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Mucosal Delivery of Drugs and Biologics in Nanoparticles

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Preface

The last two decades have seen a significant increase in nanotechnology research for drug and vaccine delivery for various diseases. However, the promise of nanotechnology as a tool for delivering therapeutic agents and their clinical translation has been slow. The delivery of nanoparticles by the mucosal route of administration further complicates their safety and efficacy due to mucosal barriers and variable clearance rates. Their complex nature and the requirement of novel characterization techniques have made the regulatory approval of products containing nanoparticles difficult. We have arranged this book into three parts with each section discussing various aspects of nanotechnology.

Part I of the book encompasses chapters that will provide the reader with a basic understanding of nanomedicine in drug delivery and the different characterization methods used for nanomedicine, which is often challenging in order to ensure their purity, safety, and effectiveness when administered by mucosal routes.

Part II of the book describes the various mucosal routes used for nanoparticle administration, their advantages and disadvantages, and the progress made in delivering nanoparticles using different mucosal routes. Individual chapters then focus on the buccal, respiratory, and oral routes of administration. The final chapter in this section discusses the role of nanoparticles in delivering vaccines and biologics and products that are currently undergoing human trials in the United States.

Part III of the book is devoted to the host-cell interaction with nanoparticles. A chapter discusses how these particles interact with epithelial and immune cells after mucosal delivery. Another chapter discusses the toxicity of nanoparticles to the host and the environment, while the final chapter discusses the biodistribution of nanoparticles after mucosal delivery.

We owe immensely to all the authors in this book who agreed to give their time and effort to write a chapter based on their experience in the nanotechnology field. We would not have successfully completed this book without their insight and enthusiasm.

Lastly, it was a pleasure working with various staff members at Springer Nature for the past 3 years on the preparation of this book. We are particularly grateful to Carolyn Spence, Sanjana Meenakshi Sundaram, and Cathrine Selvaraj for their contributions. We would also like to thank Dr. Yvonne Perrie, series editor of AAPS Advances in the Pharmaceutical Science Series, for giving us the opportunity to work on this volume. Although it was a long and sometimes challenging undertaking, it has also been an immensely rewarding experience. We thank them for helping us along the way and for their patience and understanding throughout this lengthy process.

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Contents

Part I
Background

Introduction to Nanomedicine in Drug Delivery

Tejashri Chavan, Pavan Muttil, and Nitesh K. Kunda

Abstract Recent advances in the field of nanotechnology have given a boost to the academic researchers, the pharmaceutical and biomedical industry, allowing its use in drug delivery and medical diagnosis. Nanotechnology allows the formulation of drug delivery carriers in the nanometer range that helps in overcoming disadvantages associated with conventional drug delivery systems by being small, target-specific, improved drug encapsulation, stable and less toxic at the same time. With nanotechnology, many drugs especially oncogenic molecules that are toxic or are difficult to deliver have been formulated and delivered successfully and are currently in the market, for example, Myocet® (2000) (Doxorubicin) and Marqibo® (2012) (Vincristine). Nanoparticle-based drug delivery carriers can be classified into two types; organic and inorganic. Organic nanoparticles are mostly used for drug delivery, while inorganic nanoparticles are majorly involved in diagnosis. Organic nanoparticles generally involve but are not limited to liposomes, dendrimers, polymeric micelles, polymeric nanoparticles, and solid lipid nanoparticles. Inorganic nanoparticles involve metals such as gold, silver, and iron oxide. In this chapter, we will discuss the advantages and disadvantages of nanoparticles and various materials that are used for making different types of nanoparticles with relevant examples. Further, we will discuss the recent developments in this field with some examples pertaining to each type of nanoparticles.

