolecules [72]. In combination with various synthetic materials such as fluorescence dyes, oligonucleotides, polymers, and quantum dots, graphene oxides can form gold-based hybrid nanomaterials providing multifunctionality in molecular detection. Due to the localized surface plasmon resonance (LSPR) phenomenon, which is observed in gold nanoparticles suspensions, the development of a large number of colorimetric bioassays, exploiting the color shift, detected when the aggregation or redispersion of the nanoparticles is induced by the presence of an analyte [73]. Various bioassays based on gold nanoparticles have been utilized. One of the first attempts was testing cerebrospinal fluid from patients with syphilis. In pathogenic samples, gold nanoparticles aggregated, while they remained dispersed in normal samples. Also, antibody-functionalized gold nanoparticles have found extensive commercial applications. The pregnancy test and SARS-CoV-2 rapid antigen test are representative examples of their wide use. DNA-conjugated

gold nanoparticles are used for colorimetric detection of non-nucleotide analytes (metal ions, growth factors, narcotics, etc.). Real-time screening of endonuclease inhibitors is allowed through the use of DNA-crosslinked gold nanoparticles that can be disassembled by endonucleases, cleaving the double-stranded DNA in a site-specific manner [72, 74]. Functional peptide-gold-based hybrid nanoparticles have also been proposed as promising candidates in the field of molecular imaging (biosensing/diagnostics and cell targeting/imaging) [75].

Magnetic nanoparticles, quantum dots (QDs), and plasmonic noble metal nanoparticles are among the most common nanoparticles that are used in biomedical applications intended for diagnostics, due to their unique physical and chemical characteristics, their small size, and their large surface to volume ratio [29]. These groups of nanoparticles are utilized in various applications in vivo and in vitro. In cases of non-transparent, colored, or turbid biological solutions (blood, serum, etc.), gold nanoparticles are not suitable. However, magnetic nanoparticles, composed of iron oxides, metallic iron, and copper, are advantageous. Their response is detected through modalities that are based on magnetic resonance [76].

QDs are crystalline semiconductors with sizes ranging between 2 and 10 nm in diameter. They are a great alternative to organic dyes, due to their optical properties (broad-spectrum, narrow emission profile) and their chemical stability. PbS, CdSe, and InP are among the most common combinations for QDs [77]. The combination of magnetic nanoparticles and QDs improves the sensitivity in the detection of proteins and nucleic acids even at very low levels (bio-barcode assays), avoiding the conventional drawbacks, such as photobleaching, broad bands, etc. that are observed in other bioassays using fluorescent dyes [29].

Recent progress in the production of polypeptide-based hybrid nanomaterials that transduce molecular interactions, exploiting their magnetic and optical properties, leads to their utilization in bioanalytical applications, instead of the conventional biomolecular receptors, such as antibodies [78].

Also, nanomaterials that are constructed through metal-ligand coordination bonds are commonly used in diagnostic applications. Nanoscale metal-organic frameworks (NMOFs) and nanoscale coordination polymers (NCPs) are among those that are widely used as imaging and contrast agents. Complementary enhanced techniques might enable the multiple functionalities of those materials to be used also in therapeutics [1].

4.1 Imaging Modalities

Precise diagnosis of tumors requires detailed image information. Conventional imaging cannot provide comprehensive data. Nanomedicine has received extensive attention to cover this gap. A single injection of a nanostructures contrast agent might provide complementary information. The imaging modalities that have been studied for the possible incorporation of nanopharmaceuticals include positron emission tomography (PET), single-photon emission computed tomography

(SPECT), computed tomography (CT), and magnetic resonance imaging (MRI), optical imaging, and ultrasound imaging [79].

4.1.1 Positron Emission Tomography (PET) Multimodalities Based on Nanomedicine

Whole-body imaging is allowed through PET scans, routinely. PET is utilized in nuclear medicine for various organ imaging, using β^+-emitting radionuclides such as ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{64}Cu, ^{68}Ga, ^{82}Rb, and ^{124}I. PET sensitivity is higher than conventional, single-photon nuclear imaging [80]. Nanoparticles might improve the biodistribution, targeting, accumulation, and contrast capabilities of the conventional radiopharmaceuticals, acting as amplifiers. Nanocarriers can contribute to the maximization of the efficacy of the radiopharmaceutical in each injection. Typically, five radiolabeling strategies are applied (Fig. 5), chelator-mediated labeling, specific trapping, ion-exchange method, hot and cold precursor labeling, and activation via proton beam.

PEGylated liposome-based ^{18}F [81], solid lipid nanoparticles-based ^{64}Cu [82], polyaspartic acid (PASP)-coated iron oxide (IO)-^{64}Cu, ^{64}Cu-CuInS/ZnS nanoparticles, and [^{64}Cu]CuInS/ZnS QDs are among the studied hybrid nanomaterials that are used in PET modalities, offering bifunctional imaging probes, to perform several diagnostic functions simultaneously. Particularly, iron oxide nanoparticles (IONPs) can be used in tandem for both MRI and PET or hybrid, synergistic multimodal systems (PET/MRI). Silica (SiO_2)-based core-shell nanoparticles have been studied in patients with melanoma. ^{124}I-Cy5 dye(organic fluorophore)-PEG-SiO_2 have also been tried for PET imaging for the detection of integrin-expressing lesions [83]. Amorphous dense silica nanoparticles have also served as general substrates for chelator-free radiolabeling of ^{22}Na,^{68}Ga, and ^{64}Cu [84]. In nuclear medicine, both single-walled (SWNTs) and multi-walled (MWNTs) carbon nanotubes have been studied for in vivo use in PET or SPECT, using ^{125}I, [85], ^{111}I [86], and ^{99m}Tc [87] offering rapid clearance from blood circulation.

4.1.2 Single-Photon Emission Computed Tomography (SPECT) Multimodalities Based on Nanomedicine

SPECT is routinely utilized in nuclear medicine for various organ imaging [80]. It commonly uses γ-emitters, such as ^{99m}Tc, ^{123}I, ^{67}Ga, ^{111}In, and ^{201}Tl [88]. SPECT can be used for monitoring biodistribution, pharmacokinetics, and target site accumulation because of its penetration depth and sensitivity [79]. SPECT is often used in parallel with CT or MRI. Nanocarriers have been used in SPECT imaging, forming nano-probes. Liposomes conjugated with ^{99m}Tc [89], ^{111}In-radiolabeled pluronic nanocarriers [90], Cy5-Gd_2O_3 cores within a polysiloxane shell [91], ^{99m}Tc-Annexin V-gold nanoparticles for SPECT/CT imaging of atherosclerosis plaques [92], ^{99m}Tc-bevacizumab-loaded human serum albumin pegylated nanoparticles [93],

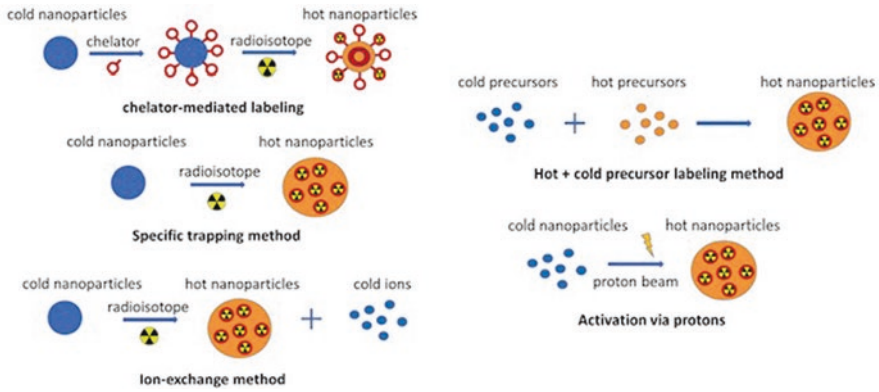

Fig. 5 The common strategies for radiolabeling nanomaterials

Gd-nanopartices labeled with ^{111}In [94], ^{99m}Tc-labeled multifunctional polyethyleneimine-entrapped gold nanoparticles for SPECT/CT imaging [95], 99mTc- folated-γ-glutamic acid, and 99mTc-chitosan [96, 97] are among the promising attempts in molecular imaging based on SPECT.

4.1.3 Computed Tomography (CT) Multimodalities Based on Nanomedicine

Computed tomography is one of the most common X-ray tomographic techniques. Generally, CT provides high-contrast images due to the inherent distinctions in X-ray absorption and attenuation by different organs and tissues of the body. Soft tissues with similar texture cannot be imaged clearly, and for this reason, several contrast agents are used (iodine, platinum, gold, tantalum, bismuth, etc.) [79]. High Z-nanoparticles have been studied for their performance as contrast agents for CT, providing extended circulation lifetime and accumulation in the regions of interest. Gold nanoparticles are among the most famous nanostructures for CT and hybrid modalities (PET/CT, CT/MRI, etc.) due to their high X-ray attenuation coefficient and their biocompatibility. Bismuth sulfide nanoparticles coated with bovine serum albumin for multimodal imaging and liposome-coated metallic core nanoparticles are among the common agent choices for CT imaging [98].

4.1.4 Magnetic Resonance Imaging (MRI) Multimodalities Based on Nanomedicine

MRI is an imaging method, based on nuclear magnetic resonance and provides details images with high contrast, and spatial resolution for this reason, and is widely used in clinical diagnosis. Early diagnosis of tiny tumors is still a challenge even for this advanced technique. Magnetic nanoparticles might be used to enhance

MRI detection accuracy. Manganese (Mn^{2+}), iron (Fe^{3+}), and gadolinium (Gd^{3+}) are among those ions that are used as contrast agents. Superparamagnetic nanostructured iron oxide has been used in MRI due to its effect on the reticuloendothelial system and its biocompatibility [79]. Gd-embedded iron oxide nanoparticles acquire T1 (positive) and T2 (negative) contrast agents to enhance the contrast in the regions of interest, improving the diagnostic results that are obtained. Metal-chelating lipids can conjugate to Gd^{3+}, $^{64}Cu^{2+}$, or $^{111}In^{3+}$. Liposomes can be rendered T1 MRI active by the conjugation with the bilayer of Gd (Gd–DTPA). Superparamagnetic liposomes, the so-called magnetoliposomes, can be used as T2 contrast agents. Thus, various nanoparticles and complexes have been studied as MRI contrast agents, and fortunately several of them have been approved for clinical use [15].

4.1.5 Optical Imaging Multimodalities Based on Nanomedicine

Optical imaging gathers various advantages and, for this reason, is commonly used. Its simplicity, and the fact that it allows multiple marker detection and observation of a wide range of tissues as well as subcellular structures, makes optical imaging an integral tool in clinical routine [99]. Biological experiments, histopathology, fluorescence-guided surgeries, and endoscopy are some of the main applications of optical imaging.

Nanomaterials enhance optical imaging, due to their properties. Quantum dots have been regarded as innovative optical biomedical probes. Magnetic nanoparticles conjugated with quantum dots are also an interesting combination for optical imaging [77]. Poly(lactic-co-glycolic acid) (PLGA) is applied as a template to bind quantum dots and iron oxide for multifunctional optical imaging, since the quantum dots can enable optical imaging, while iron oxide is used for magnetic targeting, allowing optical imaging in parallel with MRI [100]. Upconverting nanoparticles have also been considered as promising agents for optical imaging since they emit higher-energy visible light when excited by NIR light [101]. Also, if upconverting nanoparticles conjugate to Gd^{3+} and ^{18}F can also be used for MRI and PET and if it binds to lanthanides, then it becomes a CT contrast agent.

4.1.6 Ultrasound (US) Imaging Multimodalities Based on Nanomedicine

The US is a widely used technique that is based on the difference in the ultrasound impedance through tissues and allows real-time and safe imaging. It lacks sensitivity, resolution, and penetration capability [79]. Nano-emulsions (liquid-liquid, gas-liquid), as well as solid nanoparticles, can enhance the contrast of a US image. PLGA has been used to encapsulate nanoparticles for US imaging or hybrid modalities (US/MRI). The core-shell choice seems also efficient. The shell, consisting of surfactants, polymers, or proteins, allows stability and durability, whereas the core (usually SF_6 or C_3F_8) determines solubility as well as acoustic properties [102]. Focused ultrasound (FUS)-mediated drug delivery, using thermosensitive

liposomes, is another promising technique [103]. Thus, nanosized ultrasound contrast agents have gained great scientific interest.

5 Hybrid Nanomaterials for Therapeutic Applications

Nanoparticles can deliver single or multiple therapeutic agents to tumor tissues and thus improve cancer treatment efficacy. The design and development of those nanomaterials focus on the improvement of the therapeutic processes [100]. Photodynamic therapy, photothermal therapy, immunotherapy, gene therapy, drug delivery systems, and applications related to regenerative medicine and tissue engineering are the main therapeutic fields that have incorporated nanotechnological achievements [104].

5.1 Therapeutic Applications

5.1.1 Photodynamic Therapy

Photodynamic therapy (PDT) is also known as photoradiation therapy, phototherapy, or photochemotherapy [105]. PDT is widely used in dermatology, treating precancerous cells, sun-damaged skin, acne, rosacea, onychomycosis, as well as other inflammatory disorders and cutaneous infections. It is also used in various neoplastic diseases, such as squamous cell carcinoma, actinic keratoses, basal cell carcinoma, and cutaneous T cell lymphoma [106].

A photosensitizer, a light source, and oxygen are the three required components of a PDT (Fig. 6). The photosensitizer localizes to the target tissue and is activated upon irradiation with light in the presence of oxygen [107]. Then, the photosensitizer changes from the "ground state" into the "excited state," and returning to the initial condition, an amount of energy is released, generating reactive oxygen species (ROS). ROS can oxidize essential cellular components, causing apoptosis or necrosis [108]. The phototoxic effects typically occur through intracellular localization of the photosensitizer, which consequently triggers surrounding immunologic effects, including the production of interleukin 1-beta, interleukin 2, tumor necrosis factor-alpha, granulocyte colony-stimulating factor, and others [105].

Many types of photosensitizers are commercially available, allowing topical, oral, or intravenous administration. Porphyrin is one of the common photosensitizing agents, leading abnormal cells to apoptosis [109]. Nanotechnology-based drug delivery systems can improve PDT, providing accurate targeting of the region of interest and minimizing the accumulation in healthy tissues [110]. Encapsulation of photosensitizers in nanomaterials, such as poly(lactic-co-glycolic acid (PLGA) or PEG-b-poly(aspartate) (PEG-b-PAsp), has been studied. Also, lanthanide ion-doped upconverting nanoparticles (UCNPs) have received attention as a means of

controlling the limitations of conventional PDT [111]. Furthermore, pH-sensitive smart photodynamic nanomaterials, which consisted of self-assembled photosensitizers grafted to pH-responsive polymeric ligands (PLLs) and UCNPs, demonstrated a great killing effect in the target area. Cyanoacrylic nanospheres (~100–200 nm) or nanocapsules (15–400 nm) have been synthesized developing an oil-in-water emulsion incorporating phthalocyanine or naphthalocyanine [112]. Biopolyesters, natural biocompatible polymers (e.g., poly(hydroxyalkanoates) (PHAs)), and synthetic polymers (e.g., poly(orthoesters), PLGA, etc.) are usually used for micelles preparation, acting as nanocarriers [113]. PEGylated PLGA nanoparticles have been shown to have enhanced circulation time, remaining in the blood for an extended period. The famous liposomal Visudyne was a great choice in the 2000s for patients suffering from age-related macular degeneration or subfoveal choroidal neovascularization secondary to pathological myopia [114]. Dendrimer-based nanoparticles, natural macromolecule-based nanoparticles (chitosan, gelatin, etc.), magnetic nanoparticles, and fullerenes are among the common nanomaterials that are used for PDT. Moreover, silica, titanium dioxide, and zinc oxide are used as photosensitizers due to their photocatalytic potential. Gold nanoparticles, CdS quantum dots, and hybrid porphyrin-decorated quantum dots have been thoroughly studied [115]. Polymeric nanovesicle poly(ethylene glycol)-block-poly(D,L-lactic acid) [PEG−PDLLA] loaded with doxorubicin and lipophilic PS hematoporphyrin monomethyl ether (HMME) allowed combined PDT and chemotherapy in HepG2 human hepatocellular carcinoma cells [116]. Hence, hybrid nanomaterials can surely contribute to the improvement of the conventional PDT.

5.1.2 Photothermal Therapy

Photothermal therapy (PTT) refers to efforts to use electromagnetic radiation (NIR near-infrared) for the treatment of various diseases. The heat-shock response that is induced by hyperthermia (≤43 °C) is significant and can kill cancer cells since they

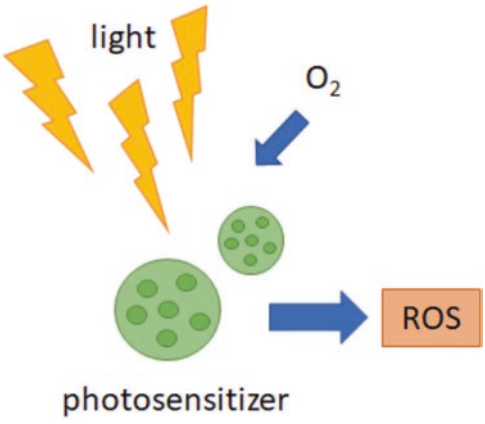

Fig. 6 Schematic representation of photodynamic therapy

are more vulnerable to hyperthermal damage than normal ones [117]. Nanoparticle-mediated hyperthermia can actively target the tumor, enhancing the tumor-to-normal tissue nanoparticle accumulation ratio. Gold nanorods, gold nanoshells, metallic ultrasmall nanoparticles, graphene and graphene oxide, iron oxide, and conjugated polymers (e.g., polyaniline (PANI), polypyrrole (PPy), polythiophene (PTh), polydopamine (PDA)) are among the available choices for the development of hybrid nanosystems for PTT [26].

5.1.3 Immunotherapy

Nanoparticles have served as vehicles, transferring immunostimulants that induce the generation of endogenous cancer antigens, eliciting an adaptive immune response. Hybrid nanosystems improve the antitumor immune responses (e.g., T- and B-cell response) compared to conventional antigens and adjuvants [118]. Gold nanoparticle-based vaccines seem to stimulate CD8+ T-cell responses through antigen delivery to lymph nodes [119]. Other studies focus on the parallel synergistic action of immunotherapy and magnetic hyperthermia, applying iron nanoparticles conjugated with anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA4) checkpoint inhibitor to enhance the therapeutic effect [120]. Encouragingly, nanotherapeutic strategies can transform cold into hot tumors, in tandem with PDT. Nanosized metal-organic frameworks (MOFs) allow this approach. Tumor hypoxia can suppress antitumor immunity, promoting tumor development and recurrence. Alleviating hypoxia might lead to the improvement of the efficacy of PDT-driven immunotherapy. Hemoglobin, catalase (CAT), manganese dioxide nanoparticles, and gold nanocages are among the common choices to develop efficient hybrid systems for cancer immunotherapy, based on nanomedicine [104]. pH-responsive copolymer (PDPA) conjugated to PDPA, and PD-1-PD-L1 interaction inhibitor (siRNA), is a nanosystem that has been studied with encouraging results. GSH-responsive heterodimers, mesoporous silica nanoparticles, micelles, and liposomes can be used as parts of hybrid nanocomplexes for immunotherapy [120].

5.1.4 Gene Editing/Gene Therapy

Editing technology of the genome is based on the general fundamental of induction of controlled double-strand breaks and the modifications, replacement, or insertion of genes with desired characteristics in the selected sequence. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins, as a part of the bacterial adaptive immune system, are considered an efficient gene-editing tool [121]. The CRISPR/Cas9 system can contribute to the knock-out of disease-related genes and edit genetic defects in abnormal cells. Nano-based CRISPR/Cas9 using cationic arginine gold nanoparticles was proven to increase the efficiency of the whole system to be delivered in the targeted area [122]. Several

similar attempts show the significant positive effect of nanotechnology in biomedicine.

5.1.5 Drug Delivery Systems

The development of delivery systems that can provide therapeutic agents to a targeted area is a field of research and development in nanomedicine [123]. Nano-based drug delivery systems focus on improving properties like solubility, immunogenicity release profiles, diffusivity, and bioavailability of the conventional delivery systems. Hence, smart hybrid drug delivery systems allow spatial-temporal release control, convenient administration routes [124], low toxicity, extended drug life cycle, and better biodistribution. In this way, they can maximize the efficacy of a drug in the region of interest, minimizing the side effects. Various bionanomaterials can be used as nanocarriers in drug delivery systems, such as chitosan, alginate, xanthan gum, cellulose, liposomes, polymeric micelles, and dendrimers. Inorganic nanoparticles (iron oxide and silica nanoparticles, etc.), nanocrystals, metallic nanoparticles (silver, gold, etc.), quantum dots, protein and polysaccharides nanoparticles, natural extracts, oligonucleotides, peptides, pharmaceuticals, and radioisotopes are among the vast variety of the available candidate for the development of hybrid nano-based drug delivery systems [24].

5.1.6 Regenerative Medicine/Tissue Engineering

Nanocomposite materials can be utilized in biomedical applications related to scaffolds for tissue engineering, medical devices, vascular stents, and dental materials. The development of nanocomposite scaffolds can minimize the limitations of the conventional polymer scaffolds, mimicking the physical and chemical properties of normal tissues [125]. Silicate-based, carbon-based, and metal/metal oxide nanoparticles can be combined with synthetic or natural polymers, creating advanced materials with great properties, allowing better interaction between cell and matrix, controlled migration, cell growth and proliferation, and cell differentiation. Artificial vascular stents coated with surface-modified $Mg(OH)_2$ nanoparticles and heart valves, hip and knee joints, and dental implants are those applications that have incorporated nanotechnology, in the last decades with significantly better performance than the conventional biomaterials.

6 Hybrid Nanomaterials for Health Prevention

Nanovaccines and sensors for rapid screening of the general population for a wide range of diseases are the two main aspects of the applications of nanomedicine for the needs of health prevention.

Vaccination is perhaps the most effective tool that we can use to control the disperse of infections, and nowadays it is more reasonable than ever before, due to the pandemic of COVID-19 [126]. Nanoparticle (NP)-based carriers, such as micro/nanoemulsions, liposomes, micelles, dendrimers, virosomes, and nanogels, offer improved efficacy compared to the traditional vaccine adjuvants. Nanovaccines allow targeted delivery, stimulation of the body's innate immunity, and strong T-cell response [127, 128].

Biosensors, as is already mentioned, offer accurate and rapid detection of a wide range of diseases at the very early stages. Breath analysis is also among those promising tools for detecting important biomarkers for various disease types [129].

7 Hybrid Multifunctional Nanomaterials for Theranostics

Theranostics is a promising field that combines therapeutic and diagnostic applications, by exploiting the properties of multifunctional systems and devices. Nanomedicine can improve the field of theranostics since it allows precise molecular imaging and targeted and individualized treatment [130]. Cytotoxicity, immunotoxicity, and genotoxicity issues are still among the limitations that need to be improved before nanotheranostics become a part of the everyday clinical routine [5]. Thus, functionalized nanomaterials have been highly investigated as promising platforms for disease diagnosis and therapy or palliative treatment (Fig. 7).

The commonly applied imaging modalities of the theranostic procedures include magnetic resonance imaging (MRI), computed tomography (CT), optical imaging (fluorescence and bioluminescence), and radionuclide imaging (PET and SPECT), while the widely used therapeutic approaches include photodynamic therapy, photothermal therapy, and gene therapy [19]. Various organic and inorganic nanoparticles can be used in theranostic systems (e.g., proteins, antibodies, polymers, lipids, dendrimers, gold, and iron oxides). The size of the whole system does not exceed 150 nm. High stability and homogeneity are the main characteristics of the theranostics and also their ability to be further functionalized for direct binding and optimized efficacy, as well as the controlled, slow, and often stimuli-responsive release on the target area. Hybrid multifunctional nanomaterials are very promising for the development of advanced theranostic systems [5, 24, 131].

8 Conclusion and Future Perspectives

The evolution in the field of nanomedicine has led to the development of hybrid multifunctional nanomaterials that gather the benefits of all their components, providing also optimized characteristics, regarding their performance in biomedical procedures. Hybrid nanomaterials are designed and synthesized to include both inorganic and organic components. These materials are utilized in diagnostics, as

imaging and contrast agents, enabling precise visualization at a molecular level, exploiting the potential of various imaging techniques, and reducing any side effect in healthy tissues. Moreover, they are used in therapeutics, particularly in anticancer therapy, such as photodynamic and photothermal therapy, hyperthermia and radiotherapy, drug delivery, and gene therapies. Furthermore, hybrid nanomaterials can be incorporated with several components that can be used for simultaneous diagnostics and therapeutics (theranostics), and thus they can be considered as multifunctional nanomaterials. It is expected that in the near nanomedicine will attract scientific interest and if some of the present limitations can be controlled, then it will be part of the clinical routine.

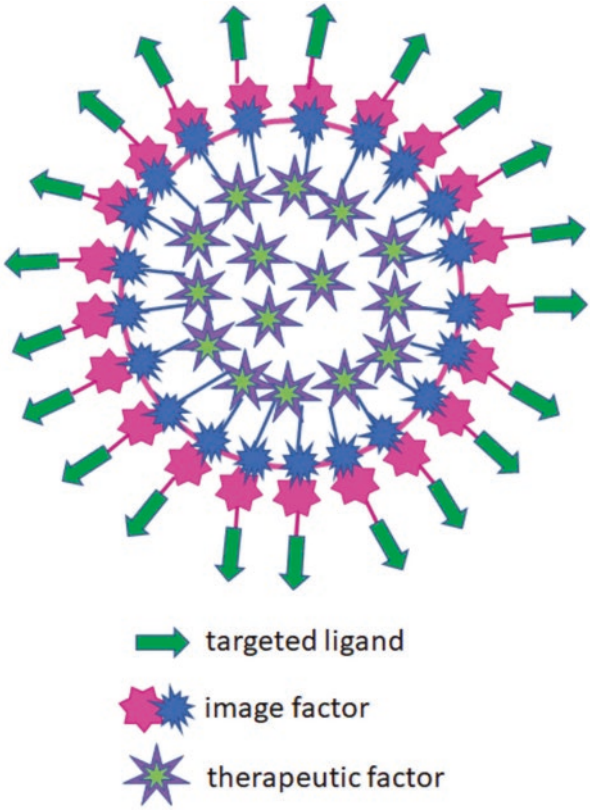

Fig. 7 Schematic representation of a typical theranostic system

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Polymeric Micelles for Targeted Drug Delivery Systems

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Abbreviations

ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
CDs	Cyclodextrins
CMC	Critical micelle concentration
DDS	Drug delivery system
DOX	Doxorubicin
PBLG	Poly(γ-benzyl L-glutamate)
PEG	Polyethylene glycol
PEG–PLA	Polyethylene glycol–polylactide
RES	Reticuloendothelial system

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1 Introduction

In current medicine and pharmacology, targeted drug delivery has gained importance as a method that enables one to increase the concentration of delivered drugs in a certain place and block or strongly limit their accumulation in healthy organs and tissues. Targeted delivery makes it possible not only to increase the duration and efficacy of drug action but also to largely reduce side effects. Nanotechnology and nanoproducts play a special role in this regard. The interest in the development of delivery systems is due to many reasons, above all to the enormous potential benefits, both medical and economic.

After a "normal" drug is injected or administered orally, it enters the bloodstream, which distributes the drug molecules more or less evenly to all organs and tissues of the body. A small portion of the drug (0.001–0.01%) enters the lesion site, whereas the bulk of it is eliminated from the body and also causes toxicity. Targeted delivery implies a different scheme, in which the carrier with the drug enters the bloodstream, circulates in the body, and accumulates only in the lesion site. However, so that this scheme could be implemented, the following requirements must be satisfied (Fig. 1).

2 Stages in the Preparation of Drug Delivery Systems

The postulate that a drug (agent), for its effect to take place, must first bind to the corresponding receptors in the cells of the pathological focus (target) was formulated by John Langley in 1878 [1]. The idea of targeted delivery of drugs (the so-called magic bullet) belongs to the German scientist Paul Ehrlich, who put it forward in 1906. In his opinion, the "magic bullet" is an ideal medicine "capable of independently finding the source of disease or the focus of disease and striking them without affecting healthy organs and tissues of the body" [2].

The "target agent" concept is based on the fact that a drug, to implement its effect, must first bind to the corresponding receptors located on the target cells. The selectivity of the drug action is determined by its pharmacodynamics and pharmacokinetics and by its metabolism and excretion from the body. There are four levels of drug action:

(a) Molecular level, at which protein molecules are direct targets for most drugs.
(b) Cellular level, at which biochemical and other components of the cell participate in transduction (generating a biological response to a certain external action).
(c) Tissue level, at which changes occur in the functions of the heart, skin, lungs, etc.
(d) Systemic level, at which changes occur in the functions of the cardiovascular and nervous systems, the gastrointestinal tract, etc.

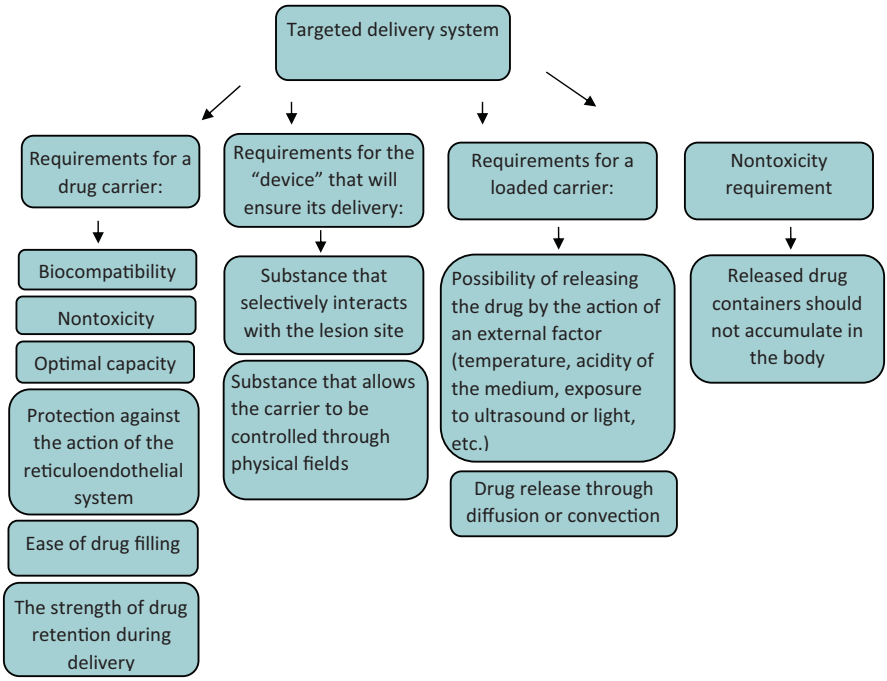

Fig. 1 Basic requirements for targeted drug delivery

The first drugs based on lipid emulsions were proposed in December 1932 by J. Johnson, when a British patent was registered on behalf of the I.G. Farbenindustrie Aktiengesellschaft. The patent stated that "pharmaceutical preparations for subcutaneous and intramuscular injections may be obtained by combining drugs with liquids such as fats or oils, if necessary together with waxes or wax-like substances, with water or other fluids, and a dispersing agent." These pharmaceutical preparations can rightfully be considered prototype drug delivery systems [3].

Lipid molecules are capable of forming two types of structures in an aqueous medium: liposomes and micelles. Usually, the terms "liposomes" and "lipid vesicles" are used synonymously. However, liposomes were first described as particles formed by the mechanical dispersion of a suspension of swollen phospholipids in water.

Liposomes are closed bubbles formed by one or more lipid bilayers, inside which there is a space, usually filled with water with substances dissolved in it. In fact, liposomes are spherical vesicles with one or more lipid bilayers. They are formed in mixtures of phospholipids with water. The inside of the liposomes contains water or a solution in which ultrasound treatment was done (Fig. 2).

Micelles (a diminutive from Latin *mica* "particle, grain") are aggregates of surfactants in a colloidal solution (sol), consisting of a large number of amphiphilic molecules. Figure 3 shows a schematic representation of a phospholipid micelle in an aqueous solution.

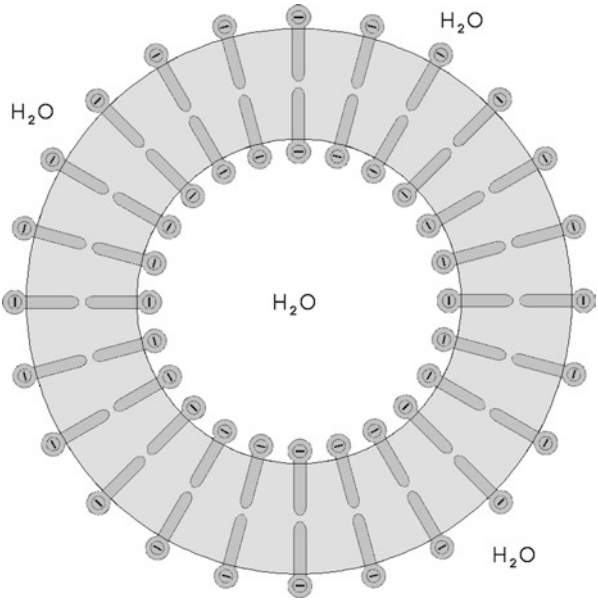

Fig. 2 Lipid bilayer and its closure, forming a liposome (https://commons.wikimedia.org/w/index.php?curid=9035720)

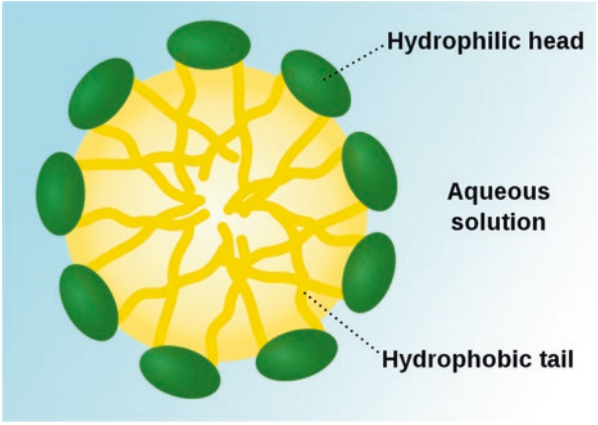

Fig. 3 Scheme for a phospholipid micelle in an aqueous solution (https://en.wikipedia.org/wiki/Micelle)

The first idea of the target polymer–drug conjugate was proposed by G. Ringsdorf in 1975 [4]. In 1976, English researcher Gregory Gregoriadis suggested placing drugs inside liposomes to promote their penetration into the body. This can be considered the beginning of the use of nanocontainers.

The widespread use of liposomes with encapsulated drugs led to an increase in their concentration in the pathological focus, increasing the time of drug action and facilitating the release of the drug in a particular location. In the case of local administration of liposomal drugs, no specific delivery of liposomes is required; they act by slowly releasing the drug into the environment. Therefore, these cosmetic products based on liposomes were introduced to the market: in particular, as cosmetic products by Christian Dior, L'Oreal, Procter & Gamble, Johnson & Johnson, Avon, etc. [5].

At the beginning of the twenty-first century, synthesized conjugates are being actively tested in preclinical and clinical trials. Many liposomal preparations successfully pass clinical tests and reach the market [6].

In particular, during the COVID-19 pandemic, Pfizer/BioNTech, and Moderna mRNA vaccines were developed, consisting of nucleoside-modified mRNA encoding the SARS-CoV-2 spike protein, which is encapsulated in lipid nanoparticles [7, 8].

3 Approaches to Making Targeted Drug Delivery Systems

The agent in a targeted drug delivery system can be a drug that binds to a target through a pharmacophore. A target is a specific molecular structure that is the aim of targeted drug delivery. Molecular targets include hormone and neurotransmitter receptors, enzymes, ion channels, transporter molecules, and nucleic acids. The receptor is that part of the target where the binding to the drug occurs, which is followed by activation of specific biochemical processes.

3.1 Active and Passive Transport

It is known that cellular metabolism, bioenergetic processes, the formation of biopotentials, the generation of a nerve impulse, and other processes are related to the transfer of substances through the membranes. In many cases, therapeutic therapy requires drug delivery through the cell membranes. The effectiveness of a drug depends largely on how permeable the membrane is to it.

Targeted delivery can be subdivided into passive and active transport. Transport by simple and facilitated diffusion is called passive; in it, substances are delivered along the concentration gradient. Under nonequilibrium conditions, the directional movement of particles is initiated by various mechanisms. The existence of an artificially maintained concentration gradient or the presence of stationary external forces of various natures leads to the stationary drift of particles. It is this drift that is considered the main mechanism of particle movement through biological membranes and is called passive transport [9, 10].

Passive diffusion across cell membranes plays a large part in the delivery of many pharmaceutical agents to intracellular targets. For measurements, biomimetic systems have been combined with advanced methods; so, attention is paid to time-resolved fluorescence, surface plasmon resonance, light scattering, etc. [11].

Active transport is possible only in conjunction with the hydrolysis of adenosine triphosphoric acid. Concentration, electric potential, pressure, and other gradients, which support life processes, are generated owing to active transport [11, 12].

Advances in drug delivery have prompted researchers to consider two important strategies in the development of new multifunctional liposomal particles: passive and active targeting strategies. In passive targeting, owing to the physical properties of the nanocontainer, the liposome-encapsulated substance accumulates in a certain affected body area and interacts selectively with the anatomical structures of the target tissue vessels while producing its pharmacological effect. Another drug delivery variant is the use of a guiding vector—for example, monoclonal antibodies, receptor ligands, enzymes, or glycoproteins [12–14].

3.2 Vector Use in Drug Delivery Systems

A vector is a compound that ensures drug delivery to the pharmacological target. After the vector is attached to the nanocarrier–drug conjugate, the resulting structure must be stable and nontoxic, and the ability to recognize the target and the efficiency of nanocarrier loading must be preserved. Specific proteins (transferrin, the peptide hormone gonadoliberin), radiolabeled monoclonal antibodies, viruses, and folic acid all can be used as vectors.

An experiment using a vector as a delivery system was first done in 1958. For targeting a drug (methotrexate), conjugation to an antibody was done. However, the term “vector” did not exist at that time; it appeared only in the 1970s. In 1975, immunologists S. Milstein and G. Koeller developed a method for generating hybrid somatic cells for antibody production. This opened up the possibility of wide application of antibodies in various fields of biology and medicine.

The first vectors used for targeted delivery were antibodies bound to liposomes. The method of their attachment was relatively simple and allowed the grafting of a sufficient amount of antibodies onto the liposome surface without violating the liposome integrity or changing the affinity and specificity of the antibodies.

Viruses such as the adenovirus, the vesicular stomatitis virus, the cytomegalovirus, the lentivirus, and the retrovirus are widely used vectors because they ensure highly contagious, efficient delivery [15–17]. However, viral vectors have several limitations, such as toxicity, immunogenicity, carcinogenicity, high cost, and difficulty of large-scale production in clinical practice [18–20]. Consequently, increasing scientific attention has been given to the development of nonviral vectors and carriers [21–23]. Recent studies have shown that nonviral vectors have the following advantages: low immunogenicity, biodegradability, easy synthesis, low production cost, and absence of restrictions on the size of injected molecules [24–28]. The

most widely studied nonviral vectors are polymers, liposomes, and inorganic nanoparticles [29–33]. The main vectors used as drug delivery systems are listed in Table 1.

Nonviral vectors can prevent premature degradation of nucleic acids, proteins, or drugs and can prolong the therapeutic effect and reduce side effects. Biomedical applications place high demands on the physicochemical properties of vectors. Meanwhile, the residual toxic effects of catalysts, solvents, and other substances in the synthetic process cannot be ignored. Therefore, it is necessary to standardize toxicological tests and determine safe exposure limits. Despite these difficulties, the widespread use of nonviral vectors to improve the efficiency of drug delivery and gene therapy is expected in the near future.

The key role of vectors can be understood as increasing drug pharmacokinetics to improve therapy. The growing number of gene therapy and vaccine vectors and drug delivery carriers has been extensively investigated because of their ease of use, targeting ability, high bioavailability, and good biocompatibility [35–37].

For example, a system was described that delivers doxorubicin (DOX) to the lungs and is based on the macrophages of active [38] and inactive cells [39]. Efficient and minimally invasive drug delivery systems have been developed for the treatment of persistent human diseases. These are based on chimeric vector systems combining at least two different vector systems. For example, chimeric drug delivery systems combining viral and nonviral features have been developed. Fusigenic nonviral particles have been constructed by conferring viral fusion proteins onto nonviral vectors. HVJ (hemagglutinating virus of Japan; Sendai virus) liposomes were constructed by combining DNA-loaded liposomes with a fusogenic envelope derived from the HVJ. The resulting HVJ envelope vector efficiently and rapidly introduced plasmid DNA into both cultured cells in vitro and organs in vivo. In addition, proteins, synthetic oligonucleotides, and drugs were also efficiently introduced into cells with the HVJ envelope vector. The authors have shown that the HVJ envelope vector is a promising tool for gene therapy experiments both ex vivo and in vivo [40].

Table 1 Characteristics of several nonviral vectors and methods used to make them

Vector	Characteristics
Polymers	Easy to synthesize, cheap, biodegradable, nonimmunogenic, can be extensively modified
Liposomes	Low-toxic, has good biocompatibility and improved pharmacokinetics, easy to synthesize
Gold nanoparticles	Stable and biocompatible, has a high surface-area-to-volume ratio, easy to modify
Mesoporous silica nanoparticles	Has a substantial surface area, a large pore size, low density, adsorption ability, and tunable pore size, easy to modify, highly biocompatible
Carbon nanotubes	Has a good adsorption ability, excellent chemical stability, high tensile strength, and significant electrical and thermal conductivity

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3.3 Cellular Penetration of Delivery Systems and Release of Drugs

Separate attention should be paid to the release of the drug and its penetration into the cell when the delivery system enters the pathological focus. This process depends largely on the nature of the interaction between the carrier (e.g., liposomes) and the cell membrane. The following kinds of the interaction of the carrier with the cell membrane can be distinguished (Fig. 4) [41]:

(a) Liposomes are adsorbed (attached) on the cell surface.
(b) The drug goes directly into the cell through the uptake of liposomes by the cell (endocytosis).
(c) Liposome membranes may fuse with cell membranes and become part of them, in which case the properties of the cell membranes may change. This includes an increase in the cell membrane permeability owing to the formation of additional membrane channels.
(d) Sometimes the cell membrane and the liposome exchange lipids.
(e) Liposomes may fuse with cell membranes and become part of them. The properties of the cell membranes, such as their viscosity and permeability and the amount of electric charge, may change. The number of channels crossing the membranes may also increase or decrease.

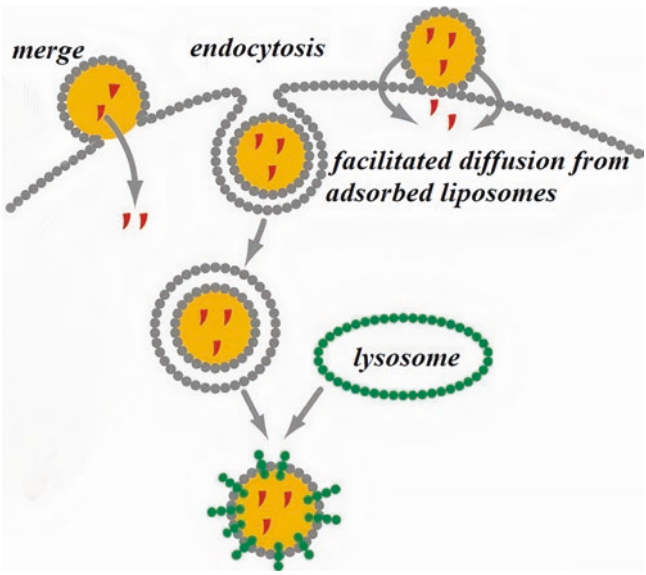

Fig. 4 Ways of penetration of the liposome contents into the cell. (Reprinted under the Creative Commons Attribution (CC BY SA 3.0) [41])

Thus, liposomes have paved the way for a new method of cell targeting, which can be called "membrane engineering." A multiscale diffusion model was developed incorporating chemical properties of material and geometry of microstructure, which is especially useful in predicting mass release from drug vectors. Owing to this model, it is possible to predict mass distribution in a flow similar to the one found in capillaries [42].

3.4 Development of Liposomal Drugs for Targeted Delivery

Liposomal drug delivery systems have proven breakthroughs and are innovative in the treatment of many diseases [43]. The main factors behind the development of liposome-based drug delivery systems were the use of three key methods.

The first method is the coating of liposomes with polyethylene glycol (PEG). This coating makes liposomes better protected from the reticuloendothelial system by reducing their recognition by macrophages, which allows liposome elimination from the body to be slowed down.

PEG also generates increased osmotic pressure around the liposome, preventing the approach of other cells. As a result, PEGylated liposomes circulate longer in blood and accumulate in tumor tissues in larger amounts than conventional liposomes.

The second method is the use of antibodies as vectors, which ensures the possibility of targeted drug delivery owing to the interaction of the antibody attached to the particle with the cell membrane receptor.

The third method is related to the fact that the endothelial cells of tumor vessels proliferate 30–40 times faster than do the endothelial cells of normal tissue vessels, so tumor capillaries are characterized by larger pores. These pores are used for the passive delivery of liposomal drugs to the tumor.

The drug inclusion in liposomes enhances patient tolerability of the encapsulated drug and enables the drug therapeutic index (ratio between therapeutic effect and toxicity) to be increased. The reduction in systemic toxicity is based on the preferential accumulation of medium-sized (50–200-nm) particles in tumor tissue owing to enhanced permeability and retention (increased capillary permeability and impaired lymphatic drainage in tumor tissue) [44, 45]. To be well transported to tumor tissue, liposomes can be equipped with carbohydrate ligands of the sialyl Lewis family, which are specific to selectins—carbohydrate-binding lectins involved in the primary interaction of blood leukocytes with endothelial cells and thus involved in various inflammatory and metastatic processes. Selectins are promising targets for the delivery of therapeutic agents to the vessels of tumor tissue [46].

The liposome membrane is usually formed from the same phospholipids that are part of the biological membrane: phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Depending on the method of liposome preparation, the liposome size may range from a few microns to tens of nanometers (nanosomes). If an aqueous drug solution is used in the production of liposomes, part of this solution

is closed inside the liposomal container and is introduced into the human body in just such form of dosage. This is important when a toxic compound, such as an anticancer agent, is used or when the drug substance needs to be protected from degradation until it is delivered to the target. The attachment of PEG molecules protects the liposomes themselves from capture by the immune system cells and thus increases the time for which the liposomes remain in the bloodstream. Liposomes deliver the drug to the cells either by fusion with their membrane or by endocytosis. On the basis of different targeting strategies, liposomes are classified into passive, active, physicochemically targeting, and multifunctional. The manufacturing technology of drug liposomes is mature and consists of film dispersion, reverse-phase evaporation, chemical gradient loading, and other methods such as the following:

(a) Heating to produce empty liposomes by hydration of phospholipids in an aqueous solution containing 3% glycerol by increasing the temperature to 60 °C or 120 °C
(b) Lyophilization of a monophasic solution for the encapsulation of heat-sensitive drugs such as DNA [47]

Liposomal drug delivery systems are believed promising in the treatment of cancer and other diseases. Owing to the development of pharmaceutical technologies, a new type of liposome, the double functional liposome, has been proposed. The advantage of these new liposomes is their expanded function, which enables the elimination of drug-resistant cancer, destruction of cancer stem cells and mitochondria, induction of apoptosis, regulation of autophagy by using the microenvironment, and suppression of resistant cancer genes [48].

A targeted liposome is a drug delivery system that selectively localizes the drug in the target tissue, organ, cell, or intracellular structure through local or systemic administration. Homobifunctional and heterobifunctional cross-linking approaches are used to prepare antibody–drug conjugates and antibody-mediated targeted liposomes. For the preparation of antibody-mediated liposomes, antibodies are conjugated directly to the distal end of PEG, which is already bound to the liposome membrane [47, 49].

For the preparation of ligand-mediated liposomes, a chemical targeting ligand is often used. Such a ligand forms a complex with a specific protein on cells or organelles to reach a target; for example, dequalinium is used to deliver drugs to mitochondria [49, 50].

3.5 Drug Release from the Delivery System

Targeted drug delivery leads to the preferential accumulation of the drug in the target area, which does not depend on the method and route of drug administration. On the other hand, targeted therapy or targeted medicine means a specific interaction between a drug and its receptor at the molecular level. Effective targeted drug

delivery systems imply four basic requirements: preservation of the therapeutic dose, avoidance of prior drug degradation, targeting, and release of the encapsulated drug.

Three controlled release technologies are currently available including (a) periodic release (a constant amount of the drug is released at a constant interval); (b) feedback release (the drug is released on command from a physical signal); and (c) continuous release (the drug is released at a constant rate) [51–56].

The methods of drug release from the polymer are divided into physical and chemical. In the physical method, the release is controlled by diffusion of the drug/solvent or penetration of the drug through the membrane [57]. The drug is placed in a container with a membrane wall. The rate of its release from diffusion-controlled systems depends strongly on the physical properties and size of the drug molecules and the loading level. The surface area of the membrane and the length of the diffusion pathway are also important. The chemical method consists in the hydrolytic or enzymatic cleavage of the main chain or the detachment of a side chain from a biodegradable polymer. The drug release from the delivery system is controlled by diffusion control. Functionalization of the surface of the drug delivery system and selection of the correct membrane material is crucial for the kinetics of drug release.

The controlled-release frame/membrane material should be nonimmunogenic, nontoxic, and biocompatible, have reduced tensile strength, retain large drug amounts, and, most importantly, allow controlled release of the drug. Typically, nanoparticles with a specific drug are loaded into the reservoir, and then the reservoir surface is coated with a programmable-rate membrane, which can be further engineered to control the drug release behavior.

The release of a particular drug from the drug delivery system (DDS) can be modulated by external or internal stimuli. The rationale behind activation-modulated drug delivery systems is that different organs have different biological environments (physical, chemical, electrical, and biochemical). For example, different organs/parts of the body have different pH values, such as blood (pH 7.4), tumor tissues (pH 6.5–7.2), lysosomes (pH 4.5–5.0), and the gastrointestinal tract (pH 6.2–7.9). Therefore, a pH-sensitive delivery system releases the drug only in its target area. Thus, the pH becomes an intrinsic stimulus for the targeted delivery of therapeutic agents [58–60].

The reduced cell cytosolic environment, as compared to body fluids, becomes a stimulus for redox-sensitive drug delivery systems to release active agents only in the cytosol and not in body fluids [61]. Tumor tissue suffers from hypoxia owing to impaired metabolism, and hypoxia-sensitive drug delivery systems serve as excellent modulated activation (in this case, hypoxia) of targeted drug delivery systems [62]. Magnetic nanoparticles loaded with therapeutic agents can be directed by an external magnetic field to a specific organ and can be stimulated to release the drug only at that location—an ideal example of an externally controlled/modulated drug delivery system [63].

Recent advances in microfabrication have made it possible to develop controlled-release systems for drug delivery. Two types of delivery devices are most popular:

microvessels and micro-/nanofluidic ones. Much promise is held by controlled-release chip-based drug delivery systems [64].

4 Selection of Carriers for Delivery Systems

Most low-molecular-weight drugs used in clinical practice have high hydrophobicity and low bioavailability. Therefore, nanotechnology development can increase drug bioavailability.

Nanoscale systems, depending on the material from which they are made, can be divided into lipid, polymeric, inorganic, peptide, and viruslike [65]. Lipid-based formulations include liposomes, self-assembled colloidal structures consisting of lipid bilayers surrounding an aqueous core, and solid lipid nanoparticles. Among polymeric nanosystems designed by using natural or synthetic polymers, one can find several structures used in pharmaceuticals and medicine (e.g., nanoparticles, nanocapsules, micelles, and dendrimers), whereas inorganic nanocarriers such as gold, silica, and silver are used in both imaging and therapy [66, 67]. Figure 5 shows structures composed of a core material containing both a hydrophobic and a hydrophilic region, surface modifiers (biocompatibility modifiers and targeting moieties), and a therapeutic payload [68].

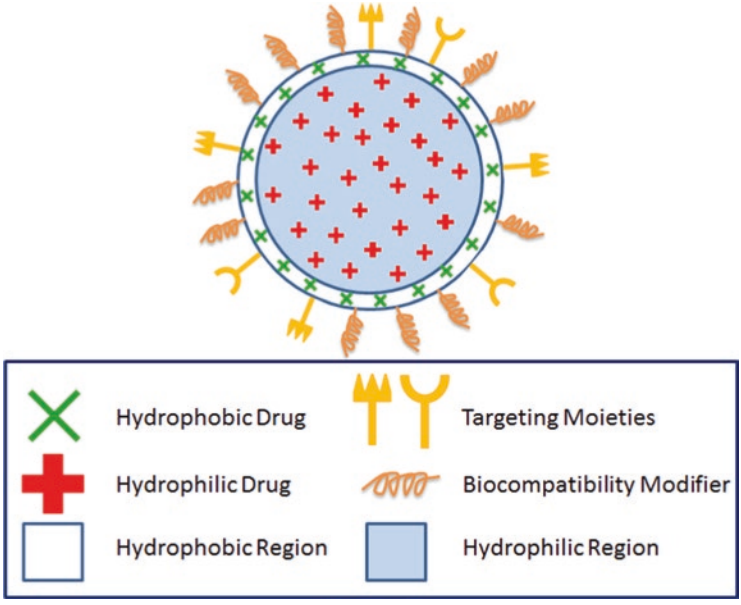

Fig. 5 Generalized nanomedicine carrier. Structure composed of a core material containing both a hydrophobic and a hydrophilic region, surface modifiers (biocompatibility modifiers and targeting moieties), and a therapeutic payload [68]

Nanocomposites include polymers or lipid-based systems to form bubbles and physically or chemically trap the drug in them. Nanocomposites solubilize drugs and impart stability to them, increasing their circulation in the bloodstream and accumulation in the target organs [65].

In the selection of nanocarriers, a key part is played by drug optimization through the improvement of the water solubility of hydrophobic drugs [69, 70] and the stabilization of easily degradable compounds [71, 72]. In addition, one should consider the role of nanosystems in ensuring drug retention in tissues, protection of drugs from enzymatic degradation, and enhancement of cellular absorption and high-precision delivery [73]. All aspects have a substantial impact on treatment efficacy [74].

4.1 Carriers: Their Advantages and Disadvantages

The use of a carrier is extremely important to ensure drug efficacy and safety. Some substances cannot withstand the journey through the digestive or circulatory system and will be inactive by the time they reach the disease area. Others can be hazardous to healthy tissues, so their potential must be unlocked at the disease site. Solid dosage forms (tablets, capsules, etc.) account for up to 90% of medications used as research objects and are the most popular and convenient dosage forms. The choice of a carrier for dosage forms containing high-molecular-weight components as an active pharmaceutical substance or an additive requires a comprehensive approach, with account taken of the effect of the carrier on the physicochemical and adsorptive properties of the active material. The following requirements are imposed on carriers:

(a) They should not interact with the drugs.
(b) They should ensure drug stability for the required period and contain the required amount of the dispersed phase in the dispersion medium.
(c) They should be nontoxic.
(d) They should ensure the optimal therapeutic effect of the drug [75].

All carriers can be divided into three types: artificial, natural (biological), and hybrid. They can also be divided into three generations on the basis of their chronological appearance and physical size:

(a) First-generation drug carriers (microcapsules, microspheres; size, 1–2 microns), produced as various dosage forms: powders, tablets, capsules, suspensions, emulsions, and so on. In pharmaceutical technology, microencapsulation began to be used in the late 1950s–early 1960s.
(b) Second-generation drug carriers (nanocapsules, nanoparticles, nanotubes, dendimers, liposomes, polymer conjugates, etc.; size, <1 micron), which are collectively called colloidal carriers. Nanocapsules are intended for parenteral administration near a specific organ or tissue. The size is less than 100 nm [76].

(c) Third-generation drug carriers, involving the use of nanotechnology, biotechnology, genetic engineering, and other fields. These include antibodies [77–80], glycoproteins [81, 82], cellular markers and receptors [3, 83–86], viruses and oncolytic viruses [87, 88], and other materials. Third-generation carriers open new possibilities for high levels of selective action and targeted drug delivery. Different types of stimuli-responsive nanocarriers are shown in Fig. 6 [89].

Nanoscale drug delivery agents (1–250 nm) can change the therapy of various diseases, owing first of all to the increased ability to overcome biological barriers, increased half-life, and targeted drug delivery. Currently, several nanotechnological platforms are used for targeted drug delivery that differ in their physical and chemical structure. These include polymersomes, nanoshells, dendrimers, polymer micelles, and polymer–drug conjugates [55, 90, 91]. Liposomes as a dosage form have numerous advantages. The most significant of them include (a) the unique ability to deliver drugs into cells, biocompatibility; (b) the absence or the minimal possibility of allergic reactions (invisible liposomes for the immune system—stealth liposomes); (c) protection of drugs from degradation in the body; (d) improving the pharmacokinetic profile of drugs and increasing their therapeutic efficacy; and (e) reduction of the general toxic effect on the body, versatility and the ability to modify the liposome structure so as to achieve specific properties [92, 93]. The main difficulty with liposome design is related to their in vitro and in vivo stability, which varies with nanoparticle size, surface charge, lipid composition, number of lamellae, and surface modifications (with ligands or polymers). All these factors make liposomes difficult to manufacture and use [94]. Liposomes can hardly penetrate tissues with severe microcirculation disorders; they can block pulmonary capillaries (leading to microvascular embolism), can cause an increase in blood glucose levels, and lead to impaired blood clotting and cholesterol metabolism. Nanosized polymer particles (nanocontainers, nanospheres, dendrimers) are loaded with drugs either by drug absorption or by conjugation with side acid groups and end of groups. For example, OH groups of polyethylene glycol are associated with vector molecules.