Keywords Nanomedicine · Drug delivery · Mucosal · Liposomes · Lipids Polymer · Metallic · Nanoparticles

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1 Introduction

Nanotechnology is the technology conducted in the nanoscale, typically between 1 and 100 nm, and could potentially transform the pharmaceutical and biomedical industry. Nanomedicine is a branch of science wherein nanotechnology is applied in the medical field to diagnose, prevent and treat diseases, repair tissue, relieve pain, and improve overall human health. For the new generation as well as existing drugs, conventional drug delivery systems provide limited biodistribution, undesirable side effects, and limited efficacy. With the advent of nanotechnology, novel drug delivery systems using this approach offer enhanced bioavailability, tissue selectivity, reduced toxicity, enhanced formulation stability both *in vitro* and *in vivo*, and the ability to overcome physiological barriers to reach target tissues.

Nanoparticles are colloidal particles consisting of macromolecular substances in the size range of 1–500 nm. These particles are carriers wherein the active substance is either uniformly dissolved, encapsulated, or adsorbed onto the matrix. Nanoparticles can be broadly classified into two categories: (i) inorganic nanoparticles and (ii) organic nanoparticles. Inorganic nanoparticles (gold, silica, iron oxide etc.) have applications mainly in biomedical imaging and thermal ablation of tumors; whereas, organic nanoparticles (polymeric, micelles, liposomes etc.) have applications in drug delivery systems for prevention and treatment of various diseases. Nanomaterials studied for drug delivery include; quantum dots, carbon nanotubes, graphene derivatives, polymeric nanoparticles, dendrimers, metal and metal oxide nanoparticles, liposomes, nanoporous materials and many others [1–8]. This chapter will introduce different types of organic and inorganic nanoparticles, their preparation and examples in drug delivery. Figure 1 shows Food and Drug Administration (FDA) approval timeline of various nanoparticles since their first approval in 1957 [9–11].

2 Organic Nanoparticles

2.1 Polymeric Micelles

Polymeric micelles are nanoscopic core-shell structures formed by the self-assembly of amphiphilic copolymers in an aqueous environment [12, 13]. Polymeric micelles are formed when the hydrophobic segments of the amphiphilic copolymer self-associates to minimize contact with water molecules [14]. The hydrophobic core of the polymeric micelle allows entrapment of poorly water soluble drugs, while the hydrophilic shell offers colloidal stability to the formulation and intrinsic stealth effect [15]. Polymeric micelles can be used for the delivery of various drugs (e.g. anti-cancer), proteins, peptides, and genetic material such as DNA and siRNA [16].

Micelles can be composed of di-block, tri-block, amphiphilic graft polymers, and ionic polymers with ionic and hydrophilic segments. Physical and biological

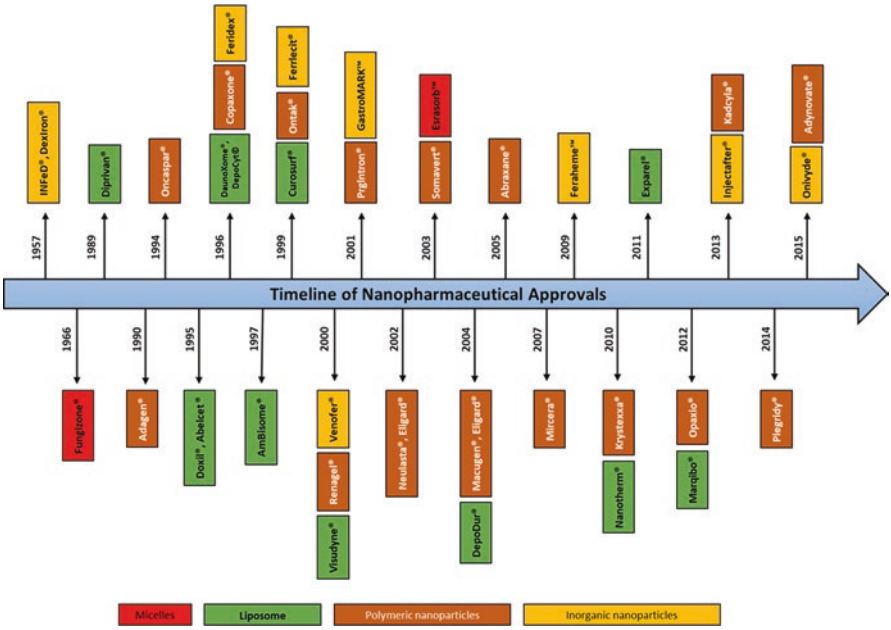

Fig. 1 Timeline of Food and Drug Administration (FDA) approved nanopharmaceuticals