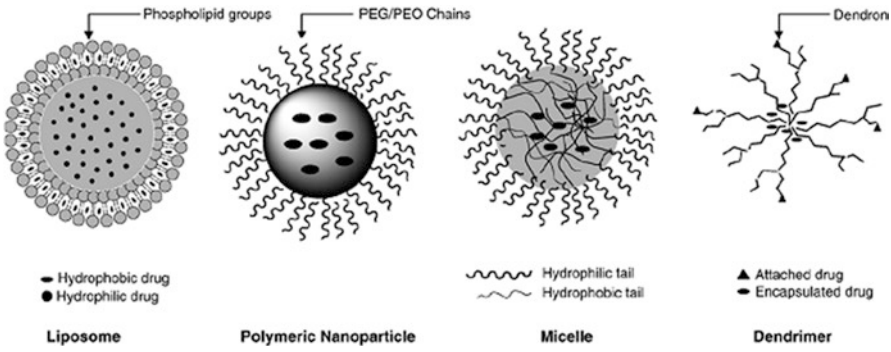

Fig. 6 Different types of stimuli-responsive nanocaries [89]

Among various drug nanocarriers, carbon carriers such as fullerenes, graphene and its oxide, carbon nanotubes, and detonation nanodiamonds are considered very promising. The prospects for the use of these carriers are due to their physicochemical characteristics, the possibility of targeted modification of the surface, and variation in particle size. Two main approaches have been adopted for modifying the surface of carriers: adsorption and covalent grafting.

For DDS as a carrier, it was proposed to use not graphene itself but its oxide. This is due to the possibility of chemical modification of its surface. Because anticancer drugs are usually water-insoluble, graphene oxide overcomes this disadvantage. It has been proposed as a platform for doxorubicin, camptothecin, cisplatin, etc. Graphene oxide can be used to track tumor angiogenesis. However, when graphene toxicity studies were carried out, it turned out that graphene and its derivatives can be dangerous to biological systems. Therefore, the use of graphene and its oxide as a carrier for delivery systems requires additional toxicological studies. There are two known ways of using carbon nanotubes as a carrier including (a) the attachment of drug molecules to the outer surface of the tube; (b) the placement of drug molecules inside the tube, after which it acts as a container.

Carbon nanotubes, like other pure carbon materials, have an inert hydrophobic surface, as a result of which they are poorly dispersed in water, stick together, and form very large aggregates. However, data on the toxicity of carbon nanotubes themselves have been obtained, and their further application requires additional studies. Inorganic materials such as gold, iron, and silicon dioxide are used in nanoparticle synthesis that has various applications in biology and medicine. The synthesis of inorganic nanoparticles makes it possible to control the size, structure, and geometric shape of the final product. Colloidal gold nanoparticles are also considered a promising platform for making drug delivery systems. They are easily synthesized and biocompatible, and their surface can be functionalized. For example, gold nanoparticles (AuNPs), which are the most studied, are used in various forms such as nanospheres, nanorods, nanostars, nanoshells, and nanocells [95]. In addition, inorganic nanoparticles have unique physical, electrical, magnetic, and optical properties owing to the properties of the base material itself. For example, AuNPs have free electrons on their surface that continuously vibrate at a frequency that depends on their size and shape, which gives them photothermal properties [96]. AuNPs are also easily functionalized, which makes it possible to give them additional properties and delivery capabilities [95]. Iron oxide is another widely studied material for the synthesis of inorganic nanoparticles, and the resulting nanoparticles based on it make up the majority of FDA-approved inorganic nanomedicines [97]. Other common inorganic nanoparticles include calcium phosphate and mesoporous silica nanoparticles, which have been successfully used for gene and drug delivery [98, 99]. Owing to their unique properties, inorganic nanoparticles have good biocompatibility and stability and fill a niche where properties are required that are unattainable for organic materials. However, their clinical use is limited because of their low solubility and toxicity, especially in formulations containing heavy metals [100].

Polymeric nanoparticles can be synthesized from natural or synthetic materials, as well as from monomers or preformed polymers, providing a wide range of possible structures and characteristics. When choosing polymers as drug carriers, investigators rely on their physical properties, which determine the release rate of drugs. Thus, it is preferable to use hydrophobic polymers that decompose to small water-soluble molecules, which ensures their rapid clearance. When hydrophilic biodegradable polymers are used, owing to their high affinity for water, when chemical bonds are broken, rather large molecules pass into the environment, which complicates their participation in metabolism. The most common polymers used as platforms for building delivery systems are polyesters. They are biodegradable, biocompatible, and easily degraded owing to the hydrolysis of the ester bond. Typically, polyglycolic, polylactic acids, and copolymers of lactic and glycolic acids are used.

Despite being biocompatible and biodegradable in vivo, polymers have disadvantages such as lack of stability, sterilization difficulties, and scaling up problems. One of the modern variants of polymeric nanocarriers is dendrimers. The most commonly used are polyamidoamine dendrimers. The unique properties of dendrimers are due to the radial symmetry of their molecules, highly ordered structure, and tree-like branching. They have sizes of 1.1 nm and above, depending on the number of stages of synthesis, and a strictly fixed molecular weight.

The drug is encapsulated inside the dendrimer molecules (Fig. 7), which increases its solubility and stability under physiological conditions. In addition, drugs can be grafted to the surface of dendrimers. This makes the use of dendrimers as carriers for targeted transport promising.

From many types of nanosized particles and materials studied, cyclodextrins (CDs), natural cyclic oligosaccharides, and molecular nanocontainers have attracted researchers' attention. CDs were discovered in 1891 by M.-A. Villiers, and the first

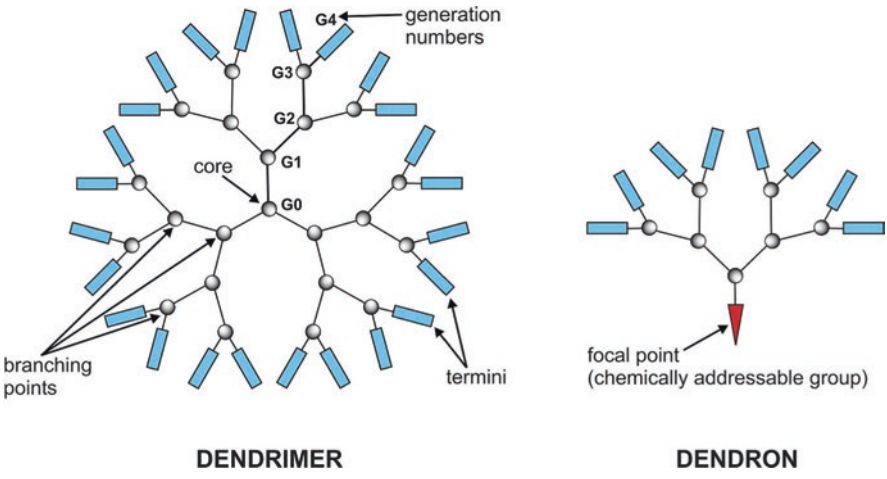

Fig. 7 Schematic representation of the internal cavities of a drug-filled dendrimer (https://en.wikipedia.org/wiki/Dendrimer)

detailed description of their preparation from starch was given in 1903. F. Kramer in 1954 was the first to show that CDs can form molecular inclusion complexes with a wide range of substrates of the "guest-host" type, in which CD molecules with their internal hydrophobic cavity play the role of hosts. The formation of inclusion complexes by CDs can radically change the physicochemical and biological properties of the included molecules, which has led to their demand as an object of modern chemical and pharmaceutical technologies. CDs can increase the solubility of poorly soluble drugs in water, as well as enhance the penetration of drugs through biological membranes [101]. CDs are of great interest because they are non-toxic [102, 103].

The disadvantage of many drugs based on CDs is a rapid decrease in their concentration in plasma blood owing to metabolic destruction in the body itself, which necessitates an increase in dose loads and, accordingly, increases the likelihood of side effects. Cases of damage to the auditory nerve have been described [104], and nephrotoxic effects have been observed after CD application [105–107].

Special attention should be paid to the direction in which natural containers are used as carriers—blood cells of humans or animals. They have the following advantages: a wide range of delivery targets, a decrease in immunogenicity and an increase in drug circulation time in vivo, biocompatibility, and controlled release of drugs. In addition, owing to the specific receptors on the membrane surface, they can be used in targeted delivery. Delivery systems based on blood cells are divided into systems using erythrocytes, platelets, and leukocytes.

The disadvantage of erythrocytes as carriers in delivery systems is their large volume (90 μm^3). Because of this, the erythrocyte cannot penetrate the tissues, and the area of its delivery is limited only to the affected foci accessible to the bloodstream.

Platelets have a short lifespan (7–10 days), but despite this, they are also considered promising drug carriers. Platelets specifically target the sites of damage and contain many biologically active proteins, and during pathological processes, these proteins are released from platelets by exocytosis. Owing to this feature, drugs are released locally at the sites of platelet activation.

Leukocytes, unlike other blood cells, have adhesive properties. In inflammatory conditions, leukocytes can adhere to the endothelium, which contains the protein E-selectin, which is synthesized in response to inflammation. Thus, leukocytes can be used in antitumor therapy owing to their similarity in adhesive properties to tumor cells. Leukocytes are used as carriers for the targeted transport of antibiotics to the site of inflammation owing to the slow release of the drug from cells in the vascular bed.

The most common forms of polymer nanoparticles are nanocapsules (cavities surrounded by a polymer membrane or shell) and nanospheres (solid matrix systems). Within these two broad categories, nanoparticles are divided into polymersomes, micelles, and dendrimers.

In general, polymeric nanoparticles are ideal candidates for drug delivery, because they are biodegradable, water-soluble, biocompatible, biomimetic, and storage stable. Their surfaces can easily be modified for additional targeting [104],

which allows them to deliver drugs, proteins, and genetic material to target tissues, making them useful in oncological medicine, gene therapy, and diagnostics. However, the disadvantages of polymer nanoparticles include an increased risk of particle aggregation and toxicity [108].

5 Polymer Carrier Micelles in Delivery Systems

Polymeric nanoparticles are nanosized drug delivery systems characterized by a core–shell structure that results from the self-assembly of amphiphilic block copolymers in an aqueous solution. In a dilute aqueous solution, the amphiphilic molecules exist separately, and the amphiphiles act as surfactants, lowering the surface tension at the air–water interface. If more chains are added to the system, adsorption at the interface becomes higher until unimer aggregation occurs owing to the saturation of the solution volume. At this stage, the critical micelle concentration (CMC) is reached. Thus, this variable is defined as the minimum concentration of polymers in solution leading to the formation of micelles. According to this, micelles are stable at a concentration of polymer chains higher than the CMC, whereas disassembly of the system is observed after dilution below the CMC [109, 110].

Owing to the presence of various functional groups (hydroxyl, carboxyl, and amino groups) in their molecular chains, micelles can be chemically altered and modified with side chains. The incorporation of hydrophobic drugs into the micellar core can further enhance the stability of the micelles. This is an important feature for injectable biomedical applications.

Polymeric micelles are of interest for drug delivery, because the hydrophobic drug loaded into the micelle core is protected by an external hydrophilic corona. This corona hinders micelle removal by the mononuclear phagocytic system. Because of this system, the stability of the active substance is increased, and its circulation time in vivo is extended.

The driving force behind the formation of micelles is a decrease in the free energy of the system owing to the removal of hydrophobic segments from the aqueous medium with the formation of a micellar core. An important factor in drug delivery is the relative thermodynamic (possibility of disassembly) and kinetic (disassembly rate) stability of the substance. The relatively small size of micelles allows them to accumulate passively in neovascularized or poorly vascularized tumors, which may lead to reduced systemic toxicity [111, 112].

Structural features of polymeric micelles (hydrophilic shell) help to avoid unexpected loss of drugs from serum components and prevent rapid elimination of drugs from systemic circulation [113, 114]. Ideal polymeric micelles are expected to reduce the toxicity of therapeutic compounds.

5.1 Brief History of Obtaining Polymer Micelles

The term "micelle" was first introduced by McBain in 1913. According to modern concepts, micelles are aggregates of long-chain amphiphilic molecules or surfactant ions that spontaneously appear in their solutions at a certain concentration. The last property essentially depends on the nature of the polar group and, especially, on the length of the molecular chain [115].

In the past decade, polymer micelles have been studied in nanotechnologies, biotechnologies [116], biomedical engineering [117], and environmental technologies [118]. In biomedicine, especially in the detection and treatment of cancer, polymer-based micellar systems have been widely studied owing to their success at the clinical level. In 1984, polymer micelles (~200 nm) were first used by Bader et al. [119] to deliver anti-cancer molecules.

The term "micellar nanoparticles" has been mentioned in publications since the mid-1990s [120], especially in transdermal therapy [121]. Micellar nanoparticles are used in veterinary medicine; for example, Scott-Moncrieff et al. [122] showed that whereas insulin in combination with mixed micelles is completely absorbed in dogs, its bioavailability is much lower than in similar studies in rats. It has been shown [123] that the rate of insulin release from micelles can be controlled by changing the concentration of glucose.

Comparative studies of the biodynamic parameters of the aqueous form of diminazene and diminazene enclosed in water-dispersed micelles in ram erythrocytes and sheep blood plasma showed that surfactants improve the intracellular penetration of the active substance owing to the interaction with the cell membrane [124, 125].

Vail et al. [126] found the efficacy and safety of water-soluble micellar paclitaxel (Pascal Vet), as compared with free lomustine, for the treatment of inoperable grade 2 and 3 mast cell tumors in dogs.

Oral intake of natural vitamin E, contained in micelles in racehorses, effectively increased the concentration of α-tocopherol in blood plasma, as compared with the control [127]. Another study by the same authors in adult and weaned piglets showed that oral administration of micellized natural vitamin E to sows (75 mg/day) and piglets (1.7 mg/day) altered the fatty acid profile in piglet tissues and improved their oxidative status [128]. Micelles were also used for the oral delivery of vitamin B12 [129].

The bioavailability and pharmacokinetic parameters of tilmicosin (a semisynthetic antimicrobial agent) were studied in broiler chickens by oral administration by using various micellar nanoparticles (solid lipid nanoparticles, nanostructured lipid carriers, and lipid core nanocapsules). Al-Qushawi et al. [130] showed that lipid nanoparticles improved the bioavailability and pharmacokinetic parameters of tilmicosin in broiler chickens. Troncarelli et al. [131] described the importance of various nanoparticles as antimicrobial agents in veterinary medicine.

Micellar nanoparticles have a greater loading capacity and excellent stability and can be considered safer for parenteral administration.

5.2 Basic Principles for the Preparation of Polymer Micelles

Drugs may be encapsulated depending on the preparation method and the drug physicochemical characteristics. The simplest preparation method is direct dissolution. Other methods include dialysis, evaporation of the emulsion with a solvent (or co-solvent), and pouring of the solution, followed by hydration of the film. The choice of method depends on the polymer characteristics and the drug, as described in [132, 133].

Because the properties of micelles (such as polarity and degree of hydration) are not uniform inside the carrier, a drug, depending on its properties, can be placed either close to the surface or in the inner core [134]. Typically, hydrophobic drugs are loaded and placed in the inner core. In certain cases, a drug may also be covalently linked to a polymer (polymer–drug conjugate).

The hydrophilic part usually consists of PEG, but other polymers can be used: poly(vinylpyrrolidone), poly(acryloylmorpholine), or poly(trimethylene carbonate). The hydrophobic segment may consist of poly (propylene oxide) or polyesters (poly (ε-caprolactone) or polymers and copolymers of glycolic and lactic acids [109].

Most examples of clinically approved polymer micellar drugs or polymer micellar drugs in clinical development are in the field of cancer therapy. There have been reports of preclinical studies using small molecule drugs in polymeric micelles for the treatment of autoimmune, cardiovascular, skin, and eye diseases; dementia; microbial infections; pulmonary arterial hypertension; and spinal cord injury and wound healing. Polymer micelles significantly improve drug solubility, stability, and bioavailability.

5.3 Stability of Polymer Micelles

Drug release from polymeric micelles can occur either by drug diffusion from intact micelles or by disassembly of micelles. Micelles must have a good thermodynamic and kinetic stability to avoid uncontrolled drug release [135–137]. Therefore, several physicochemical strategies have been proposed to stabilize the encapsulated drug in the micellar core to avoid the rapid disaggregation of the system [138].

It is known that the CMC can be decreased by increasing the length of the hydrophobic part of the unimer [135, 139]. Block copolymers conjugated with lipid molecules were synthesized. Other strategies include hydrophobic block functionalization, cross-linking of the micelle core, or formation of a conjugate between the polymer and the drug [140].

The structural stability of micelles should be investigated under relevant conditions, because proteins of plasma or intracellular fluids can be absorbed on the surface of micelles, which leads to the formation of the so-called protein corona. Such a corona partially masks the functional groups of the outer shell, modifying the physiological response of the nanocarriers [141, 142].

Serum proteins play a key role in the stability of micelles by promoting their degradation or aggregation [143]. Micellar disaggregation can be observed as a result of interaction with mucus, epithelium, lipids of the stratum corneum, and sebum [144, 145].

The use of hydrophilic blocks with "anti-fouling" properties reduces the binding of serum components (serum proteins and the complement system) and protects the encapsulated drug from loss of cargo in the circulatory system. Polymer micelles should be designed in such a way as to resist their excretion from the body owing to the adsorption of plasma proteins and/or activation by the reticuloendothelial system (RES) [146]. For imparting "anti-fouling" properties to polymer micelles, several hydrophilic blocks were added to the structure of block copolymers, as described in [147]. It has been established that the physicochemical properties of hydrophilic polymers (molecular weight and surface density) are closely related to the stability, system circulation time, and biodistribution of polymer micelles in vivo [148].

The possibility of regulating drug release depending on the pH medium or under the influence of ultrasound, magnetic field, or temperature changes has been shown [149].

The amount of drugs loaded into micelles also can affect the stability, morphology, and size of micelles in an aqueous solution. Hydrophobic interactions between drugs and the hydrophobic block of amphiphilic block copolymers are some of the main factors of drug solubilization in polymer micelles. Additional molecular interactions that exist in the core, such as hydrogen bonds, are no less significant, because they can enhance the molecular interactions between the polymer and the drug in the core.

5.4 Polymeric Micelles and Questions of Kinetics and Biodynamics of Drugs

The purpose of studying micelle pharmacokinetics is to quantitatively characterize micelle absorption, distribution, and elimination (metabolism and excretion). Pharmacokinetic data are needed to establish a relationship that "concentration effect" is less than a "dose effect." The results of pharmacokinetics help to choose an approximate dosing regimen.

As mentioned above, micelles are nanosized colloidal particles with a hydrophobic interior (core) and a hydrophilic surface (shell). The polymer micelle consists of two separate regions: an inner hydrophobic region of the polymer chain (central region) and an outer region of well-solvated hydrophilic polymer chains (crown or shell region), which imparts colloidal stability to the system [150, 151]. Drugs and contrast agents can either be placed into the micelle lipid core or covalently bond to its surface. Micelles are somewhat smaller (about 50 nm) than liposomes. To ensure the long-term circulation of micelles in the bloodstream, various modifications of their shells have been proposed, making them thermodynamically stable and

biocompatible [152]. The kinetics of drug release from polymer micelles is highly dependent on many factors: the size of the micelles, the length, crystallinity, and polarity of the hydrophobic block, as well as the compatibility between the micelle core and the drug molecules. The larger the micelles, the slower is the release of the drug [153]. Long hydrophobic blocks cause a slow drug release rate, and the closer is the temperature to room temperature, the higher is the viscosity of the medium and the slower is the release [154]. A larger core diameter may result in higher core crystallinity, which slows drug release [155]. In [156, 157], it has been established that the greater is the poly(γ-benzyl L-glutamate (PBLG) content in the copolymer, the larger is the micelle particle size. It has been found that the length of the hydrophobic block of a micelle is not significant in measuring the release rate. Also, the higher is the hydrophobicity, the slower is the release rate [158, 159]. The rate of drug release from micelles decreases with increasing drug/polymer ratio at a constant copolymer concentration [160].

Drug release is highly dependent on where the drug molecules are located [161]. If the drug is located predominantly in the crown, then the length of the core of the forming block, the micelle size, and the molecular volume of the drug are less important for determining the release rate [162]. The amount of drug loaded into the micelle core is the determining factor for its release rate. Gref et al. [163] observed a faster release of lidocaine if it was dispersed in the micelle cores. In [156, 157], it was shown that the release of adriamycin and clonazepam from micelles was slower at higher concentrations of the respective drugs in the micelle core. At high loads of lidocaine, crystallization of the drug in micelles is observed [153]. Drug release is possible only after the drug dissolves and diffuses into the external solution, so crystallization slows down drug release [164]. So far, there has been no clear picture of how the drug is released from micelles and freely diffuses from the core of an intact micelle, or release is observed after rupturing of the micelle. Some researchers have reported a biphasic release profile [111, 155]. Studies to analyze the release of drugs in vitro in an environment that mimics physiological conditions have been conducted [153, 154, 156, 165–167]. The decisive factors influencing the kinetics of drug release from a polymeric micelle include (a) the stability of micelles; (b) their compatibility with the main drug; (c) the molar volume of drugs; and (d) physiological conditions. Mechanical forces acting on polymer micelles in veins and small capillaries can also have a strong effect on drug release rates [111].

5.5 Preclinical and Clinical Trials Using Polymeric Micelles

Preclinical studies are mandatory for all micellar medicinal products, regardless of whether the original or known pharmacological substance is used to make them [168, 169]. When combining several pharmacological substances in one dosage form, the toxicity of the combination as a whole and of each ingredient separately is studied, if it has not been previously approved for use in medical practice [169,

170]. The strategy for the preclinical testing of micellar medicinal products should be a three-tiered approach, which includes (a) physicochemical characteristics of the micellar drug; (b) interaction of medicinal substances in micellar form at the cellular level in vitro (rate of cellular uptake and intercellular persistence); and (c) safety assessment of a micellar medicinal product in laboratory animals [171].

The manifestation of the toxic properties of polymer micelles largely depends on the route of their entry into the body [172–174]. Therefore, the acute toxicity of polymer micelles can be judged only from the results of studies using the route of administration that is supposed to be used in clinical trials. On the first day after polymer micelles with the drug are administered, animals should be under continuous observation. The total duration of acute toxicity observation should be at least 30 days. The cumulative properties of pharmacological substances contained in polymer micelles are evaluated in chronic toxicity studies. Three doses of polymer micelles are used in chronic toxicity experiments. Doses are calculated by the amount of active substance in the composition of the dosage form. The route of administration is similar to the clinical one. If multiple routes of administration are recommended, then all routes used should be evaluated [169, 171, 174–176]. Tests to assess the functional state of phagocytes, antioxidant system, expression of inflammatory markers, level of oxidative processes, and related damages are also used [177]. Siegrist et al. [171] recommend the following parameters for assessing the damaging effect of potential drugs contained in polymer micelles: complement activation, platelet aggregation, hemolysis, oxidative stress, cell viability, phagocytosis, inflammation, and DNA damage. Mandatory assessment of the damaging effect should be subject to the structure and function of the nervous system, kidneys, and liver as possible main targets of the toxic action of polymer micelles [178]. For the first phase of clinical trials in humans or target animals, on the basis of preclinical studies, the maximum recommended starting dose is calculated [177, 178].

5.6 Prospects for the Use of Micelles in Diseases, Including Cancer

Polymeric micelles are of interest as carriers of hydrophobic drugs. In particular, micelles can be used for the parenteral administration of drugs such as amphotericin B, propofol, and paclitaxel [179]. Like liposomes, micelles can be used for targeted drug delivery to target cells. This is achieved by attaching pH-sensitive elements to the surface of micelles.

Compared to other nanocarriers, polymer micelles are smaller, have a simple sterilization preparation process, and have a good solubilization property. The latter, unfortunately, is associated with lower stability in biological fluids. Especially difficult is the study of micelle interaction with the biological environment, which is necessary to predict the drug behavior after administration in vivo.

The use of nanoplatforms can lead to both therapy and diagnostics, hence the term “theranostics” [180]. The polymer micellar base was first used for anti-cancer drugs by Professor Kataoka in the late 1980s or early 1990s to increase the accumulation of drugs in tumor tissues. The size of micelles can be adjusted in the diameter range of 20–100 nm, ensuring that they do not pass through the walls of the vessels, so a reduction in the incidence of side effects can be expected [181].

The successful development of theranostic nanoplatforms requires the concomitant development of quantitative imaging techniques that will enable early disease detection and measure therapeutic response. Depending on the ratio of phospholipids and hydrophobic compounds, the micelle core can be adapted to accommodate several single hydrophobic molecules/nanoparticles. In [180], the encapsulated drug showed sustained release from the micellar core for 7 days. The biocompatibility of the micellar system was confirmed by cell viability analysis. The great potential of these theranostic micelles has been established for the imaging and therapy of various diseases, including cancer. Three widely studied classes of block copolymers are characterized by the presence of hydrophobic blocks and are poly(propylene oxide), poly(L-amino acids), and polyesters. These classes of block copolymers have been applied to some complex molecules in the pharmaceutical industry. Polymeric micelles can reduce toxicity, improve delivery to desired biological sites, and improve the therapeutic efficacy of active pharmaceutical ingredients [182].

Numerous types of biodegradable and synthetic block copolymers with different architectures (diblock, triblock, and graft copolymers) and physical properties (charged and neutral) are used to obtain various nanostructures such as vesicles [183–185] and spherical and rod-shaped micelles for targeted drug delivery [111].

Most polymeric micelles are designed to deliver hydrophobic anticancer drugs, which often have to be infused with surfactants and organic solvents. When administered systemically, such low-molecular-weight antitumor agents are distributed throughout the body, reducing the effective dose in target tissues and causing intoxication. In addition, the rapid clearance of anticancer drugs from the body leads to repeated administration of an effective drug concentration, which increases chronic toxicity and leads to acquired drug resistance. Thus, polymer micelles are much more beneficial for stabilizing drugs under aqueous conditions, protecting these agents inside their core from the external environment, circulating stably in the bloodstream, and selectively accumulating in tumors, where they can release drugs in a programmed manner [186].

Polymeric micelles can be designed to respond to specific stimuli to achieve spatiotemporal control over their functions, such as reporting on the conditions of their environment, releasing their cargo, and exerting therapeutic effects. Such signals may be endogenously present in the body and enhanced in affected tissues. For example, compared to healthy tissues, the tumor microenvironment provides unique stimuli for selective micelle activation, including acidic pH between 6.5 and 7.2 through aerobic glycolysis and lactate production and an altered redox potential. Moreover, endosomal/lysosomal pH (pH 6.5–4.5), enzymes, adenosine

triphosphate (ATP), intracellular ROS, and redox potential can additionally be used to control the action of micelles inside cells [186].

The promising therapeutic potential of micelles loaded with oligonucleotides, especially for cancer therapy (particularly for RNAi-based cancer therapy), has been described in [187]. Shen et al. [188] developed an oligonucleotide delivery system using pH-sensitive polymeric micelle-like nanoparticles and showed that this system effectively delivers oligonucleotides of various lengths (20,100 bp) into cells and has significant potential for cancer treatment.

Micellar forms have been proposed to create new forms of drugs:

1. *Anticancer drug*
Doxorubicin [89]
Paclitaxel [189]
Camptothecin [68]
Carboplatin [68]
Docetaxel [190]
Cisplatin [191]
Methotrexate [192]
Ethaselen [193]
5-fluorouracil [194]
Indisulam [195]
Disulfiram [196]
Amifostine [197]
Cyclosporine [198]
Gemcitabine [199]
2. *Hormones*
Estradiol [190]
Dexamethasone [200]
3. *Anti-infective (antiviral, antibacterial, antifungal, antiparasitic) drugs*
Adamantane [201]
Ciprofloxacin [202]
Amphotericin B [203]
Ivermectin [204]

Also, small organic molecules, siRNA, aptamers, peptides, carbohydrates, and antibodies have been proposed [64].

5.7 Current Status and Prediction

Among the nanoparticles used in pharmaceuticals, polymer micelles have clear advantages, because they contain amphiphilic polymers that self-assemble in an aqueous medium. These amphiphilic polymers are built with different polymer blocks. These blocks can be selected depending on the stability of the hydrophobic/lipophilic balance, size, ability to use the drug, the ability of micellization, and

stability in the systemic circulation. The nano-size of micelles allows more efficient exit through the vasculature, as compared with other drug delivery systems. Hydrophilic polymer coating will remain unrecognized by the reticuloendothelial system during circulation [205]. Therefore, micelle-based nanoparticles can be considered a system with unique characteristics, as compared with other nanocarriers. These characteristics include their smaller size, which allows passive targeting of target organs (even poorly permeable ones) [206] and more efficient cell internalization [141]; good solubilizing properties of the hydrophobic compounds assimilated into the lipophilic core; and increased circulation time in blood [207, 208]. Micelles are characterized by simple preparation and high scalability, as compared with polymeric nanoparticles and liposomes requiring complex, time-consuming, and costly manufacturing procedures [141, 208]. Despite the disagreement among scientists about the degree of accumulation of nanodrugs in tumors [209], it is important to point out that several clinically successful drugs, such as doxil and abraxane, are used to provide therapeutic assistance and effectively deliver a sufficient amount of active drugs to target tissues [210].

In the past few decades, polymer micelles have emerged as one of the most promising nanodelivery systems for cancer treatment. Micelles on PEG–PLA are well studied owing to their excellent biodegradability and biocompatibility. Genexol-PM is already approved for the treatment of breast cancer in South Korea [211]. With the progressive development of cancer therapies, the PEG–PLA micelles are increasingly used in combination with photodynamic, photothermal, gene, and immune therapy. The controlled delivery of therapeutic agents is an actively developing field and depends on the uniqueness of the tumor, the microenvironment around the tumor, and the combination with different therapeutic loads.

Nevertheless, the safety of new concepts being developed should not be forgotten, because there are data that are related not only to the toxicity of micelles [212] but also to their ability to cause neuroendocrine disorders [213]. Thus, polymeric micelles still need to be carefully studied in animal models before they can be recommended for human treatment.

6 Conclusion

More than 100 years have passed since the date of the emergence of the idea of targeted drug transport. Today, in pharmacy chains in different countries, we can buy drugs with targeted delivery ability. Such drugs are still few, but there is no doubt that their number will constantly grow. Most of the drugs that have reached the pharmaceutical market are made by using liposomal technology or CDs. An even greater number of drugs are at the stages of preclinical and clinical trials. The main trends in the development of research in the field of targeted drug transport are as follows:

(a) Development of effective vectors.

(b) Development of non-liposomal carriers with low toxicity. For example, the use of blood cells is attractive but requires the establishment of special units in medical institutions, because each patient needs an individual remedy.
(c) Determination of the optimum between the loaded amount of drug carrier and the necessary therapeutic minimum.
(d) Optimization of drug release methods.
(e) A significant increase in the stability of drugs and an increase in their shelf life.
(f) Cheaper drug production.
(g) Development of legislative norms for the regulation, certification, and production of directed transport systems.

As already mentioned, there is considerable interest in the development of polymeric micelles capable of acting as true delivery vehicles for various potent drugs that are not found in therapeutic formulations owing to their water-insoluble, hydrophobic nature. Micelles have advantages as nanocarriers for drug delivery and treatments owing to their excellent physicochemical properties, drug loading and release capacities, facile preparation methods, biocompatibility, and tumor targetability.

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Polymeric Nanoparticles for Targeted Drug and Gene Delivery Systems

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1 Introduction: Ideals of Nanoparticle Drug Delivery Systems

Polymeric nanoparticles (NPs) are nanostructured materials ranging from 1 to 1000 nm in diameter [1]. They have been used in a multitude of areas including agricultural and environmental applications [2–4], electronics and photonics [5–8], bioimaging [9–12], chemical catalyst formation [13], and nanomedicine [14]. Polymeric NPs were first proposed for medical applications in the 1970s [15–17]

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with growing interest given their potential for targeted delivery of drugs to cells and tissues across a range of pathophysiologic opportunities. Polymeric NPs can be loaded with therapeutic compounds to act either as drug delivery systems [18, 19] or targeted gene therapy [20, 21]. Polymeric NPs can take two morphologic forms – nanocapsules with the drug dissolved and retained in an inner core surrounded by a polymeric shell [1, 18–22] and nanospheres which appear as a continuous and homogenous polymeric matrix in which drugs can be adsorbed, trapped, or cross-linked (Fig. 1).

Polymeric NPs present many characteristics that make them ideal candidates for medical applications. These NPs display high kinetic stability and rigid morphology. They can incorporate hydrophilic or hydrophobic drugs [18, 19, 23, 24] and nucleic acids [20, 21] to protect their payloads from undesired degradation [25, 26]. Their heterogeneous chemical surface offers multiple possibilities for functionalization and structural versatility [1, 14, 18] enabling site-specific targeting to minimize both immune response and side effects.

Polymeric NPs comprise an extensive family. In this chapter, we will focus mainly on the most common polymer-based NPs used for targeted drug and gene delivery to date. We will discuss the general and ideal forms of drug and gene-encapsulated polymeric NPs, the main preparation methods, and biomedical applications for the more commonly used materials: poly(lactic acid) and poly (lactic-co-glycolic acid), polyurethane, poly(alkyl cyanoacrylate), polycaprolactone and poly(β-amino ester) NPs, and polymeric NPs derived from natural compounds. Other materials such as polyethyleneimine, poly(acrylamidoethylamine), poly(aspartic acid), poly(N-isopropylacrylamide), and poly(propylene sulfide) have been proposed and may well appear more often in the future [27].

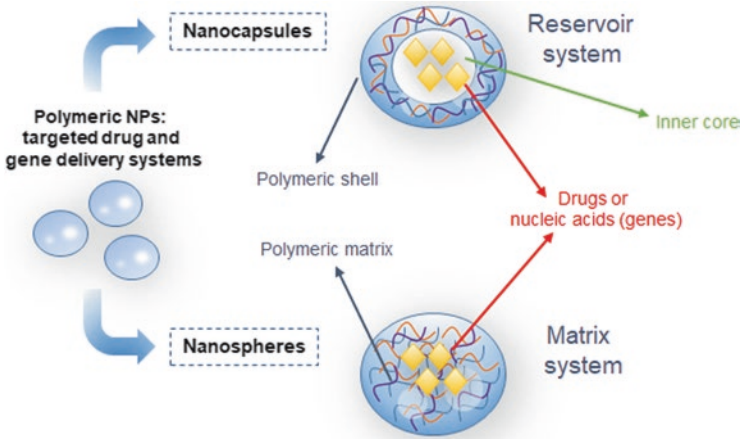

Fig. 1 Schematic representation of polymeric NPs for targeted drug and gene delivery. Polymeric NPs are divided into two large groups considering their morphology. Nanocapsules act as reservoir systems where the active compound is contained in the inner core surrounded by a polymeric shell. Nanospheres result from active compounds included within polymeric matrices. Abbreviations: NPs nanoparticles

2 Poly(Lactic-Co-Glycolic Acid) and Poly(Lactic Acid) Nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) and poly(lactic acid) (PLA) are biodegradable and biocompatible synthetic polymers that have the approval of the US Food and Drug Administration (FDA) [28]. These materials have been employed as surgical sutures since the 1960s, and their initial success led to their use as polymeric biomaterials for other applications [29]. They have the license for human use (e.g., surgical sutures, bone implants, screws, and drug-eluting targeted implants) and specifically for the synthesis of polymeric NPs for drug delivery [28, 30]. These polymeric NPs have proved extraordinary properties for the targeted delivery of hydrophilic/hydrophobic drugs, proteins, peptides, vaccines, and nucleic acids [30, 31]. Moreover, they have shown wide applicability to oral delivery, subcutaneous injection, and sustained delivery of drugs [32].

PLGA/PLA NP synthesis can be divided into the bottom-up and top-down techniques [33]. The bottom-up techniques employ a monomer as a starting point, whereas the top-down employ a pre-formed polymer. PLA formation requires the polymerization of lactic acid, and PLGA is synthesized by copolymerization of two different monomers: glycolic and lactic acid [30, 34]. The forms of the PLGA are identified by the monomer ratio; thus PLGA 50:50 identifies a copolymer whose composition is 50% lactic acid and 50% glycolic acid [35]. The monomer ratio in the polymer chain, the molecular weight of the polymer, the degree of crystallinity, and the glass transition temperature (Tg) of the polymer are factors that influence the biodegradation rate and the characteristics of the synthesized NP [29, 36].

Owing to their subcellular and submicron size, PLGA NP delivery systems have distinct advantages for drug delivery, such as reducing the dosage, ensuring the pharmaceutical effects, minimizing side effects, protecting drugs from degradation, and enhancing drug stability [35]. Surface modifications, the preparation method, particle size, molecular weight of the drug, and the monomers' ratios are parameters influencing the drug release profile and the effective response of NPs [37].

2.1 Characteristics that Awarded the Approval of the US Food and Drug Administration

PLA and PLGA have been approved by the FDA for human use in sutures, bone implants, and screws, in formulations for sustained drug delivery and in vaccine formulations [38]. More than 20 PLGA-based drug products have received regulatory approval to date, with the first PLGA-based product approval occurring in January 1989 [39]. The first FDA-approved implant system of PLGA microparticles was the Lupron® Depot that releases human growth hormone for a month [40].

The characteristics of these materials that approved drug delivery were their biodegradability, biocompatibility, mechanical properties, and controlled and sustained

release [35]. These polymeric NPs are biodegradable because they are easily degraded by hydrolysis into nontoxic products [35, 41]. PLA polymers are hydrolyzed into lactic acid monomers and PLGA copolymers into lactic acid and glycolic acid [42] (Fig. 2a). These monomers are by-products of various metabolic pathways, so they can be easily metabolized by the body via the Krebs cycle and eliminated in the form of carbon dioxide and water (Fig. 2a) minimizing systemic toxicity [30, 43]. PLA and PLGA are considered biocompatible because they do not elicit any immunological reactions or inflammatory responses [44]. However, some toxicological studies with PLGA particles suggest that local mild tissue reaction at the side of application may occur [42]. Furthermore, their mechanical and physical properties can be engineered via a selection of polymer molecular weight, monomer ratio, and functionalization to suit multiple applications [38]. Indeed, they can be easily modified to optimize their targeting characteristics [45].

PLGA polymers have also been used to sustain the release of drugs in various long-acting controlled delivery systems approved by the FDA [39]. Some advantages of sustained-release are reduced dosing frequency, decrease of the incidence of side effects, maintenance of stable plasma concentrations, and better patient compliance [39]. PLGA NPs enter cells via endocytosis and can escape the endolysosomal compartment if modifications in the polymer structure are incorporated to change the surface charge in the acidic milieu [46]. In the cytoplasm, PLGA NPs

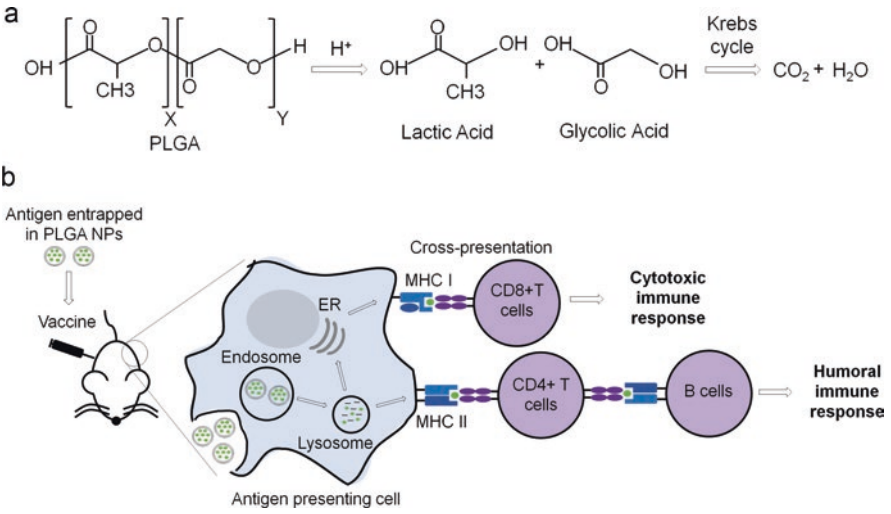

Fig. 2 PLGA NP synthesis and application. (**a**) PLGA copolymers are hydrolyzed into lactic acid and glycolic acid in aqueous conditions. These monomers are then metabolized via the Krebs cycle to carbon dioxide and water. (**b**) In this illustration, it is described how PLGA NP-based vaccines can provide a humoral and cytotoxic immune response simultaneously. PLGA NPs are internalized by antigen-presenting cells, and the antigen is processed to be presented by MHC-II. MHC-II activates CD4+ T lymphocytes that provide help for the humoral immune response by stimulating B lymphocytes. PLGA NPs can also release the entrapped antigens into the cytosol and allow the cross-presentation by MHC I and the stimulation of CD8+ T lymphocytes. Abbreviations: PLGA Poly(lactic-co-glycolic acid, NPs nanoparticles, ER endoplasmic reticulum, MHC major histocompatibility complex

undergo degradation by hydrolysis and deliver their cargo following a biphasic curve pattern [42, 47]. They often exhibit an initial burst release, followed by a very slow and prolonged release that leads to a sustained therapeutic effect [48].

2.2 Basic Principles of Preparation

Many methods are available to synthesize PLGA NPs [33]. Each has its advantages and disadvantages, but the choice of method depends on the chemical characteristics of the active component and its interactions with the organic solvents, polymer, and surfactant, as well as the final use of the NPs [28, 35, 43].

PLA and PLGA NP preparation is dictated by the solubility of the incorporated drug. Emulsion solvent evaporation is the most common technique used to encapsulate hydrophobic drugs (soluble in a water-immiscible solvent) [28, 30, 35, 43]. In this technique, the polymer and the drug are mixed in a water-immiscible organic solvent. Then, a large volume of water containing a surfactant is added to create an emulsion of oil-in-water (O/W), and the polymeric NPs are formed by homogenization or sonication. The solvent is then evaporated and the NPs collected after centrifugation. Nanoprecipitation is used to encapsulate hydrophobic drugs soluble in a water-miscible solvent [49, 50]. Here, the polymer and the drug are dissolved in a water-miscible solvent [28, 32, 33]. The organic solvent is injected in a controlled manner into the aqueous phase, and the particles are immediately formed. Then, they can be collected via removal of the solvent by evaporation or dialysis [28, 50]. Other techniques such as diafiltration or salting-out can be also applied. In the salting-out process, the polymer and the drug are mixed in a water-miscible organic solvent [33, 51]. Then, an emulsion of the organic phase in an aqueous phase containing a stabilizer and a highly concentrated electrolyte is obtained. The rapid addition of pure water to the O/W emulsion, under mild stirring, induces the formation of NPs. In contrast, the double emulsification solvent evaporation technique is used to encapsulate hydrophilic drugs such as peptides, proteins, and nucleic acids [30]. In this technique, an aqueous solution containing the drug is added to an organic solution of the polymer to create an O/W emulsion [52]. Then, the emulsion is added to a large volume of water with an emulsifier to create a water-in-oil-in-water (W/O/W) emulsion. The NPs are obtained by centrifugation after the solvent has been removed by evaporation. Other techniques have been described for the efficient entrapment of hydrophilic drugs such as spray-drying [30, 33]. Polymer concentration, polymer molecular mass, the organic solvent, the ratio of the organic and aqueous phase, the surfactant characteristics, homogenizer and agitation speed, and thermodynamic parameters are some factors that dictate NP size and characteristics [33, 53].

2.3 Main Applications in Medicine and Biomedicine

PLA and PLGA NPs have been used to deliver a wide range of hydrophilic/hydrophobic drugs, proteins, peptides, vaccines, and nucleic acids [30]. Functionalization of these NPs with ligands with high affinity for cell surface receptors can enhance the targeted cargo delivery [54]. Size, shape, surface charge, hydrophobicity, and hydrophilicity can be also manipulated on PLGA NPs to modify the uptake of NPs by antigen-presenting cells (APC) [36].

PLGA NPs are the most used polymeric NPs for vaccine delivery [55, 56]. PLGA NPs have already been successfully used for the delivery of a broad range of antigens including hepatitis-B virus antigens [57], *Bacillus anthracis* antigen [48], tetanus toxoid [58], and ovalbumin [59]. These NPs can provide continuous in vitro release of entrapped antigens for long periods and optimize the desired immune response by improving antigen uptake via selective targeting of antigen-presenting cells [60]. These NP-based vaccines provide a humoral and cytotoxic immune response and immunological memory [59, 61] (Fig. 2b). In this scenario, polymeric NPs provide a viable alternative to single-dose vaccines [62]. Furthermore, several studies focus on the treatment of bacterial infections by the development of antibiotic-loaded PLGA NPs [63–65].

PLGA NPs are also good candidates for cancer immunotherapy. They can encapsulate tumor-associated antigens alone [66] or associated with adjuvants [67] to induce long-lasting anti-tumor cytotoxic responses. They can also encapsulate signal transducers, transcription factors, or their inhibitors to restore immunosuppression in the tumor environment [43, 68]. PLGA NPs can be used in cancer chemotherapy as well. They can encapsulate anticancer drugs such as doxorubicin or paclitaxel and target the tumor to produce a cytotoxic effect and decrease tumor growth [69–73]. In the past years, PLGA NPs have also been used in gene therapy as carriers of plasmids or small interfering ribonucleic acid (siRNA) therapeutics against cancer-associated targets, among others [43].

Oral administration or injection of PLGA NPs has demonstrated good therapeutic results to treat inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis, and inflammatory lung disease, among others [74, 75]. PLGA NPs have been designed to encapsulate siRNA against pro-inflammatory cytokines [76] or chemokine receptors responsible for monocyte recruitment [77]. PLGA NPs have also been used to treat diabetes and have been proposed as potential carriers for the oral delivery of insulin because they help avoid degradation and improve its intestinal absorption [78].

3 Polyurethane Nanoparticles

Polyurethane (PU) was first synthetized in Germany by Otto Bayer and coworkers in 1937 [79]. Since then, this polymer has been industrially exploited in foams, varnishes, adhesives, thermal insulation, and more recently in biomedical devices.

For example, this polymer has been included in biologically compatible tissue adhesives, scaffolds for tissue regeneration, or foam dressings to facilitate wound healing and prevent future complications after an injury or surgery [80]. There is a growing interest in this polymeric material for the design and functionalization of PU-based NPs as drug delivery systems to specifically recognize target organs, tissues, or cells [81] due to the PU synthetic versatility and excellent mechanical properties [82, 83].

PU polymers are typically composed of organic units linked by carbamate (urethane) bonds, which are formed by chemical reaction (nucleophilic attack) between the carbonyl groups of diisocyanate monomers and hydroxyl, amine, polyols, or polyamine groups [84, 85] (Fig. 3a). Diisocyanates may also react with other polymers such as poly(ε-caprolactone) or polysaccharides to achieve diverse biocompatible polymeric NPs [86]. Most PU NPs present favorable biocompatibility, an acceptable half-life, and nontoxic degradation products gradually cleared from the body [84, 87]. Apart from PU potential to encapsulate drugs, they can also encapsulate RNA and DNA oligonucleotides with preserved bioactivity and efficient targeted delivery to specific cells, for example, to HEK293T kidney cells [88]. This highlights PU potential for gene therapy applications.

3.1 Monomers and Polymers: A Versatile Chemistry for Drug Delivery Systems

PU polymers are formed of diisocyanates and polyols or polyamines joined by urethane or carbamate bonds (—NH—CO—) (Fig. 3b). Diisocyanate monomers are a family of molecules with high reactivity and a vast variety of tailored structures (radicals). This high chemical reactivity can be explained in part by the resonance structure in the isocyanate group (—N=C=O). Isocyanate monomers are usually toxic but lead to biodegradable and biocompatible polymers when they polymerize. Diisocyanates that are frequently used for the synthesis of PU for biomedical applications include isophorone diisocyanate (IPDI), 2,4- and 2,6-toluene diisocyanate (TDI), 4,4′-methylenediphenyl diisocyanate (MDI), lysine- diisocyanate (LDI), 1,6-hexamethylene diisocyanate (HDI), and 4,4′-methylene bis (cyclohexylisocyanate) (HMDI) [81]. Monomers of diisocyanates, polyols, and polyamines should be chosen according to the final physicochemical characteristics of PU NPs that are pursued [81, 89]. PU NPs are generally obtained from polyaddition or polycondensation reactions from polyol or polyamine and diisocyanate monomers [84, 90].

The enormous array of combinatory reactions between different polyols, amines, and diisocyanates using different natural or synthetic molecules highlights the synthetic versatility and customization possibilities of PU-based NPs for a multitude of applications. Indeed, the word “versatile” has been strongly associated with PU [89]. PU polymers are characterized by distinct domain structures, including soft and hard segments, which are responsible for the typical PU properties such as flexibility and stiffness (Fig. 3b). Polyol long chains are the macromolecule elements

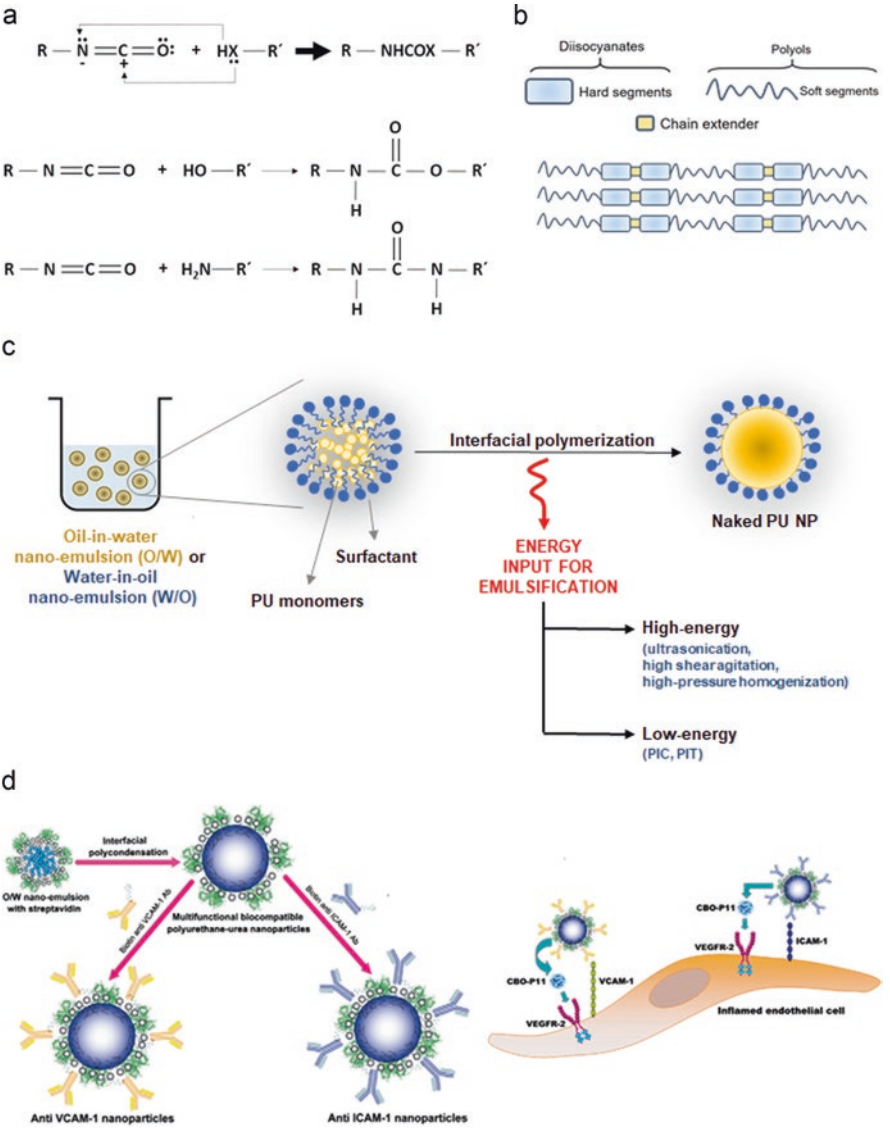

Fig. 3 Basic chemistry, synthesis from nano-emulsions, and applications in endothelial cell targeting of PU NPs. (**a**) Nucleophilic attack of the carbonyl groups of diisocyanate monomers and hydroxyl or amine groups. (**b**) PU polymer distinct domain structures, soft and hard segments, responsible for PU properties such as flexibility and stiffness. (**c**) Schematized synthesis of PU NPs from nano-emulsions. (**d**) Functionalization with VCAM-1 and ICAM-1 antibodies and specific targeting of multifunctional PU NPs loading an antiangiogenic drug, CBO-P11, on inflamed endothelial cells. (Adapted with permission from Ref. [112]). Abbreviations: PU polyurethane, NP nanoparticle, PIT phase inversion temperature, PIC phase inversion composition, Ab antibody, VCAM-1 vascular cell adhesion protein-1, ICAM-1 intercellular adhesion molecule-1, VEGFR vascular endothelial growth factor receptor

that define the soft segments and flexible properties. Isocyanates and chain extenders are the elements that define the hard segments. Isocyanates are short molecules that exhibit high crystallization capabilities. Hard segments are responsible for the mechanical strength of the final macromolecule. The combination of soft and hard segments explains part of the high versatility of PU to obtain products with an extremely wide range of elasticity [81, 91]. Several PU-based NPs for biomedical applications have been synthesized using the polymerization of diisocyanates and polyols or polyamines. However, there is a growing interest in non-isocyanate PU-based polymers for the synthesis of PU NPs [92–94] explained in part by the non-selective side reactions that may occur during PU polymerization using isocyanates [93]. The isocyanate can avidly interact with the solvent (water molecules) and disturb the main reaction [93]. The synthesis of PU polymers through non-isocyanate polymerization is based on thermally stable transcarbamoylation reactions, such as the polymerization between diamines and pentafluorophenyl dicarbonates [92]. For example, non-isocyanate PU-based polymeric NPs have been used for targeted drug release to the mitochondrial matrix [94]. Therefore, non-isocyanate chemistry reveals new possibilities for PU-based NP synthesis.

3.2 Synthesis of the Polymeric Shell from Nano-Emulsions

Nano-emulsions are emulsions with droplet size on the nanometric scale, usually between 20 and 200 nm. They are formed by two immiscible liquids, one of which is uniformly dispersed in the other, with an appropriate surfactant [95, 96]. Nano-emulsions are transparent or translucent to the eye [97]. They are classified as oil-in-water (O/W), if the internal oil phase is dispersed in an aqueous solution, or water-in-oil (W/O) if the internal aqueous phase solution is dispersed in an external oil phase [95]. Nano-emulsions present high kinetic stability and offer the possibility to obtain NPs with a very large surface area, which is ideal for drug solubilization [98]. This points to nano-emulsions as an attractive and ideal colloidal system for the synthesis of drug and gene delivery systems. Indeed, the synthesis of nano-emulsions is one of the most common approaches to the synthesis of PU-based NPs for biomedical applications [84].

PU nanocapsule synthesis is usually carried out by interfacial polymerization in droplets. Interfacial polymerization/polyaddition reactions allow the polymerization of PU monomers with different molecules to offer a variety of functionalities to PU NPs [99]. Both hydrophilic and hydrophobic drugs can be encapsulated into the droplets depending on the internal phase and composition. The polymeric shell in nano-emulsions is formed by step-growth polymerization at the interface between two immiscible phases. Nano-emulsions are thermodynamically unstable, and they require an energy input for their formation. Thus, typical methods used to synthesize PU NPs from nano-emulsions by interfacial polymerization are generally classified as high-energy emulsification methods (ultrasonication [90, 100–102], high shear agitation [103, 104], or high-pressure homogenization [104]) and low-energy

emulsification methods. Low-energy emulsification methods allow the formation of nano-emulsions with smaller and more uniform droplet sizes rather than high-energy methods. Low-energy emulsification methods include phase inversion temperature (PIT) and phase inversion composition (PIC) approaches. In PIT, a phase transition occurs when the system is speedily cooled and heated in a constant composition. In PIC, the temperature is maintained constant, and phase transitions occur when the nano-emulsion composition is modified [99, 105, 106]. The NP synthesis method is tuned to the desired properties of the therapeutic nanosystems since the emulsification process may affect drugs or bioactive compounds (Fig. 3c).

3.3 Applications for Targeted Drug and Gene Therapy in Endothelial and Cancer Cells

PU has been widely used as a biocompatible scaffold for vascular grafts and as a platform to stimulate neovascularization, inhibit platelet adhesion, and enhance cell adhesion and proliferation [107–110]. During the last decades, PU has also been employed in the synthesis of PU NPs for drug and gene delivery systems to target and treat cells and tissues in a variety of diseases. For example, some reports have investigated the use of PU NPs to target endothelial cells (ECs) [111–113]. ECs play critical roles in inflammation through the recruitment of leukocytes via synthesis and exposition of cell surface adhesion molecules. The bioadhesion properties of biotin-modified PU NPs have been demonstrated in porcine and human endothelial cells and porcine aorta [113]. Furthermore, ECs contribute to the progression of multiple human cancers since they are closely involved in the promotion of angiogenesis, suppression of apoptosis, invasion, and metastasis. Accordingly, streptavidin-coated PU NPs have been designed to target ECs to inhibit the vascular endothelial growth factor (VEGF) signaling pathway [112]. These NPs incorporated CBO-P11, which is a VEGF receptor inhibitor. Biotinylated antibodies against vascular cell adhesion protein-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were attached to the surface of the polymeric matrix to achieve selective targeting of the inflamed ECs (Fig. 3d). CBO-P11 delivery through streptavidin-coated PU NPs efficiently inhibited the activation of VEGF receptors and proliferation of inflamed ECs [112]. This report highlights the potential of PU NP for the design of nanotherapies to arrest the vascular network in tumor tissue. PU NPs have also been extensively investigated as drug and gene delivery systems to target and treat cancer cells [113–125]. PU NPs improve the pharmacokinetics of some encapsulated chemotherapeutic agents such as doxorubicin or cisplatin, among others [117, 118, 120]. Biotinylated PU NPs linked to a DNA plasmid encoding green fluorescent protein and two anticancer drugs, phenoxodiol and sunitinib, have been designed for the treatment of liver cancer cells [121]. Biotinylated PU NPs reduced the viability of hepatoma cells with high specificity and simultaneously allowed the identification of cancer cells, thereby acting as a theranostic (therapy and

diagnostic) tool. In another study, black phosphorus quantum dots (BPQDs) were encapsulated into PU NPs to improve their stability and dispersibility [122]. PU NPs improved the photothermal and photodynamic efficiency of BPQDs and increased the cellular growth arrest of HeLa, MCF7, and HepG2 cancer cells through reactive oxygen species formation. In conclusion, PU NPs have demonstrated interesting properties for the targeted delivery of drugs and nucleic acids to treat ECs and cancer cells.