properties of micelles depend on the block polymer selected for preparation [16]. Poly (ethylene glycol) (PEG) with a molecular weight of 2–15 kDa is the most commonly employed polymer for forming the hydrophilic shell and offers the stealth effect to the micelle [12, 16]. Other polymers studied for preparing polymeric micelles are poly(vinyl alcohol) (PVA), poly(ethylenimine) (PEI), poly(2-ethyl-2-oxazoline) (PEOz), poly(N-vinyl-2-pyrrolidone) (PVP), dextran, poly(acrylic acid), poly(aspartic acid), poly(D,L- lactic acid) (PLA), poly(D,L-lactic-co-glycolic acid) (PLGA) poly-N-(2-hydroxypropyl) methacrylamide (HPMA), and poly(ε-caprolactone) (PCL) [12, 16].

Polymeric micelles offer several advantages such as small size (20–100 nm), exhibit greater stability, are biocompatible, improved drug loading, prolonged circulation, and their amphiphilic nature allows increased solubility of poorly water soluble drugs [15]. Table 1 lists some of the methods that have been used for micellar preparation including dialysis, sonication [13], and thin film hydration technique [17]. Wang et al. 2018 have reported soluplus and solutol®HS15 based self-assembled micelles for delivery of curcumin [18]. Curcumin is poorly soluble in aqueous media and has limited oral bioavailability [18]. Soluplus and solutol®HS15 based self-assembled micelles were made using ethanol solvent evaporation method [18], and curcumin encapsulated in soluplus and solutol®HS15 based micelles had improved solubility (4200 fold), Caco-2 membrane permeability, and oral bioavailability in rats compared to free curcumin [18].

Table 1 Commonly used methods for the preparation of polymeric micelles

Thin-film hydration technique	Dialysis technique	Sonication
1. Drug(s) and polymer(s) are dissolved in chloroform.	1. Drug & polymer or drug polymer complex is dissolved in dimethyl sulfoxide (DMSO) and is added drop wise to water/ buffer under continuous stirring.	1. Drug and amphiphilic polymer or drug polymer complex is dissolved in water.
2. Chloroform is evaporated by rotary evaporation to obtain thin film.		2. The solution is ultrasonicated for a predetermined time to achieve desired sized micelles [13].
3. The thin film is hydrated in water/ buffer to achieve micelles.	2. This solution is allowed to stir for a predetermined time to form micelles.	
4. The obtained micelles can be further sonicated to reduce the particle size [17].	3. Micelles are then dialyzed against water to remove DMSO and un-entrapped drug [13].	

Self-assembled micelles generally suffer from poor *in vivo* stability below the critical micellar concentration (CMC), leading to dissociation and early drug release following administration, ultimately producing drug-related toxicity [19]. To improve micellar stability in extracellular environment and to achieve targeted drug delivery, stimuli-responsive cross-linked micelles (SCMs) have been designed [12, 20]. Covalent cross-linking is considered an effective way of imparting stability to polymeric micelles [12]. In addition, cross-linking of core prolongs circulation time and maintains the sturdiness of the shell [12]. Further, covalent cross-linking can be employed to the hydrophilic shell or hydrophobic core [12]; however cross-linking of corona can lead to inter-micellar cross linking, loss of shell fluidity and polar affinity leading to decreased stealth effect [12, 21]. Ling et al. 2018 have reported the design of redox-responsive camptothecin (CPT)-conjugated disulfide core-cross-linked micelles based on the poly (ethylene glycol)-dihydrolipoic acid (MeO-PEG 2 k-DHLA) conjugate [12]. Covalent linking of CPT to the core of dihydrolipoic acid prevented unwanted leaking of the drug or burst release and improved the stability under the mimics of physiological conditions [12].