4 Poly(Alkyl Cyanoacrylate) Nanoparticles

Alkyl cyanoacrylates are monomers with high reactivity and excellent adhesive properties [126]. In fact, alkyl cyanoacrylates are the well-known "superglue." Manufactured by Henkel company, "superglue" has been widely employed as an adhesive for do-it-yourself activities [126]. In medicine, some of the alkyl cyanoacrylate monomers have FDA approval as surgical glues in humans [127–129]. Alkyl cyanoacrylate monomers and polymers were first reported in 1949, but it was not until 1979 that Couvreur and colleagues first designed matrix-structured NPs for drug delivery using these polymers [17]. Since then, poly(alkyl cyanoacrylates) (PACA) have been investigated for the design of nanotherapies to increase the efficiency of drug delivery to target organs [126]. PACA NPs have been synthetized to deliver cytotoxic compounds to solid tumors [130] and encapsulate brain-targeted drugs able to cross the blood-brain barrier [131–133]. Many bioactive compounds have been loaded on PACA NPs, such as chemotherapeutic agents, antibiotics, bioactive peptides, antiviral drugs, nonsteroidal anti-inflammatory drugs, anti-fungal drugs, nucleic acids, etc. To date, there are many in vitro and in vivo preclinical studies and few clinical trials using PACA nanocarriers, but its use as drug delivery NPs has not yet been approved for humans [134]. Even so, the functionalization of PACA NPs with different compounds continues to be studied to obtain ligand-decorated NPs with improved cellular uptake, low opsonization by the mononuclear phagocytic system, and enhanced tissue-targeted drug release capabilities.

4.1 The Glue for Nanocarriers: Advantages and Disadvantages

PACAs are synthetized by a wide range of monomers. PACA monomers are clear and colorless liquids with a distinguished strong and irritating odor. They rapidly polymerize in the presence of humidity or trace anions [135]. The addition of polymerization inhibitors may be necessary to maintain stability, and, consequently, the physicochemical properties of the resulting polymers may be modified [126]. Alkyl cyanoacrylates produce vapors in a dry atmosphere (with 50% relative humidity) that may produce harmful respiratory effects and eye irritation. Prolonged exposure to high concentrations of these vapors could lead to the appearance of allergic

reactions. Nonetheless, PACA monomers are not considered to be toxic due to their rapid polymerization rates [136].

PACA monomers have been used since the early 1960s for diverse biomedical applications due to their excellent adhesive properties. PACA can strongly bind to different substrates including living tissues and skin [126]. Thus, long-chain PACA monomers have been used as surgical glue for the treatment of skin wounds and in sutures during endovascular surgeries [127]. PACAs are biodegradable and biocompatible polymers that have also been extensively studied to synthesize nanocarriers for the encapsulation and the controlled release of drugs [126]. For example, site-specific butyl- and isobutylcyanoacrylate-based NPs have been synthetized to avoid opsonization with promising results in the treatment of various diseases [126, 135].

One of the main disadvantages of intravenous administration of colloidal systems for controlled drug release is their rapid elimination by the action of the mononuclear phagocytic system. Nanocapsules have been functionalized with poly(ethylene glycol) (PEG) to reduce the interactions of PACA NPs with opsonins and phagocytosis [135]. This process is known as PEGylation. For example, sterically stabilized copolymeric NPs (PACA-PEG) have been used as nanocarriers to encapsulate drugs and avoid premature drug elimination [126, 137]. The synthesis of these nanomaterials involved the direct incorporation of PEG into the emulsion. The final formulation is obtained by a process of nanoprecipitation or solvent evaporation. Nanospheres and nanocapsules of PEG-PACA-co-hexadecyl cyanoacrylate synthetized with this method have been employed for the delivery of the antitumor agent tamoxifen [138]. Butylcyanoacrylate NPs coated with polysorbate 80 have also been suggested to deliver dalargin to the brain via intravenous administration. Polysorbate 80 interacts with low-density lipoprotein (LDL) receptors to stimulate the uptake in endothelial cells [139] and interferes with the action of the mononuclear phagocytic system.

4.2 Polymeric Reactions and Synthesis

Knoevenagel condensation reaction between an alkyl acetate and formaldehyde is the most common procedure to obtain PACA monomers with different alkyl side chains [126] (Fig. 4a). PACA polymers are obtained from the polymerization of these monomers through three different mechanisms: free radical polymerization, anionic polymerization, and zwitterionic polymerization. Anionic and zwitterionic polymerizations (Fig. 4b) are the most common PACA synthesis methods since they can be rapidly triggered at room temperature [126]. In anionic polymerization, the reaction is initiated by basic chemical species present in the polymerization media. Usually, OH^- ions from the dissociation of water molecules react with the electrophilic carbon of the alkyl cyanoacrylate monomer (nucleophilic attack). The formed PACA monomer radicals react with other monomer molecules to originate first oligomers and then polymers [126]. Other basic compounds and surfactants rather than water molecules can also act as catalysts of the polymerization reaction. In

zwitterionic polymerization, a nucleophilic species serves as the reaction initiator. The resulting polymer will present equivalent positively and negatively charged groups (zwitterionic material) [126, 140].

PACA NPs are composed of homopolymers or copolymers depending on the presence of one or two types of monomers [126]. Most of the formation processes of PACA NPs described in the literature are based on the polymerization of a wide array of monomers [135, 141–143]. Both nanosphere and nanocapsules can be obtained using PACAs. Several PACA nanospheres are synthetized by emulsion polymerization. Couvreur et al. described for the first time in 1979 the synthesis of PACA NPs with a matrix structure designed for drug delivery. They were obtained through the polymerization of alkyl cyanoacrylates from emulsions [17]. The reaction was initiated by OH^- ions in the polymerization media, and the polymeric chain was elongated by anionic polymerization. The size of the obtained nanospheres with this method varied between 50 and 300 nm [135].

PACA nanocapsules are formed by interfacial polymerization in O/W or W/O emulsions, microemulsions, and nano-emulsions. Synthetized nanocapsules present a vesicular structure and are suitable for the entrapment of active compounds of both hydrophobic and hydrophilic nature. Moreover, the surface of PACA nanocapsules obtained by "in situ" polymerization can be functionalized with specific molecules that react with PACA monomers. These functional groups include polysaccharides, PEG, and methoxymethyl-PEG [137].

4.3 Main Biomedical Applications

One of the main biomedical applications of PACA polymers is the synthesis of NPs that are designed to encapsulate active compounds for the treatment of various diseases. The main active compounds that have been incorporated into PACA NPs include siRNA molecules for gene therapy [144, 145], peptides [146–149], and chemotherapeutic drugs for cancer treatment [150–157] (Table 1). siRNAs are usually incorporated into PACA NPs by surface adsorption in the final steps of the synthesis [145]. Surface adsorption of these nucleic acids in NPs allows intravenous administration of chemically unmodified targeted siRNA. siRNA release from PACA NPs has been investigated primarily for gene therapy in cancer cell lines [145]. Another therapeutic strategy has been the incorporation of bioactive peptides. Low molecular weight hydrophilic peptides are typically poorly encapsulated in PACA NPs prepared from emulsions. The entrapment of these peptides in NPs could be increased using acryloyl functionalization [149]. Examples of peptides that have been encapsulated in PACA NPs include insulin for oral administration [146], long-acting somatostatin analogue [147], selegiline to treat symptoms related to Parkinson's disease [148], and dalargin [139] (Fig. 4c).

PACA NPs have mostly been investigated as drug delivery systems for chemotherapeutic agents. Chemotherapy has been widely used in cancer treatment, but conventional administration to patients presents different limitations and problems.

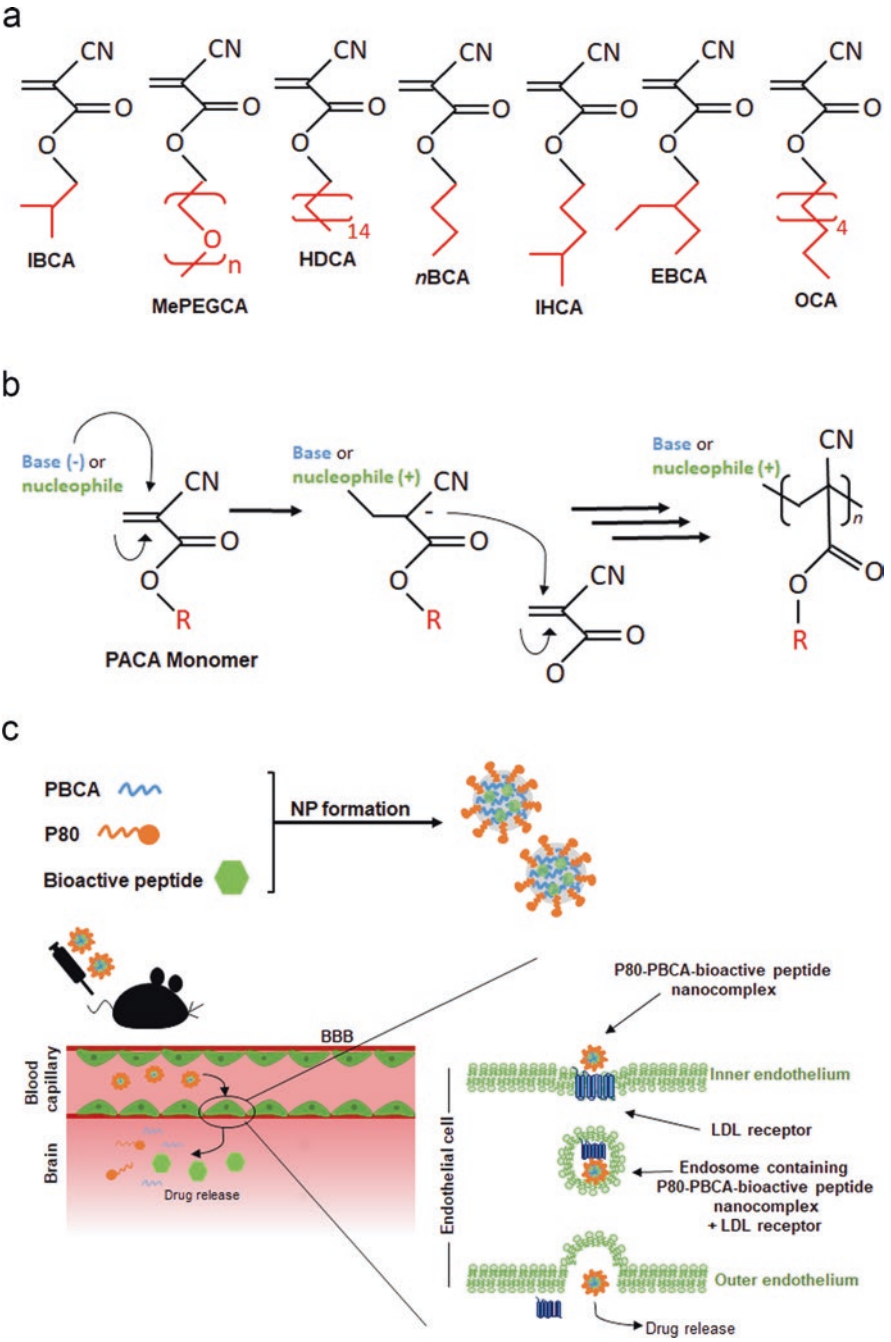

Fig. 4 PACA monomers, polymerization reactions, and applications for brain-targeted drug delivery. (**a**) Monomers used for biomedical applications with different side chains. (**b**) Anionic and

(continued)

zwitterionic polymerization reactions for PACA synthesis. In anionic polymerization, basic compounds act as reaction initiators, whereas reaction initiators in zwitterionic polymerization are nucleophiles. (**c**) Schematic representation of polysorbate 80 (P80)-coated PACA NPs used for bioactive peptide drug delivery to cross the blood-brain barrier. P80 is hypothesized to interact with LDL receptors in endothelial cells to permit the internalization of NPs to the brain from blood capillaries. Abbreviations: IBCA isobutyl cyanoacrylate, MePEGCA methoxypoly(ethylene glycol) cyanoacrylate, HDCA hexadecyl cyanoacrylate, *n*BCA butyl cyanoacrylate, IHCA isohexyl cyanoacrylate, EBCA 2-ethylbutyl cyanoacrylate, OCA octyl cyanoacrylate, PACA poly(alkyl cyanoacrylate), PBCA poly(butyl cyanoacrylate), P80 polysorbate 80, BBB blood-brain barrier, LDL low-density lipoprotein

These problems include high-dose requirements, non-specific targeting, poor bioavailability, development of multiple drug resistance, and harmful side effects [158]. PACA NPs have been suggested as chemotherapeutic drug delivery systems to successfully address the delivery-related problems. For example, cabazitaxel, a semisynthetic derivative of a natural taxoid, has been incorporated into PACA NPs to obtain efficient delivery to cancer tissue and cells [150–152]. Cabazitaxel encapsulated in poly(2-ethylbutyl cyanoacrylate) NPs showed better efficacy compared to similar concentrations of free drug in breast cancer basal-like xenografts [152]. The drug was incorporated into the oil phase during emulsion synthesis [152]. Drug encapsulation in PEGylated PACA NPs enhanced pharmacokinetics to allow two times higher drug concentrations in tumor tissue 96 hours after injection and approximately three times higher concentrations 24 hours afterward [152]. Doxorubicin has also been incorporated into PACA NPs for the treatment of glioblastoma [153, 154] and breast cancer [156]. Entrapment of this cytostatic drug into PACA polymeric nanocapsules significantly reduces the drug clearance in mice [157]. In conclusion, all these studies demonstrate that chemotherapeutic drug encapsulation into PACA NPs can effectively overcome the limitations of chemotherapy selectivity and the poor bioavailability of some chemotherapeutic agents.

5 Polycaprolactone Nanoparticles

Polycaprolactone (PCL) polymer was first synthesized by Van Natta et al. in the early 1930s using a ring-opening ε-caprolactone polymerization method [159]. PCL is a thermoelastic polymer with good stability under ambient conditions and easy processability [160, 161]. In fact, it is one of the easiest synthetic polymers to manipulate and process into a wide range of sizes and shapes since it shows a low melting temperature and very good viscoelastic properties [160]. Since its discovery, PCL has been extensively studied in the biomedical field due to its excellent physicochemical properties. Particularly, it has been considered for the synthesis of biocompatible scaffolds for tissue engineering and the synthesis of nanocarriers for drug delivery [160]. For example, PCL polymers have been successfully incorporated in implantable tissue scaffolds for cardiovascular [162, 163], nerve regeneration [164], bone [165], cartilage [166], and skin tissue engineering [167]. Some of

Table 1 Bioactive peptides, siRNA, and chemotherapeutic drugs included in PACA NPs for the treatment of diverse diseases

PACA NPs	Incorporated peptide	Bioactivity evaluation in cells, tissue, or animal models	References
Bioactive peptides			
PIBCA nanospheres	Insulin	For oral administration in diabetic rats	[146]
PIBCA nanocapsules	Octreotide (long-acting somatostatin analogue)	For subcutaneous and oral administration to rats	[147]
Copolymeric PMePEGCA and PHDCA	Seleginile	Interaction with amyloid-β peptide 1–42 for Alzheimer's disease treatment	[148]
Polysorbate-80-coated PBCA	Antinociceptive opioid peptide dalargin	Human and bovine brain capillary endothelial cells, rat astrocytes, and healthy ICR mice	[139]
siRNA			
Copolymeric chitosan-coated PIBCA and PIHCA	siRNA oriented toward RET/PTC1 junction oncogene	Papillary thyroid carcinoma bearing mice	[145]
Chemotherapeutic drugs			
PBCA, PEBCA, and POCA	Cabazitaxel	Breast (MDA-MB-231), colon (HCT116), and prostate cancer (PC3) cells	[150]
PEBCA	Cabazitaxel	MDA-MB-231, MDA-MB-468, and MCF-7 breast cancer cell lines and basal-like patient-derived xenograft	[151]
PBCA coated with poloxamer 188 and polysorbate 80	Doxorubicin	Intracranial glioblastoma in rats	[153]
Cyclosporin A loaded PIBCA	Doxorubicin	P388 leukemia-resistant cell line	[154]
PBCA	Doxorubicin	GS-9 L, F-98, and RG-2 rat glioma cell lines	[155]
PBCA	Doxorubicin	MCF-7 human breast tumor cell lines, E0771 mouse cancer cells, and immunocompetent C57BL/6 mice	[156]
PIHCA	Doxorubicin	Evaluation of distribution pattern in mouse tissues after intravenous administration	[157]

Abbreviations: siRNA small interference ribonucleic acid, *NPs* nanoparticles, *PIBCA* poly(isobutyl cyanoacrylate), *PMePEGCA* poly[methoxypoly(ethylene glycol) cyanoacrylate], *PHDCA* poly(hexadecyl cyanoacrylate), *PBCA* poly(butyl cyanoacrylate), *PIHCA* poly(isohexyl cyanoacrylate), *PEBCA* poly(2-ethylbutyl cyanoacrylate), *POCA* poly(octyl cyanoacrylate)

the PCL-based formulations are approved by FDA and have been widely included in medical products such as sutures and bone screws. The use of PCL to design polymeric NPs has also been employed for decades. However, NP synthesis from PCL homopolymers is hampered by polymer hydrophobicity [160, 168, 169]. Hydrophobic PCL confers poor stability to NPs in water and may undergo rapid elimination from the organism. Surface modifications and functionalization of PCL NPs are necessary to address these stability issues. For example, PEGylation has been proposed to obtain more efficient PCL NPs for drug delivery [170, 171].

5.1 Brief Description of Polymer Features

PCL is a linear, aliphatic, and partially crystalline polyester [160, 161]. It is characterized by a very low melting point temperature of around 60 °C and a glass transition temperature of around −60 °C [172]. PCL is chemically composed of a sequence of methylene units and ester groups with the common molecular formulation $(C_6H_{10}O_2)_n$. It is usually obtained from the polymerization of ε-caprolactone monomers, although its polymerization from methylene-1,3-dioxepane (MDO) and 6-hydroxycaproic acid has also been described [173–175]. PCL is hydrophobic, biodegradable, and bioabsorbable. It has been widely used in medicine to construct scaffolds for tissue engineering and NPs for controlled drug release [160]. Indeed, PCL-based NPs are permeable to a wide range of drugs that are uniformly distributed into the polymeric matrix. For this reason, the synthesis of polymeric NPs from PCL has received great interest in recent years.

The physicochemical properties of PCL mainly depend on the molecular weight of the polymer and the degree of crystallization. These features will also determine the degradability of the polymer (hydrolysis of ester bonds) under physiological conditions [160]. PCL degrades into fatty acids and releases nontoxic products such as succinic acid, caproic acid, butyric acid, and valeric acid [176]. Interestingly, the PCL degradation rate can be modified via modification of the molecular weight, the degree of crystallization, or the chemical structure with hydrophilic polymers such as PEG, PLA, or poly (glycolic acid) (PGA) [177]. Indeed, the degradation kinetics of PCL has been extensively studied to synthesize NPs with different degradation rates. PCL modification by copolymerization with other polymers has been suggested as one of the major mechanisms to modulate the kinetics degradation of PCL, especially for pharmaceutical applications [174]. PCL by itself is characterized by a very slow degradation rate (between 2 and 4 years) depending on the molecular weight of the starting polymer. The degradation of PCL is slower compared to other polymers that are used to synthesize NPs for drug release. This fact allows the design of pharmaceutical formulations based on NPs for long-term drug release (up to several months). Polymers with higher hydrophilicity can be used to modify PCL chemical structure. For example, higher degradation rates are obtained when hydrophilic poly(ethyleneoxide) (PEO) is attached to PCL to form PEO-PCL copolymers [178]. Longer segments of PEO increase the rate of degradation of the

copolymer [174, 178]. PLA has also been suggested as one interesting component to copolymerize with PCL to increase its degradation rate [179]. In conclusion, PCL NPs should be functionalized with hydrophilic polymers to obtain NPs with a selected biodegradation and drug release rate.

5.2 Chemical Options for the Synthesis

PCL polymers are synthetized through different chemical reactions and from different starting monomers. ε-Caprolactone ring-opening polymerization (ROP) was the first synthetic method used to obtain PCLs [159]. Since then, ROP has become one of the most employed procedures for the synthesis of these polymers. ε-Caprolactone ROP is obtained by three main different mechanisms: anionic, cationic, and coordination-insertion ROP [173, 174] (Fig. 5a). Anionic ROP is based on the reaction between anionic species and the carbonyl group of ε-caprolactone. The ring-opening reaction induces the formation of a growing alkoxide [173]. In cationic ROP, a cationic group reacts with ε-caprolactone to synthesize positively charged molecules. Positively charged monomers will then react with another ε-caprolactone monomer to induce the ring-opening of the initial ε-caprolactone [173]. In coordination-insertion ROP, the formation of PCL is obtained through the coordination of ε-caprolactone with a metal-based catalyst. The PCL chain interacts with the metal catalyst via an alkoxide bond during the polymerization [173]. There are different types of metal complexes reported for coordination-insertion ROP in PCL synthesis such as aluminum isopropoxide and stannous octoate [173, 174]. Stannous octoate has been widely used as a reaction catalyst for the ROP of ε-caprolactone in biomedical applications due to its low toxicity and high efficiency [174]. Some authors have also suggested an alternative ε-caprolactone ROP mechanism based on enzymatic catalysis (eROP) with lipases. In this mechanism, ε-caprolactone monomers react with lipase to form a lipase-activated caprolactone complex. The activated complex then reacts with alcohol groups to obtain PCL chain elongation [173].

Most of the PCLs NPs reported in the literature are synthetized from the polymerization of ε-caprolactone through different mechanisms. However, other monomers have also been employed to obtain PCLs. For example, MDO radical ROP or the condensation of 6-hydroxycaproic acid has been used to synthesize PCL polymeric chains [173, 175] (Fig. 5b).

5.3 Main Applications in the Biomedical Field

PCLs have been widely used in tissue engineering [162–164, 166, 167] and for the synthesis of NPs for drug delivery due to the extraordinary biodegradable and biocompatibility attributes of these polymers. PCL NPs have been designed to improve the pharmacokinetics of multiple drugs for the treatment of a wide variety of

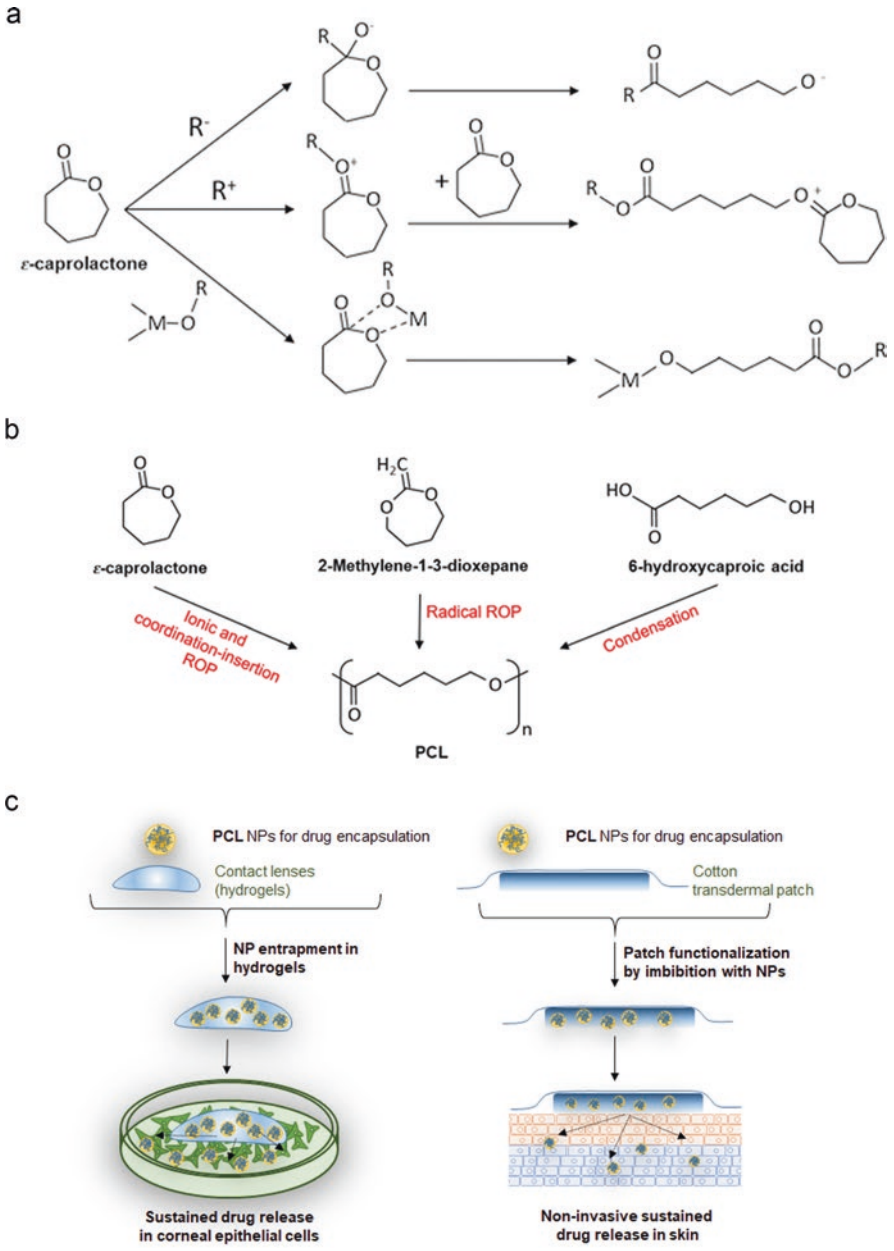

Fig. 5 Options of PCL polymer synthesis and applications of PCL NPs for long-term drug release. (**a**) Anionic (R^-), cationic (R^+), and metal (M) coordination-insertion ROP from ε-caprolactone. (**b**) Options of PCL polymer chain synthesis from different starting monomers such as ε-caprolactone, 2-methylene-1-3-dioxepane, or 6-hydroxycaproic acid. (**c**) Schematic representation of noninvasive and sustained drug release from PCL NPs incorporated in contact lenses or cotton transdermal patches. Abbreviations: ROP ring-opening polymerization, PCL polycaprolactone, NP nanoparticle

diseases, such as cancer [180–184], glaucoma [185, 186], schizophrenia [187], ulcerative colitis [188], or the encapsulation of immunosuppressive drugs [189]. PCLs have been broadly tailored with other polymers (diblock, triblock, or even pentablock) [190] to reduce hydrophobicity and improve polymer degradation. PCLs have variable degradation rates depending on their molecular structure. This offers the opportunity to exploit these properties for the design of pharmaceutical forms with variable degradation rates. Indeed, long-term degradation PCLs have been used in prolonged drug release strategies. For example, PCL nanocapsules have been employed for the long-term delivery of pilocarpine in the treatment of glaucoma [186]. The intracameral administration of PCL nanocapsules in rabbits demonstrated an effective drug release for up to 42 days [186]. Contact lenses embedded with PCL NPs have also been designed to improve topical ocular loteprednol administration [191]. PCL NP-embedded contact lenses released loteprednol up to 12 days after their incubation with rabbit corneal epithelial cells [191] (Fig. 5c). PCL NPs are also useful to encapsulate drugs such as indomethacin for topical administration and sustained drug release. Indomethacin-PCL NPs showed sustained drug release in freshly excised abdominal human skin [192] (Fig. 5c). Melatonin has also been incorporated into PCL NPs in transdermal patches to obtain a noninvasive and sustained drug release formulation [193].

Copolymerization with other types of polymers has been suggested as an alternative for the modulation of PCL degradation and the enhancement of hydrophilic properties of NPs. Some of the amphiphilic copolymers obtained from PCL chemical modifications are poly-ε-(D,L-lactide-co-caprolactone) [194], PCL-PEO-polylactide [195], PEG-PCL-LDI [196], and poly(N-isopropylacrylamide)-PCL [197]. In particular, PEGylation of PCL chains has been widely used for the design of polymer-based nanocarriers for numerous applications [195, 196, 198–200]. For example, PEG-PCL NPs have been used for the encapsulation and release of doxorubicin in breast tumor-bearing mice [200]. These NPs were linked to collagenase IV by carbodiimide bonds to promote tumor extracellular matrix degradation and more efficient drug release [200]. Doxorubicin-loaded PCL NPs have also been modified with dextran to increase the nanosystem hydrophilic properties [201].

In conclusion, PCLs offer plenty of possibilities to synthesize long-term drug delivery systems for the encapsulation of a wide range of hydrophobic drugs. Overall, researchers have been investigating for decades the optimal synthesis methods and chemical modifications to obtain biodegradable and stable PCL NPs for an effective, targeted, and long-term treatment of different diseases.

6 Poly(β-Amino Ester) Nanoparticles

Poly(β-amino ester)s (PβAEs) are cationic biodegradable polymers that were firstly described by Chiellini in 1983 [202] and some years later applied to gene delivery by Lynn and Langer [203]. PβAEs are promising non-viral gene delivery systems due to their distinctive physicochemical properties: straightforward chemical

functionalization, low size, and positive surface charge. These special features allow PβAEs the condensation of DNA into small and stable NPs, the protection from non-specific degradation, and the improvement of cellular uptake and endosomal escape [204]. Therefore, these polymers are mainly used to successfully deliver nucleic acids [205]. They are a feasible alternative to viral gene delivery systems due to their biological safety, cost-effectiveness, low immunogenicity, and larger genetic cargo [206]. PβAEs have also been applied in other biomedical areas: delivery of drugs and proteins, magnetic resonance imaging agents [207, 208], or scaffolds for tissue engineering [209].

The biocompatibility and biodegradability of PβAEs is the main benefit of these compounds compared to other available positively charged polymers (poly-cations). For example, poly-Lysine and polyethyleneimine [205] show potential cytotoxicity caused by cellular apoptosis and necrosis [210, 211]. PβAEs are easily synthetized by conjugation of amines and diacrylates, and additional amines can be incorporated to improve transfection efficiency. The presence of pH-sensitive amine groups in their structure allows buffering of acidic environments and facilitates endosomal escape through the proton sponge effect [205, 212]. Large libraries of PβAEs have been developed by the combination of different monomers [204, 213]. These libraries allow the selection of the most effective PβAEs with precise physicochemical characteristics for a specific application.

6.1 Polymer Characteristics

PβAEs are biodegradable polymers that present low cytotoxicity and good biocompatibility. PβAEs are easily degraded in aqueous conditions via hydrolysis of their backbone ester bonds to small molecular weight bis(β-amino acid) and diol products, which are nontoxic [203, 205, 209]. Their degradation rate is dependent on the hydrophilicity of the polymer at physiological pH; the more hydrophilic they are, the faster they degrade. This is because the ester bonds are more exposed and distended in an aqueous solvent [209, 214].

PβAEs interact electrostatically with the negatively charged cell membrane due to the polymer positive surface charge and this facilitates their uptake [215]. PβAE-based polyplexes can be internalized via energy-dependent endocytosis or adsorbed onto the cell membrane via physical binding [215]. PβAE-based polyplexes that are internalized via caveolae-mediated uptake do not experience endolysosomal degradation. This contrasts with the polyplexes adsorbed via physical binding [215]. PβAEs display endosomal escape via protonation at the lower pH of the endosomal compartment. This increases osmotic pressure that causes endosomal physical rupture and the subsequent release into the cytoplasm [204, 206, 216] (Fig. 6a). Screening of PβAEs libraries has shown that the increased number of secondary and tertiary amines improves the buffering capacity at low pH and facilitates endosomal escape [204, 206, 217]. The polymer pKb, given by the protonatable amines in the structure, can be tuned to influence their endosomal escape [218–220]. PβAEs are

pH-responsive polymers in a wide pH region from 3.5 to 7.2 due to the presence of ionizable moieties in the tertiary amine groups [210, 220, 221]. The PβAEs pH sensitivity is greatly affected by their solubility [220]. An increase in the hydrophobicity of the polymer backbone at physiological pH decreases pKaH in such a way that PβAE NPs dissociate in pH values under 6.5 [210, 220]. Indeed, the protonation of the tertiary amino group is essential to understand the polymer hydrophilia [221]. In conclusion, the polymer physicochemical properties affect both the pKa value and the pH sensitivity [222].

PβAEs contain many functional groups that can be chemically modified to overcome certain limitations of traditional PβAE structures. It has been demonstrated that changes in the chemical composition of PβAEs have a deep impact on transfection [219]. In fact, PβAE NPs can be functionalized with different types of ligands to target membrane receptors for cell-specific therapies [206]. The incorporation of these ligands has been accomplished by covalent attachment during polymer synthesis [223] or by the addition of the ligand after NP formation via electrostatic or hydrophobic interactions [224].

6.2 Preparation Methods

PβAEs are easily synthesized by Michael addition reaction without the generation of by-products and, therefore, without the need for further purification steps [205]. Linear PβAE polymerization occurs via the conjugate addition of amines or secondary diamines to diacrylates [209, 210, 225] (Fig. 6b). Branched PβAEs are synthesized via the conjugate addition of amines or secondary diamines to triacrylates and diacrylates [206, 217]. Their synthesis can be carried out in the absence of solvents or anhydrous solvents [203, 209]. The most common solvents used are dimethyl sulfoxide (DMSO), chloroform, and dichloromethane [209]. The main advantage of these solvents is the prevention of hydrolysis phenomena during the synthesis. In contrast, solvent-free polymerization provides other benefits such as the increase in the reaction rate and the lack of a solvent removal step [226]. The preparation of PβAEs using solvents is the most common approach worldwide. PβAEs synthetized with solvents are precipitated after polymerization in cold diethyl ether, hexane, ether, or ethyl ether and dried under vacuum [209]. Numerous types of acrylates and amines can be combined differently to prepare PβAE libraries [206, 210]. PβAEs can present different physicochemical properties including variable molecular weights, polydispersity index (PDI), hydrophobicity, surface charge, responsiveness, and tunability depending on the monomers used in the polymerization [210, 227]. Namely, PβAE transfection and toxicity properties can be adjusted by the addition of end-cap chemical groups [206] (Fig. 6b). The addition of end-cap amine groups can increase the cationic charge of PβAEs and therefore the affinity for negatively charged nucleic acids promoting enhanced cell uptake and transfection efficacy [210]. Branched PβAEs have the advantage that multiple end-groups may be chemically modified to amplify their functionality [206].

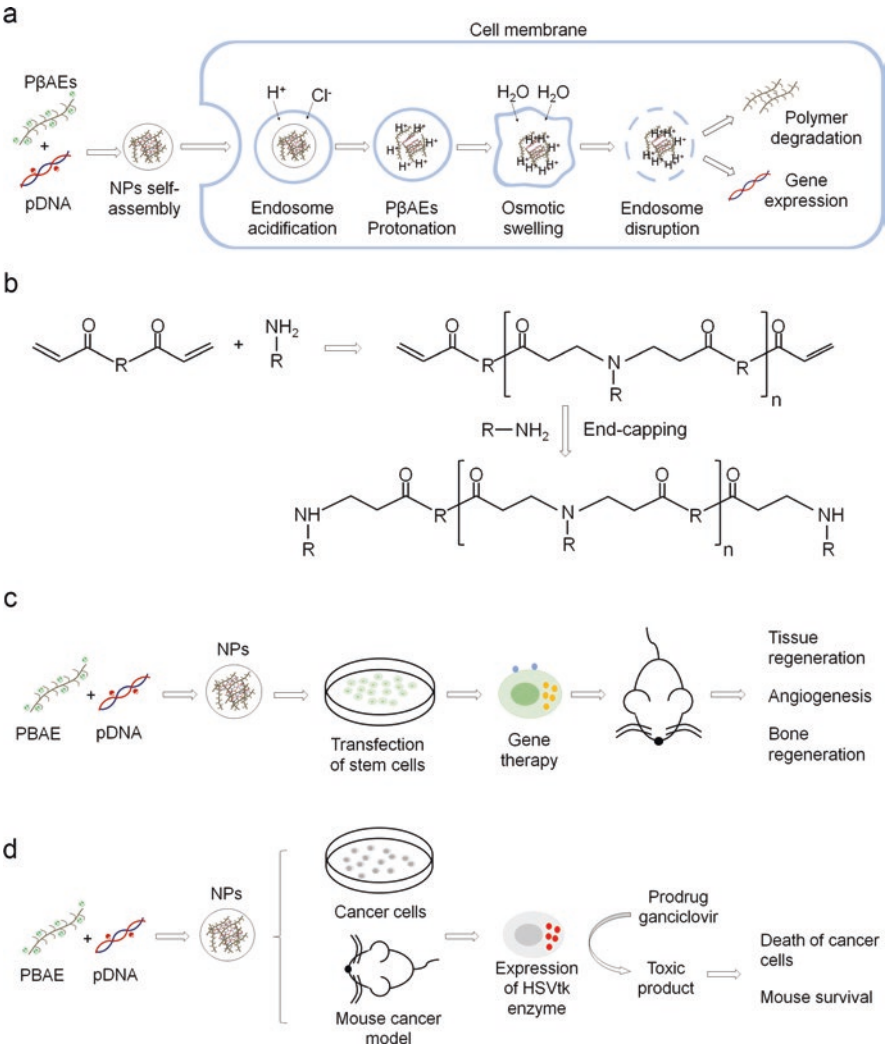

Fig. 6 PβAE NP synthesis and applications. (**a**) PβAE/nucleic acid nanoparticles self-assemble by electrostatic interactions. PβAEs escape from the endosomal cell compartment because they undergo protonation due to the acidic pH leading to osmotic pressure buildup, endosomal rupture, and release into the cytoplasm. (**b**) Example of the synthesis of linear PβAEs by conjugation of a primary amine to a diacrylate with the addition of an amine end-group. (**c**) PβAEs NPs can be used to encapsulate pDNA and achieve gene therapy in stem cells that are used for tissue engineering. (**d**) PβAEs NPs can be used to deliver a pDNA encoding herpes simplex virus type 1 thymidine kinase (HSVtk) to kill cancer cells via conversion of ganciclovir into a toxic product that induces cancer cell death. Abbreviations: PβAEs Poly(β-amino ester)s, NPs Nanoparticles, pDNA plasmid DNA, HSVtk herpes simplex virus type 1 thymidine kinase

The formation of NPs is driven by electrostatic interactions between the positive polymer chains and the negatively charged biomolecules without any chemical

modification [210]. For example, the formation of PβAE/nucleic acid NPs is based on self-assembly between cationic PβAEs and the anionic nucleic acid [206]. It has been demonstrated that polymer molecular weight, polymer structure, polymer chain end-group, and polymer/DNA ratio affect gene delivery efficacy [226]. Furthermore, other methods such as solvent displacement have also been used to efficiently synthesize PβAE NPs [228].

6.3 Applications in Targeted Cell Delivery of Nucleic Acids

PβAE NPs have been used as gene, drug, and protein delivery systems and as magnetic resonance imaging agents [207, 208] (Table 2).

PβAE NPs have been used for gene therapy in stem cells to generate customized cell implants useful for tissue engineering and regenerative medicine (Fig. 6c). For example, mesenchymal stem cells (MSC) have been transfected with PβAE NPs that encapsulated a plasmid DNA encoding and expressing VEGF [229]. Scaffolds seeded with these engineered MSC promoted angiogenesis in a mouse ischemic model. Other authors used PβAE NPs to induce CXCR4 expression with a plasmid DNA in stem cells resulting in improved tissue regeneration and angiogenesis in a mouse hindlimb ischemia model [230]. In other studies, PβAE NPs were used to modify stem cells to promote bone regeneration [231, 232]. These investigations showed that implants of adipose-derived stromal cells modified with plasmid PβAE NPs increased the levels of platelet-derived growth factor-BB and stimulated robust osteogenic effects [231]. Other groups have described the use of PβAE NPs to modify gene expression profiles in stem cells to treat cancer [233]. Interestingly, PβAEs can also be used for cell reprogramming. Primary human astrocytes transfected with PβAE NPs containing pDNA-Sox2 or pDNA-Olig2 have shown full conversion to neurons or oligodendrocyte progenitors, respectively [234]. Another group reprogrammed induced pluripotent stem cells from human fibroblasts using PβAE NPs containing a DNA plasmid expressing the Yamanaka factors [235]. Apart from pDNA delivery, other studies have also shown the effectiveness of PBAE NPs to condense siRNA [236, 237].

PβAE NPs have also been used as delivery systems for antineoplastic drugs. Potineni et al. synthesized a pH-sensitive hydrophobic PEO-modified poly-1 PβAE NPs to deliver paclitaxel to tumors [228]. These NPs were pH-sensitive and effectively released the drug into the low pH environment of the solid tumor. Tang and coworkers developed a pH-sensitive amphiphilic PβAE NPs for co-delivery of chemotherapeutic drugs and genes to treat drug-resistant breast cancer with high efficiency [238]. The chemotherapeutic agent doxorubicin was encapsulated in the hydrophobic core that was pH-sensitive. Short-hairpin RNA (shRNA) targeting the survivin gene (shSur) was incorporated within the hydrophilic shell forming a co-delivery NP to inhibit tumor growth. Another approach to treat cancer was tested by Mangraviti [239]. They designed PβAE-based NPs to deliver the suicidal gene

Table 2 Applications of PβAE-based NPs

Application	Payload	Cells or animal models	Outcome	Reference
Gene therapy of stem cells	VEGF pDNA	Mouse ischemic model	Angiogenesis	[229]
	CXCR4 pDNA	Mouse hindlimb ischemia model	Tissue regeneration	[230]
	PDGF-BB pDNA	Murine calvarial defect model	Bone regeneration	[231]
Cell reprogramming	Sox2 pDNA	Primary human astrocytes	Conversion of astrocytes into neurons	[234]
	Olig2 pDNA	Primary human astrocytes	Conversion of astrocytes into oligodendrocytes	[234]
	pDNA of Yamanaka factors	Human fibroblasts	Reprogramming of iPS cells from human fibroblasts	[235]
Cancer	Doxorubicin + shSur	Mice model	Inhibition of tumor growth	[238]
	HSV-tk pDNA	Malignant glioma model	Death of cancer cells in vitro and increased survival in treated animals	[239]
	HSV-tk pDNA	Small lung cancer cells	Death of cancer cells	[240]
	p53 protein pDNA	Rat brain tumor models	Increased survival in treated animals	[241]

Abbreviations: VEGF vascular endothelial growth factor, *pDNA* plasmid DNA, *CXCR4* C-X-C motif chemokine receptor 4, *PDGF-BB* platelet-derived growth factor BB, *siRNA* small interference RNA, *iPS* induced pluripotent stem cells, *Sh* short-hairpin, *Sur* survivin gene, *HSV-tk* herpes simplex virus thymidine kinase

herpes simplex virus thymidine kinase (HSV-tk) in a malignant glioma model. These NPs induced death in cancer cells in vitro in combination with the prodrug ganciclovir and, indeed, increased survival in treated animals (Fig. 6d). The same strategy was also used by Kim and colleagues in small cell lung cancer cells [240], and the same results were obtained by another group that used PEG-PβAE NPs to deliver pDNA encoding the tumor suppressor p53 in malignant brain tumors models [241].

7 Polymeric Nanoparticles from Natural Compounds

Polymeric materials can be classified into natural or synthetic by their origin. In this section, we will focus on natural polymers as a great alternative to synthetic polymers because they are easily obtained, renewable, and biosafe. Natural polymers are abundant in nature and can be obtained from animals, plants, bacteria, or fungi

[242]. They present unique characteristics such as biocompatibility, biodegradability, low immunogenicity, tunable properties, and straightforward preparation methods [243]. They can be classified as polysaccharides and protein-based polymers [242]. Polysaccharides are long chains of polymeric carbohydrates composed of monosaccharide repeating units linked by glycosidic bonds [244]. Polysaccharides can be obtained from plants (guar gum, pectins, and cellulose derivatives [245]), animals (chitosan, chondroitin, and hyaluronic acid (HA)), algae (alginate), and microorganisms (dextran and xanthan gum) [244]. The main polysaccharides used for the synthesis of drug delivery systems are chitosan, dextran, alginates, and hyaluronic acid. Protein-based polymers are constituted by amino acids linked via peptide bonds [246] and can have two origins: animals (collagen, gelatin, albumin, silk protein, keratin) or plants (zein, gliadin, soy protein) [247].

The main characteristics of natural polymers highlight the extraordinary potential of these compounds to be valuable candidates for NP formation. Natural NPs present different physicochemical properties depending on the chemical structure [248]. For example, polysaccharides and protein functional groups can be easily chemically modified resulting in a wide array of derivatives with interesting properties [244]. Natural polymeric NPs have been widely applied in agriculture, food processing, and the biomedical sector [243]. One natural polymeric NP called Abraxane has been approved by the FDA [249]. Abraxane is an albumin NP that encapsulates and delivers paclitaxel to treat cancer [249–252]. No other natural polymers have been approved by the FDA up to date although some of them such as chitosan, zein, and gelatin are identified by the FDA as Generally Recognized as Safe (GRAS) polymers [242, 253].

7.1 From Food and Biological Components to Drug Delivery Systems

Polysaccharides are used for the synthesis of drug delivery systems due to their biological safety, biodegradability, biocompatibility, and good stability [244]. Some of them also present mucoadhesive properties that permit their adherence to the mucus layer of epithelial surfaces thereby enhancing the drug bioavailability [254, 255]. In addition, positively charged polysaccharides such as chitosan can open the tight junctions between epithelial cells and increase the paracellular permeability and drug uptake [256, 257]. Chitosan is a linear cationic polysaccharide obtained by alkaline deacetylation of chitin, which is mainly extracted from the exoskeleton of crustaceans [258–260]. Their cationic nature is of great interest for the delivery of nucleic acids [248], and their amino groups provide pH sensitivity [261]. Alginate is a hydrophilic polymer extracted from marine brown algae or soil bacteria [262]. Alginates can form gels by reaction with divalent cations to encapsulate biomolecules [263, 264] and have pH sensitivity at alkaline conditions opposite to chitosan [265]. Dextran is a water-soluble polysaccharide composed of a linear chain of D-glucose and synthesized by either lactic acid bacteria or enzymatically [266].

Other polysaccharides such as hyaluronic acid have natural cancer cell targeting properties and are easily degraded by hyaluronidases [242]. Hyaluronic acid is a linear anionic glycosaminoglycan that is the main component of the connective tissue and synovial fluids of all vertebrates [267].

Protein-based polymers are biocompatible and biodegradable and have tunable properties for the design of polymeric NPs [246]. Most proteins are amphoteric. Their charged functional groups can become cationic or anionic depending on the pH and the protein isoelectric point (pI) [253]. Keratin proteins are negatively charged and are mainly used for the delivery of positively charged molecules. They are pH-sensitive proteins due to a large number of carboxyl groups [246, 268]. Elastin is obtained from tropoelastin. Elastin NPs display interesting thermoresponsive properties for bone morphogenetic protein (BMP) release [269, 270]. Gliadin shows mucoadhesive properties that facilitate adherence to the mucus membranes and enhance NP absorption [271]. Albumin has great properties as a drug carrier and transport of protein-derived NPs [272]. Gelatin shows great versatility and simple synthesis through collagen hydrolysis [273]. Two types of gelatins can be produced based on collagen hydrolysis resulting in proteins with different pI, molecular weight, amino acid composition, and viscosity [253, 274]. Type A gelatin is positively charged and extracted through an acidic process whereas type B gelatin is negatively charged and is processed under alkaline conditions [275]. This means that different gelatin NPs can be synthesized to encapsulate either positive or negative biomolecules. Other proteins such as zein and silk fibroin also show great properties and are great candidates for the synthesis of NPs [246, 276].

7.2 Synthesis of Nanoparticles from Natural Compounds

Polysaccharide-based NPs are mainly prepared by four mechanisms: covalent cross-linking, ionic cross-linking, polyelectrolyte complexation, and self-assembly [244, 277] (Fig. 7a). Ionic cross-linking consists of the interaction between charged polysaccharides and oppositely charged ions or polymers (polyanions and polycations) [278, 279]. Ionic cross-linking is a procedure simpler than covalent cross-linking, but it is limited to charged polysaccharides. In contrast, covalent cross-linking can be applied to neutral polysaccharides but requires complex conjugation chemistry and vigorous reaction conditions [244, 280]. To date, the most widely used ionic cross-linkers are divalent calcium ions and polyanion tripolyphosphate [278, 279]. Some covalent cross-linkers are glutaraldehyde [281, 282] and di- or tricarboxylic acids [280, 283]. Another method used to synthesize polysaccharide-based NPs is polyelectrolyte complexation. In this technique, polysaccharides with opposite charges can interact electrostatically and form polyelectrolyte complexes (PEC) [280, 284]. Most PEC NPs have been prepared using positively charged chitosan and negatively charged HA and alginate [280, 285]. A different method includes polysaccharide modifications by hydrophobic molecules (lipophilic molecules, fatty acids, cholesterol, bile acids, and oligomers) to

synthesize amphiphilic polysaccharides that can self-assemble to constitute NPs [244, 277, 286]. Other studies have also demonstrated that polysaccharide-based NPs can also be prepared by different multi-step methods of emulsification and subsequent NP formation [287].

Protein-based NPs can be synthesized using different approaches: desolvation, simple and complex coacervation, emulsification, and spray-drying [243, 246, 253]. In the desolvation process, a desolvating agent like alcohol or acetone is added to an aqueous solution of protein under stirring leading to protein dehydration and aggregation [288]. In the method of simple coacervation, environmental factors such as ionic strength, pH, and temperature are modified to reduce the protein solubility and

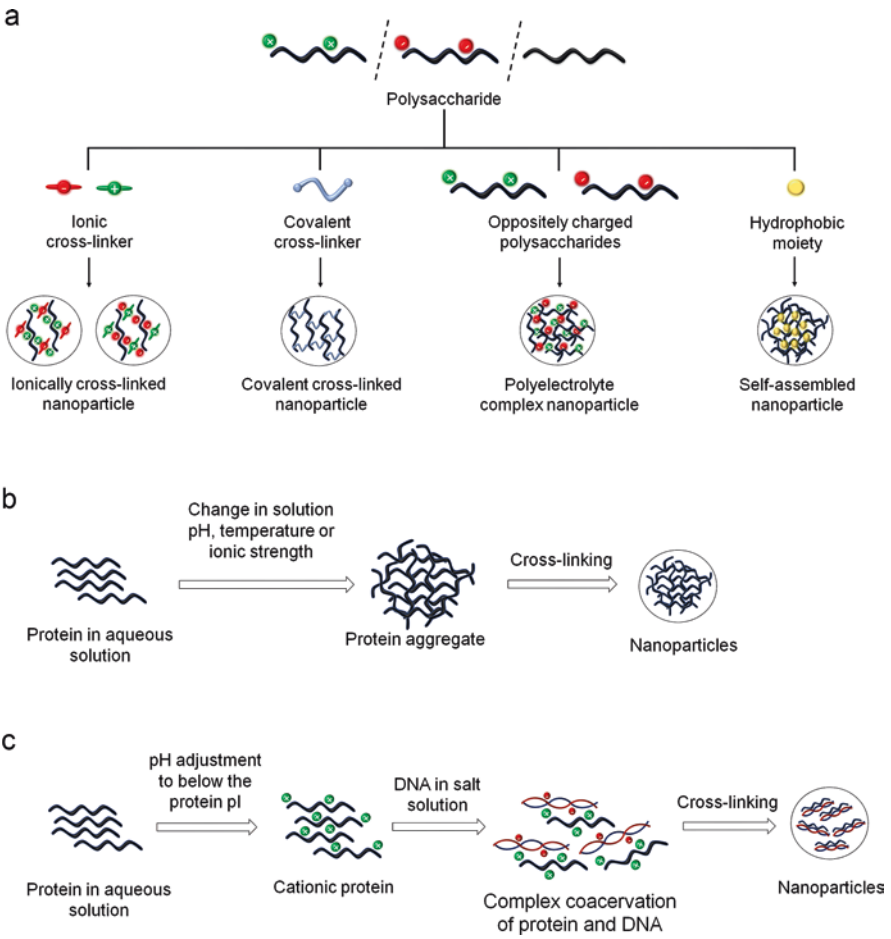

Fig. 7 Natural polymeric NP synthesis. (**a**) Schematic illustration of different strategies employed to prepare polysaccharide-based nanoparticles: covalent cross-linking, ionic cross-linking, polyelectrolyte complexion, and self-assembly. (**b**) Preparation of protein nanoparticles by coacervation. (**c**) Preparation of protein nanoparticles by complex coacervation method. Abbreviations: pI isoelectric point

promote phase separation [243, 246, 247] (Fig. 7b). In both techniques, NPs are ultimately synthesized by cross-linking. Complex coacervation is a method ideally suited for DNA entrapment. In this method, the pH is adjusted below the pI of the protein to promote the protonation and positive charge in the protein to interact electrostatically with other polyelectrolytes or DNA [253, 274] (Fig. 7c). Protein NPs can also be prepared by emulsification [253] or spray-drying. In this latter technique, a protein solution is sprayed out of a nozzle, and the contact with a hot drying gas induces moisture evaporation and NP formation [289, 290].

7.3 Commercial Formulations and Use in Medicine

Polysaccharide-based NPs are potential candidates for several biomedical applications. Chitosan NPs (CSNPs) have received increasing attention as gene delivery vehicles due to their cationic charge. CSNPs have been widely used as pDNA [291] and siRNA delivery vehicles [292]. CSNPs have been mainly used to treat cancer [293], inflammatory diseases [294–296], cerebral diseases [297–299], and infectious diseases [300, 301]. Chitosan can also be used in combination with alginate to create polyelectrolyte complexes [302]. Indeed, chitosan-alginate NPs (CANPs) have demonstrated great potential as candidates for DNA delivery [284, 303, 304], encapsulation of chemotherapeutics [305, 306], and insulin [307], among other drugs [308, 309]. Other polysaccharide-based NPs such as HA-NPs have been reported to selectively target tumor cells because many cancer cells overexpress HA-binding receptors such as the cluster of differentiation (CD) protein CD44, lymphatic vessel endothelial (LYVE)-1 receptors, and receptors for HA-mediated motility (RHAMM) [310]. Therefore, HA-functionalized nanocarriers have been mainly used in drug delivery [311, 312], gene delivery [313, 314], and diagnostic agents [310, 315] in cancer therapy. HA-NPs loaded with both doxorubicin and siMDR-1 can sensitize drug-resistant tumor cells and suppress tumor growth in vivo [316]. Finally, dextran NPs are stimuli-responsive and have been used to deliver siRNA [317], doxorubicin [318, 319], or insulin [320], among others [321].

Protein-based NPs offer a wide range of applications in medicine as both drug delivery vehicles and bioimaging carriers [322, 323]. Abraxane is an albumin NP that encapsulates and delivers paclitaxel and has been officially approved by the FDA [249–252]. Paclitaxel has also been effectively delivered to treat cancer by gelatin NPs [324]. Other antineoplastic drugs such as doxorubicin have been successfully delivered by silk fibroin [325], elastin [326], and keratin [327] NPs to cancer cells. Zein NPs have been used to simultaneously transport antineoplastic drugs and diagnostic agents [328]. Protein-based NPs have also been used to deliver bioactive molecules such as VEGF [276], antibiotics [329], antifungals [330], insulin [331], and nucleic acids. They have also been employed as plasmid DNA delivery vehicles [332–334] or more recently as a novel siRNA delivery system to treat cancer [335]. Furthermore, protein-based NPs have unique chemical properties for enhanced local delivery upon physical or chemical stimulation. Silk fibroin [325]

and keratin [327] NPs have been reported to present a pH-sensitive delivery of doxorubicin. Elastin NPs have shown thermoresponsive properties that allow the delivery of cytokines such as BMP-2 and BMP-14 [270]. Finally, gliadin NPs have been used as a bioadhesive delivery system for oral drug administration [336, 337].

8 Status and Prospects on the Use of Polymeric Nanoparticles in Medicine

Polymeric NPs are biocompatible and biodegradable colloidal systems that can be synthesized using different methodologies to obtain a wide array of chemical structures. The surface of these NPs may be functionalized to confer targeted drug and gene delivery properties using bioactive molecules or other polymers. The main advantages obtained via functionalization of polymeric NPs for targeted drug or gene delivery can be summarized as follows: reduction of adverse side effects; efficient drug and acid nucleic protection from degradation; and escape from the mononuclear phagocytic system. This chemical versatility and the many possibilities of functionalization allow the development of a wide catalogue of pharmaceutical formulations. The profound complexity of polymeric NPs emphasizes the need for an interdisciplinary team of scientists including biologists, physicians, and chemists to further elucidate optimal designs.

The administration of polymeric NPs has been mainly studied in intravenous or oral pharmaceutical formulations. However, other formulations that contain polymeric NPs have been designed in recent years to allow controlled drug release, such as nasal sprays, contact lens hydrogels, or dermal patches. In the future, we foresee drugs also incorporated into polymeric NPs for sustained release from medical devices such as stents, subcutaneous implants, or orthopedics. This scenario opens a window in medicine toward a much more precise and personalized therapy.

Since their first use in the 1970s, polymeric NPs have been extensively studied in vitro, in vivo, and ex vivo for many applications related to site-specific delivery, and yet, only PLGA NPs have been approved by FDA for drug delivery. Other polymer materials have been approved by FDA for other applications such as sutures, surgical glues, or tissue scaffolds. It is therefore expected and necessary that more polymers are accepted by FDA in the forthcoming future to apply novel drug delivery systems in clinics. The expansion of the use of these NPs may represent a deep impact on the management of a multitude of diseases, potentially becoming key components of drug and acid nucleic delivery in the future.

9 Conclusions

In this chapter, we described the general characteristics, main synthesis methods, and principal applications of the most common polymeric NPs used to encapsulate drugs and nucleic acids in the biomedical field. PLGA is the only group of polymers approved by the FDA for human use in formulations for sustained drug release and has been studied for vaccine delivery. PU NPs have great synthetic versatility and have demonstrated excellent fulfillment to target and treat endothelial and cancer cells. PACA NPs are particularly efficient for the encapsulation of chemotherapeutic agents and bioactive peptides to cross the blood-brain barrier. PCL NPs have been used for long-term drug delivery. PβAE NPs display great synthetic versatility and are mainly used for gene delivery. Natural compound-derived polymeric NPs are a good alternative to synthetic polymers since they are renewable and easily obtained from nature (animals, plants, bacteria, or fungi). All of the polymeric NPs described here display high biocompatibility and biodegradability and can be easily combined with other biomolecules or polymers for targeted drug or gene therapies. Indeed, polymeric NPs offer extraordinary advantages over other NPs in terms of the potential chemical modifications and the vast number of applications (targeted therapies, controlled drug release, tissue engineering, medical devices) that can be exploited in different fields of medicine.

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Magnetic Nanoparticles for Diagnostic and Therapeutic Applications

Parameswaran Kiruthika Lakshmi, Chockaiyan Usha, Selvaraj Meenakshi, and Muthupandian Saravanan

1 Introduction

Nanobiotechnology is an evolving tool for the development of eco-friendly and reliable methodology for the fabrication of nanoscale materials. Nanoparticles attract increased attention due to their usage in diverse areas of medical science and technology. The interaction between nanoparticles and biological structures is the most exciting component of research. Designing an eco-friendly process for the fabrication of nanoparticles is one of the key steps in the area of nanotechnology research [1].

Magnetic nanoparticles (MNPs) have been found to be the most fascinated area due to their unique magnetic properties [2]. The MNPs have developed as an effective tool in biomedical arena due to its unique potentials. MNPs are also employed in medical diagnosis in magnetic resonance imaging as agents for enhancing contrast.

MNPs have unique properties such as uniformity, size, increased surface area and biocompatibility [3], superparamagnetism, and magnetic moment that can be customized for application in varied fields [4]. These outstanding properties make MNPs suitable to be used in the separation of biomedical components, hyperthermia, catalysis, imaging, magnetic target drug delivery diagnosis, and biosensing (Fig. 1).

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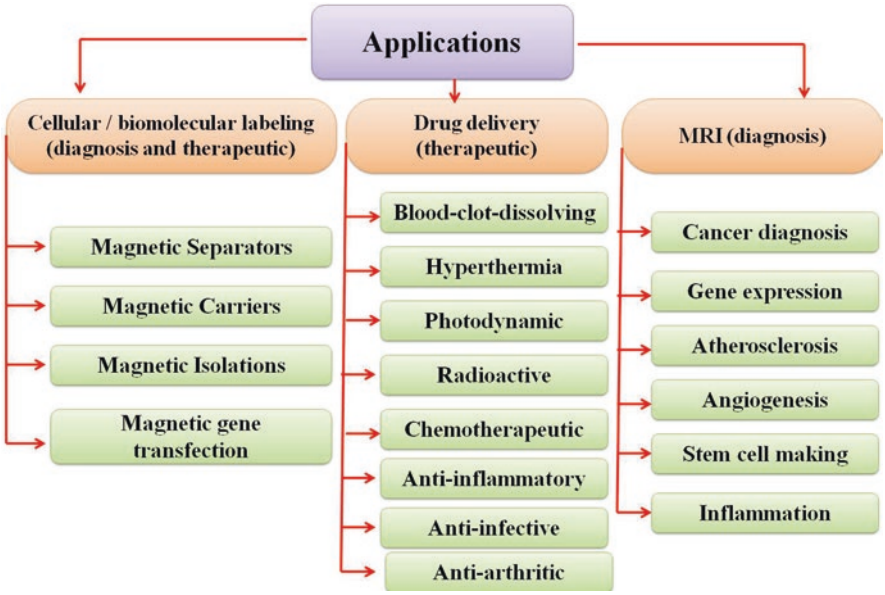

Fig. 1 Biomedical applications of MNPs

To employ MNPs for biomedical applications, parameters such as stability, size less than 100 nm, superparamagnetic behavior, and magnetic saturation should be considered. Further, MNPs must be layered with a protective coating which must be safe and biocompatible. The layer on the surface of MNPs will aid in avoiding accumulation, deposition, and biodegradation of MNPs, to confirm steadiness [5]. Modification of MNPs is reliant on specific diagnostic and biomedical applications.

MNPs are intended for the specific distribution of drugs in the incidence of the magnetic field [6]. MNPs exhibit increased magnetic sensitivity and superparamagnetic behavior in the absence of a magnetic field [7]. Several metals (Fe, Ni, Co, Au, and Ti) and metal oxides are used to synthesize MNPs [8]. Among them, magnetite is extensively used for therapeutic applications [9]. MNPs are used as a tool for diagnosis in medicine. MNPs are attached to a specific molecule of interest that results in the generation of magnetic signals which can be sensed in the incidence of magnetic field using a magnetometer.

Researchers investigated and reported the superparamagnetic property, zero coercivity and colloidal stability of maghemite nanoparticles [10]. Nanotubes and nanorods are also under research for applications in drug delivery. Among the various types of nanorods, iron oxide nanorods have fascinated consideration owing to their superparamagnetic property [11] with controlled release of nanoparticles and biocompatibility [12]. Nanotubes are widely used in the loading of large amounts of bioactive compounds in the inner cavities, while the outer surface is functionalized with the target molecule of interest [13].

Nanotubes of magnetic nature associate the magnetic properties allowing a differentialfunctionalization of inner and outer surfaces, which was reported to have potential applications in drug delivery and bioimaging [14]. Iron oxide nanotubes act as carriers in the designing of anticancer drugs and are also reported to be internalized in human cancer cell lines [15]. In the context of the current drive, this chapter describes ways of synthesizing MNPs and the usage of MNPs in the biomedical arena.

2 Designing MNPs

MNPs display a positive response to the magnetic field. In this context, many researchers proposed various types of MNPs with diverse chemical configurations for biomedical applications. To improve physical and magnetic properties, various parameters such as size, morphology, composition, and surface chemistry can be custom-made [5].

MNPs should be stable at pH 7 in a physiological environment to be used in therapeutic and medical diagnosis. Further, the stability of colloids will depend on the surface chemistry and charge resulting in steric and coulomb repulsions [16]. Generally, to use NPs in therapeutic use, they must be layered with a biologically compatible polymer to hurdle biodegradation and accumulation while they are subjected to the magnetic field. The medicine will adsorb to the NPs [17].

The two major properties of MNPs used for therapeutic purposes are nontoxicity and non-immunogenicity. Further, MNPs must be very small to sustain blood circulation and travel through the capillary system. To regulate the movement of MNPs in the blood vessels, the MNPs should have increased magnetization with an external magnetic field [18]. The fabrication method of MNPs can describe the size, shape, and the chemistry of the surface of the NPs [19].

Properties of Magnetic Nanoparticles

The performance of substances under an effect of an external magnetic field is evaluated in terms of susceptibility and permeability. Susceptibility designates the magnetization level of MNPs in the incidence of the magnetic arena. The permeability specifies the magnetic orientation persuaded by the magnetic arena. The particles with increased permeability expose a decreased confrontation in reaction to the magnetic arena [20]. Particles are categorized into diamagnetic, paramagnetic, and ferromagnetic based on their vulnerability to the magnetic arena.

Diamagnetic particles in the incidence of external magnetic arena induce weak magnetic moment. When the external arena is detached, the spins return to the original site with very few magnetic features. The key property of the diamagnetic material is that they are filled with subshells of electrons [21]. Paramagnetic particles have a weak magnetic arena analogous to the applied external arena. The vulnerability of the paramagnetic substances was found to be positive in the range of (10^{-5} to10^{3}) [22]. Ferromagnetic particles are also known as magnets. Magnets have a

huge and positive vulnerability. The vulnerabilities of ferromagnetic particles are evaluated by the external arena, temperature, and atomic structures. Unlike the diamagnetic and paramagnetic particles, the magnetic properties of ferromagnetic particles are determined to even after the exclusion of the magnetic arena. When the magnitude of the magnetic arena is decreased, the reduction of total magnetization occurs due to the recurring spins to their initial directions [23].

2.1 Iron-Based MNPs

Iron oxide NPs have been extensively used by researchers as it is a ferromagnetic substance and retains magnetization property even after the removal of the magnetic field. Iron oxide NPs (Fe_3O_4, Fe_2O_3) have been extensively used in in vitro diagnostic and therapeutic applications. Various procedures are available to synthesize magnetic NPs like nanorods, nanowires, and nanocubes that are fabricated by various synthesis methods such as "bottom-up" approaches.

Iron oxide NPs range from 1 to 100 nanometers. Iron oxide NPs have gained significance due to their magnetic properties; unique physical, chemical, thermal, and mechanical properties; and usage in the biomedical field. Magnetite is one of the most common iron oxide materials used in therapeutic applications. Magnetite NPs have been employed in many biological applications due to their biocompatibility and less toxicity to the human system [24]. Magnetite NPs are employed as vehicles in drug delivery and immunoassays.

In the magnetic resonance imaging (MRI) technique, iron oxide NPs are used in cell imaging to distinguish between healthy and abnormal cells [25]. In drug delivery, iron oxide NPs are used as vehicles. The drugs stand on the surface of the NPs. In an applied external magnetic arena, the NPs are motivated to the chosen region in the human system, and the drug-coated on the NPs gets released. This process permits the saving of the wastage of drugs as it is site-specific [26].

Magnetic iron oxide NPs are used in hyperthermia. Superparamagnetic NPs can be used to heat cancer cells to 41–45 °C under the influence of a magnetic arena. The injury to normal cells is reversible, but the injury to tumor cells is irreversible [27]. Magnetic iron oxide NPs with polymer overlays are also used in the separation of cells and the purification of protein molecules.

2.2 Cobalt-Based MNPs

Cobalt-based NPs (CoB NPs) are with high paramagnetic properties and appeal as hard magnets. Naturally, cobalt is a ferromagnetic metal, when the particle size is minimized to the nanometer range, cobalt acts as a superparamagnetic material [28]. Another distinguishing property of CoB NPs is that it confers "smart" qualities and hence it is widely used in various biomedical approaches. CoB NPs are also

under examination for the prospect of gaining multiple signals through functionalization with fluorescent dyes. Apart from the superparamagnetic signal, high sensitivity emission signals were also detected, and hence CoB NPs are been used in imaging techniques [29]. Research conducted in rats revealed that paramagnetic signals emanated from CoB NPs can be enhanced by functionalizing the CoB NPs with Ga^{68} and luciferase [30].

2.3 Nickel-Based MNPs

Nickel-based MNPs are used in various arenas such as solar cells, tissue engineering, and medical applications [31]. Nickel- and copper-based MNPs are stable and biocompatible and display magnetic properties [32]. The nickel ferrite ($NiFe_2O_4$) is a well-known spinel magnetic material prepared by solid-state reactions in which the solid reactant is heated to produce a new solid composition [33]. Nickel ferrite NPs possess appealing properties such as low Curie temperature as the particle size decreases, high electrical resistivity, and low eddy currents losses which make them suitable in the field of medicine [34].

Researchers [35] analyzed the magnetic characteristic and cytotoxic properties of NiCu NPs. Further, the researchers also evaluated the cytotoxic and magnetic properties of NiCu NPs fabricated by the mechanical milling method. Another research group [36] examined the antibacterial activity of Cu, Ni, and NiCu MNPs for possible use in the dentistry field. The antibacterial activity of NiCu NPs was assessed on the human pathogens *Staphylococcus aureus* (gram-negative) and *Escherichia coli* (gram-positive), as well as in *Streptococcus mutans* (dental pathogen). NiCu NPs have increased potential to be used in cancer therapy and are also carriers for anticancer drugs. Such collective treatments have boundless application in cancer therapy.

3 Synthesis of MNPs

Various noteworthy efforts have been devoted to the synthesis of state-of-the-art and constant methods for the fabrication of MNPs. Accuracy in fabrication and functionalization of the MNPs is perilous as it disturbs the physicochemical characteristics and stability [7]. Generally, the fabrication of MNPs is classified into (a) physical methods, (b) chemical methods, and (c) biological methods [37]. The physical methods consist of a top-down approach in which fabrication starts from bulk and reduces to synthesize NPs. Whereas, chemical and biological methods follow a bottom-up approach, where the particles are accumulated to produce NPs.

A comparative representation of physical, chemical, and biological methods for the fabrication of various kinds of NPs is depicted in Fig. 2. When compared to physical methods, the chemical method of synthesis is reported to be appropriate as

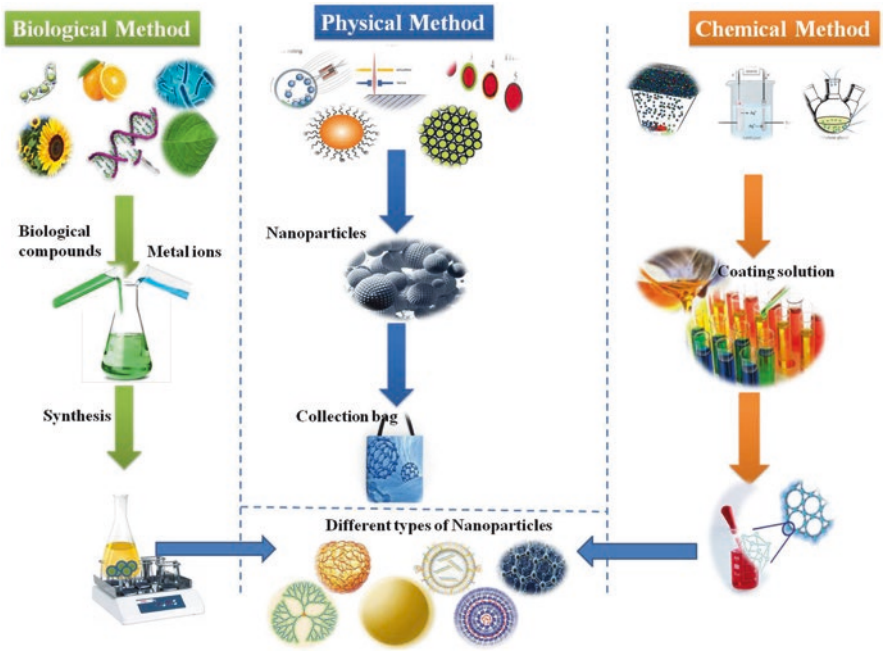

Fig. 2 Comparative representation of the synthesis of nanoparticles

they have extensive application. Nevertheless, numerous contrary imprints of the chemical methods of NP synthesis have been described, as lethal materials might get adsorbed on the surface of NPs [38]. Major disadvantages of the physical and chemical method of NP synthesis include the high rate and use of hazardous chemicals which are hazardous to the environment and humans. Furthermore, owing to technical developments, these methods (physical and chemical) are not much favored. Hence research is engrossed in the green synthesis methods of NPs.

Green synthesis methods are more preferred as they are biological/natural, for instance, the reducing agent in the chemical method is a synthetic solution [39]. Henceforth, there is a pressing necessity to advance the fabrication of NPs through gentle ways with the environment, in which lethal chemicals are not used and hence it gains significance owing to their eco-friendly features (Fig. 3) [40].

Physical Method

Ball milling is a lucrative, consistent, reproducible method used to fabricate nanoparticles. This method holds many pros such as low cost, simple procedure, and consistency. However, disadvantages such as contamination and making of asymmetrical-shaped NPs are associated with this method.

The ball milling technique is used to make powders into fine atoms [41]. In general, a ball mill comprises a container that interchanges around the axis and balls of a ball mill. The powder is treated by the interaction and friction of the balls with the walls of the container. The ball milling procedure relies on the energy released from

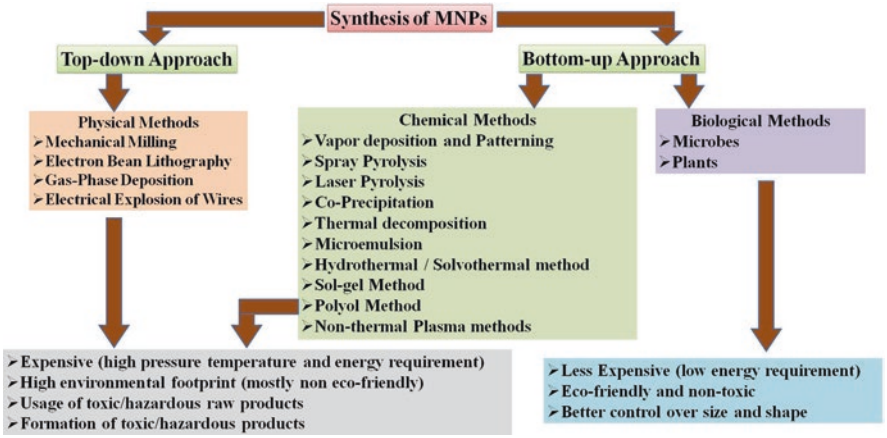

Fig. 3 Pros and cons of the various methods of synthesis of nanoparticles

the impact of balls and the powder. Finally, it results in the production of fine particles. Based on the collision of the balls and interaction with the container of the ball mill, the process is classified as vibration mill, planetary mill, and magneto-ball mill. Among these methods, the planetary mill is the common one because it is used to reduce the size of the particle. The gas-phase deposition is another physical method used for the synthesis of nanoparticles. The gas-phase deposition process comprises a catalyst-assisted chemical vapor method of molecular forerunners on a matrix comprising alumina and gold leading to the formation of nanostructure [7]. Many researchers have used the gas-phase synthesis method for the fabrication of MNPs [42].

Chemical Method

In spray pyrolysis, the fabrication of NPs is completed by the addition of chemicals onto the reactors for evaporation of the solvent. The solute is reduced inside the aerosol prospered by interruption of the particles at high temperatures [43]. Earlier findings have reported that the magnetization of MNPs gets increased by the spray pyrolysis process [44]. The laser pyrolysis process includes the use of heat through carbon dioxide laser on gases for the start and nourishment of chemical reactions. This method encourages the heating and freezing outcome of the precursors to influence reactions [45]. This process progresses the biological and morphologic features such as increased surface area, crystalline particle, stability and electrical conductivity [46], and magnetic saturation attaining up to 70 emu/g [47]. The co-precipitation method is known to be the utmost precise way of fabricating SPIONs (superparamagnetic iron oxide nanoparticles) which have an average diameter of <50 nm. This method involves a chemical process happening in an aqueous monophasic liquid medium [48]. The annealing temperature plays a vital role in the process of magnetization of the MNPs [49].

Biological Method

The biological method of fabrication of NPs is gaining attention as it is eco-friendly and could be scaled up for further studies. The restoration of the environment to its natural and pristine state over the use of green technology has expanded rapid impetus. Microorganisms and plants propose innumerable habits for the fabrication of MNPs due to their diversity in genes [50]. In general, microorganisms yield intra-/extracellular inorganic products, which are mostly in nano sizes and have clear morphologic features [51]. Besides, the use of parts of the plants such as leaves, stems, and flowers has developed as a real substitute for the fabrication of metal NPs [52]. These processes are mostly preferred as they offer increased yield, better reproducibility, and an increased degree of control over the required nano size and features of the synthesized NPs [53].

4 Physicochemical Characterization of MNPs

The nanoscale dimension of the MNPs determines their physical and chemical characteristics which makes them highly efficient and beneficial for a broad range of biomedical applications. Physicochemical properties of MNPs like shape, size, thermal, optical, magnetic, and electrical properties are the key factors influencing the suitability of MNPs for biomedical applications [54]. The superparamagnetic behavior of MNPs is size-dependent. Hence, tuning of MNPs within the size limit is essential for preserving their characteristics. For instance, the optimal size for intravenous administration of therapeutic MNPs ranged between 10 and 100 nm [37]. The geometric structure of MNPs has been reported to affect the characteristics and efficiency of MNPs. The architecture and composition of nanocomposites have a direct influence on saturation, magnetization, and structural deformity. MNPs can be synthesized in various forms like rods, spheres, wires, disks, triangles, gels, cubes, cages, polyhedrons, etc. Reduction in the dimension of MNPs offers a high surface-to-volume ratio [55]. A higher aspect ratio is needed for surface functionalization and cellular internalization of MNPs. The higher the ratio, the greater will be the surface area and extended half-life in the bloodstream.

The magnetic property of the MNPs is directly influenced by the shape and composition of the MNPs like Au-MnO flower-shaped nanoparticles, cobalt disks, ferrite cubes, Ni-Fe wires, and maghemite rods [56]. The variations in the magnetic moments are because of the shape anisotropy of the MNPs. It also leads to magnetization reversal by thermal fluctuation and influences the heating efficacy of MNPs [57]. The optical characteristics of the MNPs can be changed by tuning the size and geometry of the fabricated MNPs. It plays a critical role in biomedical applications of MNPs, particularly in the field of MRI-optical imaging systems. Strong resonance stemming caused by the transition of electrons in the monovalent metals like gold and silver is widely used to enhance the contrast in optical imaging systems [58]. The magnetic and optical characteristics can be enhanced by hybridizing metals with metal oxide NPs [55] and by adding fluorophores [59].

The surface charge of the MNP influences the electrical property of MNPs and their performance in drug delivery, imaging, cellular uptake, and localization [60]. For varying biomedical applications, the surface charge of the MNPs can be altered by coating the MNPs with suitable agents like dextran (DEX-neutral charged), carboxymethyl-dextran (CMD-negatively charged), and diethyldiaminoethyl-dextran (DEAE-positively charged) [61].

5 Surface Coating of Synthesized MNPs

Surface coating of MNPs is mandatory under in vivo conditions, to protect them from the adverse effects caused by reactive oxygen species, bioactive compounds, and dissolved oxygen. Generally, chemically stable and compact shells like gold film or silica shells are used to coat the core nanoparticles. However, for preventing nonspecific adsorption of other biomolecules, functionalization of the shell material should be considered before coating. For biomedical purposes, hydrophilic polymers are used to coat the therapeutic MNPs. This type of coating is essential to avoid the agglomeration of MNPs in a highly functional environment. The coated MNPs should have a neutral charge with limited hydrophobic and zwitter ionic properties. Different functional groups like carboxyl group, amine group, etc. are generally added to the coatings of MNPs for immobilizing the targeted ligands, drugs, fluorescent particles, adjuvants, and other agents that can enhance the biocompatibility [62]. Compounds like chitosan, polyethylene glycol, proteins, and lipids are conjugated to the MNPs to increase their stability and biocompatibility under in vivo conditions [9]. Metals, metal oxides of inorganic origin, bioactive components, organic surfactants, and polymers are the materials generally used to coat and modify the surface chemistry of MNPs for its successful application in nanomedicine.

5.1 Organic Coating of MNPs

MNPs synthesized by using organic solvents exhibited high magnetic moments because of their monodispersed and single-crystalline property. Hence additional surface coating is necessary to prevent hydrophobicity [63]. Organic coating of MNPs involves two different techniques such as absorption and covalent bonding [64]. The hydrophobic surfactants found on the surface of MNPs are replaced with hydrophilic ligands. Organic polymer like polyethylene glycol is widely used for altering the ligand on MNPs as they are biologically compatible and help to prevent the nonspecific interaction of cellular proteins with MNPs [65]. For instance, researchers [66] fabricated monodispersed iron oxide nanoparticle of size 9 nm and functionalized it by adding dopamine-terminated polyethylene glycol. DPA was first conjugated with the PEG as it contains a higher affinity toward the iron oxide

nanoparticles. Oleate and oleyamine found on the surface of the nanoparticle are then replaced by incorporating this DPA-PEG conjugate. Coating of MNPs with PEG not only prevents the agglomeration of MNPs in the physiological environment but also increases the stability and biocompatibility of MNPs so that they could be detected by the host immune response. This property of PEG-coated MNPs makes them a highly suitable candidate for targeted drug delivery.

Other organic molecules like dextran's [67], polyethylene oxides [68], and dendrimers [69] have also been applied for exchanging the ligands. Because of the smaller size and having a greater affinity to the membrane surface of MNPs, researchers [70] incorporated DPA directly on the surface of MNPs for exchanging ligands. Even though PEG played an efficient role in preventing the nonspecific binding of proteins with MNPs, it has a high propensity for oxidative degradation. To overcome this limitation, scientists [65] developed a neutrally charged polyzwitterion made of repeating units of zwitterion moieties. These zwitterions found in the cell membrane extend the half-life of circulating MNPs and reduce the cytotoxicity imposed by the MNPs on normal cells. A team of researchers [71] succeeded in the functionalization of MNPs by coating them with polydehydroalanine. It can also be performed by incorporating polyzwitterions during the synthesis of magnetic nanoparticles. Encapsulation of MNPs in a shell is yet another technique widely used to increase the hydrophilicity and biocompatibility of MNPs. Another group of researchers [72] tuned the size of MNPs by embedding the MNPs in a polymer matrix and by changing the polymer concentration, solvent type, and solvent-water ratio. They revealed the interrelationship between the concentration of polymer and the size of the synthesized MNPs. Their findings paved the way for other nanotechnologists to synthesize MNPs of varying sizes to meet the requirements in biological applications.

5.2 Inorganic Coating of MNPs

Inorganic materials like silica can also be used to coat the surface of MNPs to prevent its aggregation under in vivo conditions and for enhancing the stability of nanosuspensions under extreme environments. Silica is the widely used inorganic material for coating MNPs as they are highly biocompatible [73]. It also permits subsequent functionalization of MNPs by alkoxysilanes. Scientists [74] reported that the coating of FePt NPs using a silica shell of 17 nm thickness inhibits its cytotoxicity in the physiological environment. He also stated that the internalization of FePT NPs by tumor cells is also feasible as they are strong T2 agents. His findings on silica-coated FePT NPs made them a potential candidate for fabricating diagnostic and therapeutic MNPs for treating hyperthermic tumor ablation. Silica coating of MNPs also demonstrated its potential in magnetic resonance and cellular imaging. Silica-coated MNPs also exhibited a better control of magnetic properties even during hyperthermic therapy. Another research team [75] reported that the silica coating reduced the magnetization of MNPs by 32% under room temperature when

exposed to a magnetic field of 1 kOe because of the existence of diamagnetic shells. Interaction between silica and MNPs also reduced the Curie temperature by 7%. Hence, the magnetic properties and biological stability should be taken into consideration before coating the MNPs using silica.

Generally, for hyperthermia treatment, inorganic coating materials are preferred in comparison with organic materials, as the metallic coatings permit heat under the influence of a magnetic field. Gold is widely used for metallic coating of MNPs as it can be incorporated easily with biological macromolecules like DNA and proteins [76]. Gold coating of MNPs also facilitates the functionalization of nanoparticles, prevents the MNPs from oxidation, and alters the magnetic properties of nanoparticles. Scientists [77] investigated and optimized the number of gold coatings on the surface of FePT nanoparticles synthesized from the high-temperature solution phase. When exposed to a lower temperature, gold coating on FePT NPs decreases the coercivity of nanoparticles up to threefold when compared with uncoated nanoparticles. It also reduces the blocking temperature up to 50% because of the reduction of surface anisotropy contributed by interaction among gold atoms and FePT nanoparticles.

6 Assessment of Surface Chemistry, Stability, and Biocompatibility of MNPs

Some of the key parameters considered in the description of NPs are size and shape. It is possible to measure size, aggregation degree, surface charge, surface area, and surface chemistry. Size and ligands present on the exterior of the NPs disturb the possessions and applications of NPs.

X-ray diffraction (XRD) is the most broadly used method for the description of NPs. Typically, XRD offers details concerning the structure of crystallines, phase nature, parameters of lattice, and grain size of crystals. X-ray absorption spectroscopic technique (XAS) provides details about the absorption coefficient of x-rays of NPs as a function of energy. Fourier-transformed infrared spectroscopy (FTIR) is most extensively used for the measurement of electromagnetic radiation absorption with wavelengths in the mid-infrared region (4000–400 cm^{-1}). The nuclear magnetic resonance spectroscopic technique (NMR) provides information regarding the structure of nanomaterials. The method is based on the NMR principle displayed by nuclei that have a nonzero spin in the magnetic arena. The Brunauer-Emmett-Teller (BET) method is used for the description of nanomaterials. It is based on the phenomenon of adsorption of a gas on a solid surface. It is widely used in analyzing the surface area of NPs [78].

The photoluminescence spectroscopic technique (PL) monitors the light released from atoms that have captivated photons. PL is useful as the description technique for fluorescent NPs, such as quantum dots and metal nanoclusters. Mass spectrometry (MS) offers vital elemental and molecular details on the composition, structure, and chemical state of NPs and their conjugation to target molecules.

MNPs should be compatible with the biological systems, nontoxic and stable for in vivo usage. These characteristic structures are measured by altering the size and by coating the surface of NPs [79]. It is been reported that magnetic molecules such as iron, nickel, and cobalt are toxic due to oxidation. Hence, the MNPs have to be coated [80]. For therapeutic applications, MNPs must have the capability to leak from the endothelial system to grasp the site. Upon supervision of NPs into blood, opsonization happens. In this method, NPs are covered with plasma proteins and get removed by phagocytic cells and finally will not be able to reach the target cells [81]. To circumvent this process, NPs are layered with organic layers. This extra layer increases the time of circulation and stability of colloids of MNPs [82].

The stability of magnetic suspension outcomes from the equilibrium among magnetic dipole-dipole, van der Waals, and electrostatic, steric forces [83]. The strength of these forces is the significant property of intricate MNPs with stability. Further, to steady these MNPs in aqueous solutions, various types of polymers can be coated on MNPs by end-grafting or by encapsulation [5].

7 Functionalization of Magnetic Nanosystems

MNPs must be functionalized by conjugating them with functional groups to make use of the fabricated NPs in therapeutics. In general, the surface coating permits a base for the attachment of functional groups to MNPs. Commonly molecules like antibodies, peptides/protein molecules, and polysaccharides permit precise recognition of cells; thereby it binds to a specific receptor on the cell surface. Linker molecules such as 1-ethyl-3-(3 dimethylaminopropyl) carbodi-mide hydrochloride, N-succinimidyl 3-(2-pyridyldithio) propionate, N-hydroxysuccinimide, or N, N′-methylene bis acrylamide are also used to confer the primary hydrophilic layered molecules to the targets [5].

In preparation for MNPs, points should be considered in such a way that the targeted cell population is recognized with increased specificity. The efficacy of biomedical applications relies on cell-nanoparticle interactions. Scientists have reported that cell membranes play a vibrant part in cell-nanoparticle interactions.

8 Theragnostic Applications of MNPs

Synchronization of diagnosis and therapy offers a highly effective means of treating diseased parts without harming other healthy sites. Personalized and advanced treatment has become possible with the advent of such theragnostic approaches. MNPs play an inevitable role in theragnostic as they can be directed and functionalized within the biological system by applying a magnetic field externally. An additional advantage of MNPs is that they drop their magnetism instantly after their detachment from the magnetic field [84]. Researchers are focusing on fabricating

multilayer coating of MNPs to facilitate the controlled release of therapeutic nanoparticles to the target site specifically. Currently, these sophisticated, functionalized, multicoated MNPs are highly useful in delivering a gene to the specific target site [85], MRI [86], tissue replacement therapy [87], etc. Biomedical application of MNPs in magnetic hyperthermia treatment, magnetic resonance imaging, sensing diseased site, separating biomolecules, delivering drug and gene to the diseased site, tissue engineering and replacement, transfection, detection, and chelation of iron moieties are discussed below (Fig. 4).

8.1 MNP-Mediated Hyperthermia

Hyperthermia is a therapy used to induce apoptosis in cancerous cells by applying high temperatures to the diseased site. Generally for hyperthermia treatment, the cancer cells are exposed to a temperature exceeding 43 °C for 30 minutes [88]. MNPs when subjected to altering magnetic fields generate heat by converting magnetic energy into thermal energy [89]. The type of nanoparticle used and the strength of the magnetic field influence the amount of heat generated during hyperthermia [5]. Functionalization and coating of MPNs with silicone increases the specificity of hyperthermic treatment [90]. For instance, antibodies specific to cancer antigens when combined with MNPs enhanced their uptake at a specific target site during hyperthermia therapy [91]. However, the possibility of overheating remains a great challenge in the applicability of magnetic hyperthermia treatment. To overcome this issue and reduce the dosage, magnetic oxide nanoparticles have been designed with

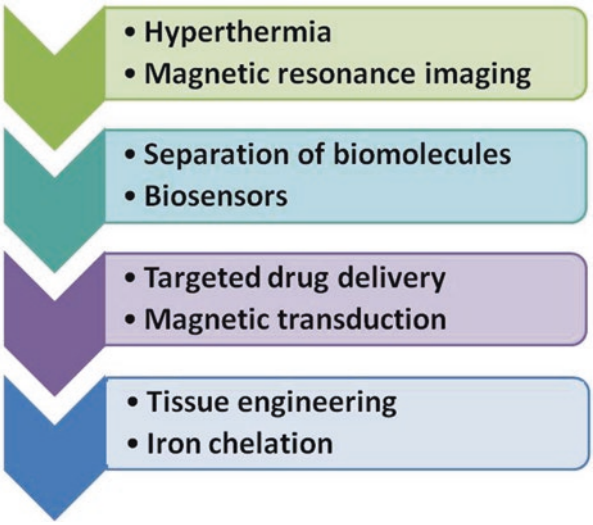

Fig. 4 Theragnostic applications of MNPs

improved heating potential and temperature controller to regulate the heat generation by itself [92]. For facilitating the retention of nanoparticles at a specific tumor site and to minimize the diffusion of nanoparticles to surrounding normal tissues, temperature-responsive agents like lipids, heat-sensitive polymers, and hydrogels were incorporated with MNPs before hyperthermia treatments [93].

During clinical trials, the combined effect of iron oxide nanoparticles and low dosage radiotherapy enhanced the survival of the cancer patients without any significant side effects, suggesting the importance of MNPs in triggering the efficacy of other cancer therapies [94]. Similarly, another group of scientists [95] reported the increased efficiency of magnetic hyperthermia when used in conjugation with gene therapy, involving tumor necrosis factor-α and GADD 153 stress-inducible promoter, in controlling the growth of the tumor. The most prominent advantages of magnetic hyperthermia over other conventional therapies are its deep penetration potential and low tissue damage. Researchers [96] documented the synergistic effect of hyperthermia and chemotherapy in the successful suppression of tumor growth. In this investigation, a nanocarrier system was fabricated using hollow porous magnetic carbon nanoparticles, and polyglutamic acid was used to cover the pores found in the shell to prevent the leakage of a chemotherapeutic agent to other unaffected sites during distribution. This type of sealed nanoparticle delivery system will help prevent the development of drug resistance.

8.2 MNP-Based Magnetic Resonance Imaging

MNP-based magnetic resonance imaging is the most advanced technique used in the diagnosis of cancer during the past few decades. In comparison with computed tomography (CT) and conventional magnetic resonance imaging (MRI), MNP-based magnetic resonance imaging does not involve any radiation and is noninvasive. In this technique, MNP-based formulations are being used as contrast agents for taking images with a high level of temporal and spatial resolutions [97]. Particularly, iron oxide nanoparticles (IONPs) and super paramagnetic iron oxide nanoparticles (SPIONs) contributed to a tremendous improvement in imaging-based cancer diagnosis worldwide. Several types of IONPs have been produced and assessed for their applicability as a contrast agent in MRI. The specificity of these IONPs has been enhanced by adding ligands that can bind specifically to target cells or on the surface biomarker of cancerous cells. For instance, antibodies, peptide molecules, and fragments of antibodies that can bind to tumor-specific receptors like EGFR, MUC-1, HER2/neu, αvβ3 integrin, and uPAR were incorporated into the surface of IONS. These conjugated IONPs facilitated the detection of tumor sites by exhibiting tissue-specific accumulation of nanoparticles and its enhanced retention in the target site [98].

The coating of MNPs with biologically compatible materials can increase the image intensity and contrast and helps in the accurate diagnosis of diseases. Various dextran-coated nanoparticles like ferucarbotran, ferumoxtran, and ferumoxides

have got approval for clinical applications as MRI contrast agents [99]. The MNP-based MRI is highly useful in the detection of lymph node metastasis from the tumor sites and to delineate solid tumors from metastases. Recently, multi-modal imaging agents received considerable attention in the field of a cancer diagnosis. For instance, pH-sensitive ligands were conjugated with magnetic IONPs for detecting small-sized tumors having a diameter below 3 mm. These magnetic nanogrenades get activated under an acidic environment in tumor sites and produce strong signals, resulting in an earlier diagnosis of tumor [100]. However, the size, shape, and toxicity of the MNPs should be considered before their utility as contrast agents. The toxicity of MNPs will be determined not only based on their surface coating but also based on the cell type. MNPs of size 10–100 nm are considered to be efficient in MRI systems, as they possess a longer half-life period and can easily penetrate through blood capillaries as well [101].

8.3 MNPs for Separation of Biomolecules

Magnetophoresis is a process of separating colloids and magnetic nanoparticles by applying magnetic field gradients. It is a widely used approach in the field of engineering and biomedicine. A modified form of magnetophoresis, namely, low-gradient magnetic separation (LGMS), can also be used for the separation of biomolecules, which works under the influence of a weak magnetic field formed by using high-power permanent magnets. The migration of magnetic particles depends on the strength of the magnetic field imposed on them as well as on the influence of suspension fluids [102]. In recent years, the magnetophoretic separation technique has become more popular among the scientific communities particularly because of the invention of magnetic nanoparticles for various applications like removal of heavy metals from contaminated water bodies, drug delivery, removing toxic algae from water resources, detection, sorting of cells, etc. [103]. Other than this, MNPs in combination with monoclonal antibodies help remove infectious pathogens from the blood [104].

Magnetic nanoparticles are considered to be efficient in the biomedical separation technique as the user can remotely control the migration of MNPs toward a particular target or to extract and separate them from the fluid by altering the external magnetic field [105]. Various types of MNPs have been used in the differentiation, detection, and separation of body cells and other analytes. For instance, magnetic nanobeads coated with streptavidin are widely applied in the in vitro selection and sorting of sensory neurons [106], stem cells [107], and other types of body cells. In the case of blood tumors or during metastases, tumor cells will be circulating in the blood and other body fluids and may result in the spreading of the tumor to other vital organs. Capturing such circulating tumor cells (CTC) is essential for primary diagnosis and treatment. For separating the CTC by magnetophoresis, the CTCs are labeled with magnetic beads. Additionally, a team of researchers [108] fabricated a microfluidic device for the separation of spiked cancer cells, endothelial progenitor cells, and hematopoietic stem cells from the blood samples. It offers a rapid,

efficient, and highly robust technique for sorting cells directly from a patient's blood. Similar to the microfluidic device, another device that can capture cancer cells like SKBR3 and COLO205 with capture efficiency of 86% and 90%, respectively, was developed by a team of researchers [109]. Another research group reported a magnetophoretic separator for the separation and detection of breast cancer cells.

8.4 Role of MNPs as Biosensors

Disease diagnosis during the early stage of onset is important for successfully providing treatment. Nanoparticle-based electrochemical and optical sensors offer a highly sensitive approach to early diagnosis of cancer. But, these sensors require preprocessing or pretreatment of the biological sample to avoid unnecessary interference by other macromolecules in the system [110]. When compared to other biosensors, magnetic nanoparticle-based sensors are highly useful in the immunological assay as the biological specimen exhibits very low susceptibility to the magnetic field, resulting in the highly sensitive detection of diseases even from unprocessed or minimally processed samples [110]. For sensing applications, MNPs with diameters ranging from 10 to 100 nm are usually embedded in a matrix made of the polymer [111].

Magenetophoretic efficiency of biomolecules under the influence of magnetic field forms the basis for the development of integrated biosensors. For instance, MNPs conjugated with antibodies are used as ultrasensitive barcodes for the detection of protein analytes as well as for removing the tagged oligonucleotide molecules [112]. The high sensitivity and specificity of MNPs make them a suitable candidate for fabricating biosensors for clinical applications. The MNP-based biosensors are majorly classified into two types. Firstly, giant magnetoresistive biosensors are made up of alternating layers of non-magnetic metal and ferromagnetic layers, which on exposure to an external magnetic field change the orientation of the ferromagnetic layer and resulted in the development of resistance in the tool [113]. The second type of biosensors is the magnetic tunnel junction biosensors which are widely used in the detection of immunological disorders. This sensor contains a thin insulating barrier packed in between two ferromagnetic layers.

Scientists [114] proposed that the synergistic approach involving magnetic bead-based cell labeling, PCR amplification-based hybridization, and inductively coupled plasma-mass spectrometry (ICP-MS)-based detection could be used for differentiation and enumeration of cancer cells. Another team [115] proposed an immunomagnetic flow system for the identification of circulating tumor cells and other cancer markers simultaneously. Similarly, a research team [116] applied MNPs for highly sensitive detection and counting of cancer cells such as human lung carcinoma cells and human hepatocellular carcinoma cells. Currently, MNP-based biosensors have been utilized for the detection of food pathogens [117], DNA [118], and viruses [119]. However, in some cases these sensors may exhibit changes in their resistance in the absence of MNPs, resulting in the generation of

false-positive signals. Hence, before the sample analysis, a strong pre-magnetizing field will be applied to the system to reduce the noise level [120].

8.5 MNPs as Vehicles for Targeted Drug Delivery

MNPs have been widely used as vehicles for delivering drugs as well as genes to the specific target site. In this technique, the MNPs are incorporated with the drug molecules and injected intravenously into the patient. Then by using an external magnetic field, these particles are channelized to the specific target site and allowed to hold for their activity and finally removed [121]. MNPs can be loaded with a high dosage of drugs to ensure effective concentration of drugs at the target site and for avoiding adverse side effects caused by dispersion of drugs to the surrounding normal tissues [122]. While developing a nano-delivery vehicle, parameters such as stability, size, coating, physiological characteristics, and biocompatibility of the MNPs should be taken into consideration [123]. For acting as an efficient carrier system, the MNPs have to be in the size below 100 nm, and they should retain their hydrophilicity under in vivo conditions [124]. Scientists [125] suggested that the hydrogels, micelles, and polymeric microspheres are highly efficient in protecting the drug from biochemical degradation, lowering systemic toxicity, increasing absorption rate, and enhancing the specificity of the drug. In addition, dendrimers and biodegradable polymers, because of their structure and size, can also act as suitable vehicles for delivering drugs [126].

The biocompatibility and stability of MNPs can be improved by the functionalization of nanoparticles using chemical or biological agents. The gold film, polyethylene glycol, carboxydextran, poly-L-lysine, and silica gel have been used as coating materials for the functionalization of MNPs [127]. The driving of the drug to the target site is influenced by various factors like temperature, pH, osmolality, etc. [128]. Factors like magnetic field strength, size of MNPs, magnetic properties, drug-loading efficiency, flow rate, and location of the target site can also affect the drug delivery system [129]. The same factors have to be considered for delivering the gene to the target site in which an oligonucleotide is incorporated with MNPs instead of drug molecules. The carrier molecule has to release the drug or gene at the specific site based on the local environment, viz., pH, GSH, etc., or by the influence of external stimuli like X-ray, heat wave, light impulse, etc. [130]. Instead of all these challenges, MNP-based drug carrier systems including methotrexate, doxorubicin, and paclitaxel have reached clinical trials for cancer treatment [124].

8.6 MNPs in Magnetic Transfections

Scientists all over the world are in the surge of finding a non-viral transfection agent for delivering genes to the target molecules. Biocompatible magnetic nanoparticles exhibited their efficacy to serve as a promising tool for transfection under in vivo

and in vitro conditions. Magnetofection is the term used to describe the gene transfection mediated by MNPs under the influence of a magnetic field [5]. In this technique, the reporter genes or therapeutic genes are incorporated into the magnetic nanoparticles and are guided to reach the target site by applying a varying magnetic field. Coating of MNPs with magnetic polycation polyethyleneimine and particular gene vectors enhanced the transfection efficiency of MNPs in non-permissible and permissible cells [131]. Such a type of magnetofection enhanced the activity of enzymes like luciferase in endothelial cells and provided a suitable means of transferring antisense RNA under in vitro and in vivo conditions [132]. Scientists [133] developed a standard protocol for transferring genes using a non-viral transfection system. The protocol starts with the synthesis of MNPs, followed by the incorporation of DNA to the MNP core, fabrication of magnetic polyplexes and lipoplexes, magnetofection, and post-transfer data analysis.

Very few in vivo studies authenticated the successful delivery of macromolecules to the tumor sites. For instance, nanocarrier-based delivery of BIRC5 siRNA to human breast cancer xenografts and survivin siRNA to colorectal cancer xenografts have been reported in mice models [134]. The incorporation of antibody EGFRVIII with MNPs enhanced the rate of survival of rats with glioma [135]. A team of researchers [136] documented the successful delivery of chlorotoxin-MNP conjugate in treating brain tumors in mice. Another research group [137] applied MNPs incorporated to dendrimers and anti-EGFRVIII siRNA for silencing genes involved in brain tumor formation in mice.

8.7 Applicability of MNPs in Tissue Engineering

Tissue engineering (TE) is a scientific discipline that aims to produce, replace, and repair damaged tissues and cells and retain their physiological functions. Unavailability of suitable biomaterials, insufficient cell growth, lack of growth factors, inability to stimulate communication at the cellular level, and lack of control over the properties and cellular functions act as a barrier to the advancement of the TE techniques. Nanoparticles because of their size-dependent characteristics aid to overcome various pitfalls faced by the TE approach nowadays. Advantages of utilizing nanoparticles in TE are their large surface-to-volume ratio and their ability to diffuse across the membrane when compared to other small proteins and peptides. Moreover, the researcher can customize the size and surface characteristics of the synthetic nanoparticles. Various nanoparticles including metallic, metal oxide nanoparticles, and carbon nanotubes have been used in TE for enhancing biological and mechanical performances [138]. Selecting appropriate nanoparticle for TE can augment the electrical, mechanical, and biological properties of scaffolds and helps in the proper functioning of the system [139].

Magnetic nanoparticles have shown promise in controlling the cellular patterns, delivering genes, and constructing solid 3D tissues and in the field of mechanotransduction. The mechanical strength of hydrogel microfibers increased when

synthesized with MNPs [140]. Researchers [141] embedded the MNPs along with gold and silver nanoparticles in the scaffolds for enhancing the scaffold mechanics. Details about cellular functions and differentiation can be obtained by integrating MNPs. For instance, a research team [142] utilized MNPs for inducing apoptosis in cell lines, while another group of scientists [143] applied MNPs for increasing calcium levels intracellularly. PEG-modified MNPs coated with aminosilane (PEG-Mags) were used for investigating cell patterns in TE [144]. He also found that the incorporation of MNPs in tissue constructs on exposure to magnetic force improved the development of skeletal tissues. The MNPs also support the thermal fluctuation at room temperature [145]. Iron oxide MNPs are widely utilized in stem cell replacement therapy for various purposes like cell sorting, engraftment, and labeling of cells [87]. MNPs have a wide range of applications in welding the tissue surfaces which involves denaturation of proteins and repolymerization of neighboring peptides [146]. Scientists [91] utilized magnetic nanoparticles for constructing multilayered keratinocyte sheet-like 3D constructs. Researchers also [147] reported the applicability of magnetic nanowire arrays in tissue engineering.

8.8 MNPs for Detection and Chelation of Iron in Biological Systems

Deposition of excessive metals may lead to various neurodegenerative diseases like multiple sclerosis, Friedreich's ataxia, Alzheimer's, Huntington's, and Parkinson's diseases [148]. Accumulation of iron in substantia nigra pars compacta confers the neurodegenerative disorder called Parkinson's disease. The severity of the disease can lead to loss of dopaminergic neurons, enhanced production of reactive oxygen species, and failure of motor control [149]. Cytotoxicity and short circulation time under in vivo conditions hindered the application of conventional iron chelators [150]. To overcome this situation, iron oxide nanoparticles with supramagnetic properties have been utilized as they are biocompatible and nontoxic in nature. Osmotin, a plant protein obtained from the tobacco plant with the potential to prevent neurodegeneration, was incorporated with dextran-coated Fe_3O_4 nanoparticles. The nanoconjugate is then injected and channelized to the brain of Aβ1–42-treated mice by applying a magnetic field [151]. The MNP-based conjugate exhibited only a minimal accumulation in other tissues, reached the brain within a few minutes, and reversed the brain damage significantly in the mouse model.

Controlled release of drugs from the nanoparticles was also achieved by utilizing the acidic pH condition in the brain of patients with Alzheimer's disease, which was neutral in the case of healthy persons [152]. Scientists [153] reported the applicability of oleic acid modified iron oxide nanoparticles conjugated with shRNA, nerve growth factor, and N-isopropyl acrylamide derivative in reversing the brain damage caused by Parkinson's disease. Iron oxide nanoparticles play an important role in the non-radioactive imaging-based diagnosis of neurodegenerative disorders. The iron-chelating potential of such MNPs can also be utilized for therapeutic

applications [154]. Further research on iron-chelating potential of MNPs could be explored to develop an efficient nanotheranostics platform for simultaneous detection and treatment of neurodegenerative diseases.

9 Biodistribution, Clearance, and Toxicity of MNPs

Therapeutic nanoparticles administered into the bloodstream of the individuals will reach the target site by a passive transport mechanism. Hence, it is foremost important to determine the pharmacokinetic properties of the synthesized nanoparticles (Fig. 5). Hydrodynamic size and surface characteristics of the MNPs are the two important features that play a vital role in pharmacokinetics [155]. The relationship between the therapeutic MNPs and the reticuloendothelial system (RES) decides the plasma lifetime of administered MNPs. During clearance by RES, the opsonin proteins circulating in the blood get adsorbed to the surface of MNPs and facilitated its recognition and removal of MNPs by tissue macrophages. The strong interaction of MPNs with the cellular surfaces and components circulating in the blood plasma may lead to a short plasma lifetime for the cationic MNPs [156]. MNPs of diameter ranging between 10 and 100 nm are considered to be highly suitable for in vivo applications as they are pharmacokinetically active in nature [157]. MNPs with a diameter greater than 100 nm can be easily opsonized and cleared from the bloodstream by the RES. Whereas, the smaller MNPs with a diameter lesser than 10 nm are generally cleared by extravasation of the tissues and by the renal system.

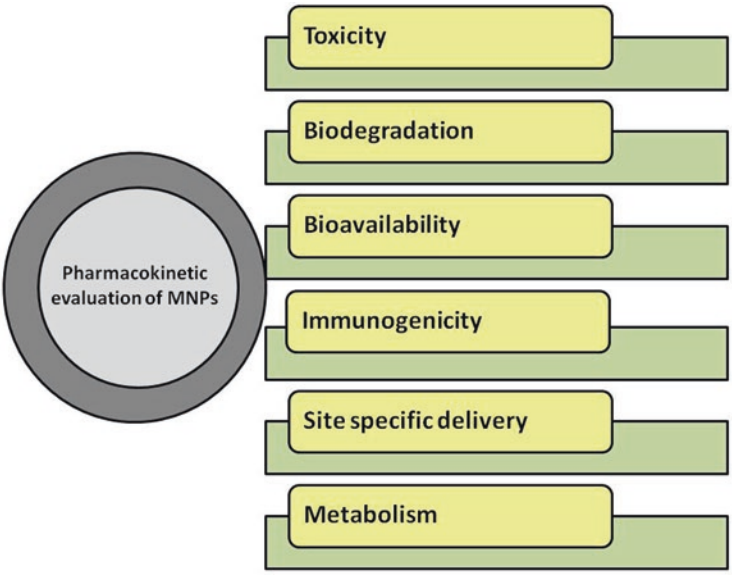

Fig. 5 Pharmacokinetic evaluation of MNPs

Surface modification and coating of MNPs can reduce the rate of clearance by resisting its interaction with RES. Particularly, coating of MNPs with polyethylene glycol provides resistance to the MNPs against opsonization by tissue macrophages and is useful for extending the plasma lifetime of MNPs [158]. Research teams [159] reported that the PEG-modification of MNPs with a hydrodynamic diameter of 170 nm resulted in the increase of MNP half-life up to 12 hours in a rat model, and it could be detected by MRI even after 24 hours in the tumor site. Such MNPs with extended circulation half-life are known to possess better therapeutic efficacy.

Assessing the biodistribution of MNPs in a living system is of utmost importance for achieving success in treatment with minimal side effects [160]. Most of the MNPs administered into the body will get distributed in the liver and spleen. Kupffer macrophages found on the sinusoids are responsible for the uptake of MNPs in the liver, while mechanical filtration and tissue macrophages regulate the distribution of MNPs in the spleen [146]. Such a type of non-target distribution of MNPs has raised the concern about cellular toxicity. However, the type and charge of MNPs, method of synthesis, characteristics of MNPs, coating materials used, dose and purity of MNPs, route of administration, and biological distribution determines the toxicity of MNPs in tissues [161]. All these factors necessitate a thorough toxicological evaluation of any MNP formulation before its administration in the living system. The toxicity evaluation of MNPs should be carried out in animal models before human trials. In vivo toxicological assessment of MNPs includes histological evaluation, cytokine detection, blood counts, lipid hydroperoxide estimation, and liver functionality tests [162]. Coated core-shell-MNPs are biocompatible and nontoxic in nature, and the toxicological characteristics are independent of the administration route. A team of researchers [163] investigated the cytotoxicity of MNPs by altering the coating materials and concluded that the coating of iron oxide nanoparticles with biocompatible poly-L-lysine hindered the uptake of IONPs by macrophages and thereby reduces the ROS production and apoptosis-mediated cytotoxicity.

10 Potentialities and Challenges in the Clinical Application of MNPs

Advancements in the field of nanotheragnostics provide a novel approach to treating complex diseases like cancer, neoplasia, and neurodegenerative disorders. Specifically, MNPs are widely applied for performing various tasks like medical imaging, drug delivery, hyperthermic therapy, gene therapy, etc. The ability of MNPs to respond to external magnetic stimuli makes them highly preferable to other nanoparticles in providing site-specific treatment and mitigating the toxic effects of drugs on other healthy tissues. Surface functionalization of MNPs aided them to get deposited in the tumor site and releasing the chemotherapeutic drug for destructing target cells. Imaging techniques with the high spatial and temporal resolution for diagnosis and grading of cancer became possible only after the advent of

the MNP-based MRI system. Coating of MNPs with carbon, silica, and gold enhances the stability and biocompatibility of MNPs in living systems.

However, the biomedical applications of MNPs consist of a few technical gaps which hinder their movement from bench to bedside. The main disadvantage in the applicability of MNPs for hyperthermic treatment includes its weak magnetic property and the requirement for large-sized MNPs (more than 250 nm). After treatment, the retrieval of such large-sized MNPs from the body is relatively difficult [164]. Hence, the development of small-sized super paramagnetic nanoparticles (about 2 nm) with high magnetic properties will be helpful in hyperthermia, magnetic particle imaging, and its subsequent clearance by the renal system.

Advancements in magnetic particle imaging techniques are needed for scanning the whole body which will be useful for detecting the tumorigenesis, monitoring medical interventions, and providing proper treatment. Further research on the development of high-power microfluidic systems is essential for detecting the circulating tumor cells, cancer biomarkers, cell sorting, and highly efficient monitoring of cancer therapy. Extensive research on biosensors and microfluidic array devices is needed for rapid detection and subsequent removal of circulating tumor cells.

As of now, targeting of MNPs is found to be effective only for treating superficial tumor-like skin cancer and is not efficient in treating tumors of deeper tissues. Limitations in the homogenous penetration of MNPs also suppress its therapeutic outcomes [165]. Other than this, approval of MNPs for human treatments requires extensive toxicological analysis. Successful translation of MNP-based treatment requires the involvement and cooperation of scientists from various fields. Scientists with chemistry and material science background are needed for the synthesis and functionalization of MNPs. Expertise in physics is required for determining the appropriate magnetic field gradient. In vitro and in vivo assessment for pharmacokinetics properties, evaluation of drug efficacy, biodistribution, and toxicity analysis necessitates the involvement of pharmaceutical scientists. For planning and execution of human trials, clinicians are essential.

Successful translation of MNP- based imaging techniques offers hope for the therapeutic translation of MNP-based technology in the near future. An increase in the number of in vivo studies using animal models and subsequent evaluation by clinical trials will translate MNP-based theragnostic approach, a practically feasible one. More interdisciplinary research is needed to address the technical gaps in the clinical translation of MNPs.

11 Conclusions and Future Prospects

During the past few decades, extensive research has been carried out for synthesizing MNPs with desired physicochemical properties for its utility in biomedical applications. There has been technological progress in terms of stability, quality, and applications of MNPs in imaging, diagnosis, and therapeutic platforms.

Currently, many nanoparticle formulations are commercially available in the market for performing highly sensitive diagnoses of diseases.

Although researchers succeeded in active targeting tumors using functionalized MNPs such as nanoformulated paclitaxel and anthracyclines under in vivo conditions (in albino rats and mice), still several key issues regarding the biocompatibility of MNPs need to be addressed before its translation into clinical trials. Other investigations related to the theragnostic applications of MNPs are still found in the preclinical stage. Particularly, issues related to the regulatory clearance of MNPs are highly dependent on their toxicological properties. Even though there are several hurdles in the clinical translation of MNPs, still they are a strong candidate for elongating the life expectancy of patients, reducing pain during treatment, and increasing the success rate of a cancer diagnosis as well as treatment. Nevertheless, interdisciplinary research involving the active collaboration of expertise from chemistry, physics, structure engineering, medicine, and pharmacy can revolutionize the field of medicine by developing advanced nanodevices and nanoformulations with biocompatibility.

Despite the abovementioned applications, MNPs can also be used to fabricate a nano-biopsy system for collecting samples for biopsy from tumors found in remote, critical parts and from those which are not easily accessible by physical methods. This nano-biopsy system will also help design specific treatments and improving therapeutic outcomes. Cancer immunotherapy is yet another area attracting the attention of scientific communities worldwide. Current strategies based on subcutaneous injection of trained cells from the patient and commercial vaccines are of least importance as they may cause several complications. Applications of MNPs as delivery vehicles for the target-specific delivery of vaccines are highly reliable as they can be removed from the site at any time point. It can be predicted well ahead of that the MNPs will revolutionize the medical field by their influence on an imaging-based diagnosis, target-specific drug delivery, gene therapy, and the development of ultrasensitive sensors and arrays for disease diagnosis.

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