2.2 Dendrimers

Dendrimers are well-defined, spherical, compartmentalized polymers consisting of hyper-branched structures with layered architecture. They form tree-like structures from monomers *via* convergent or divergent step growth polymerization. The size and shape of dendrimers can be precisely controlled by controlling the number of branching. A dendrimer consists of three domains: (i) the core, (ii) the branches, and (iii) the terminal functional groups. The branches originate from the core and progress geometrically to form concentric layers (referred to as generations). Generation

of the dendrimer is decided on the basis of how many repeating units it has and that are accountable for its spherical structure [22–25]. The core of the dendrimer is labeled as generation zero (G0) with successive layers as G1, G2, etc. The cavities or hollow spaces in the dendrimer are used to encapsulate the drug. Example of a dendrimer structure is depicted in Fig. 2.

There are two approaches for dendrimer synthesis; divergent and convergent. In the former approach, there is a radial expansion by chronological addition of monomers, with each addition constituting a new generation in dendrimer synthesis. In order to achieve stable and flawless dendrimers, it is essential to complete each reaction (individual monomer addition) prior to incorporation of new generations [25]. This approach of synthesis offers the advantage of surface modification for functionalities in the terminal step. Disadvantages include time-consuming purification process and increased risk of defect as next generation of monomers are added. Despite the disadvantages, this is the most commonly employed method of dendrimer synthesis [25]. The convergent method is the opposite of divergent method wherein the synthesis is initiated from the surface. By integrating various branching points, chains that are responsible for dendrimer formation are prepared and later attached to the common center point. This method overcomes the disadvantages associated with divergent approach, and offers easier purification, high monodispersity, and fewer branch defects. However, this method has drawbacks such as lower yield and the possibility of steric hindrance while attaching the branches to the core [25, 27].

Dendrimers have been made from a variety of polymers such as polyamidoamine (PAMAM), glycogen, poly(propylene imine) (PPI), poly-L-lysine (PLL), poly(ethylene glycol) (PEG), and the copolymers N-(2-hydroxypropyl)

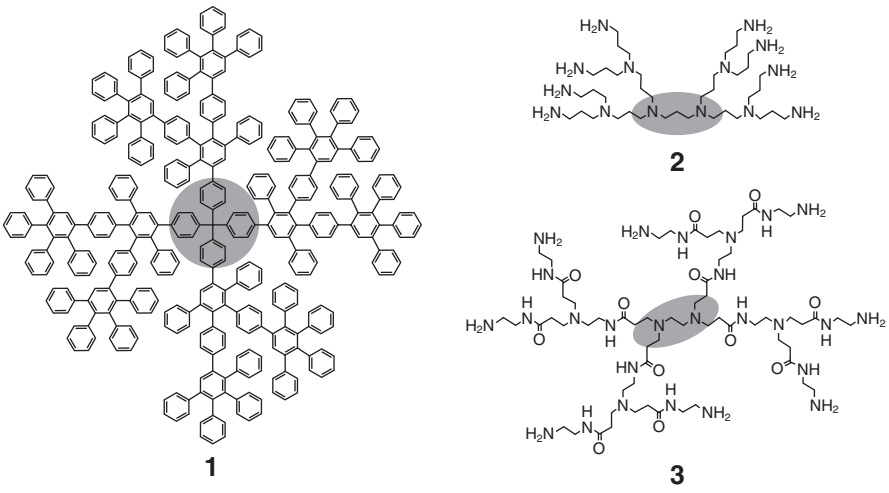

Fig. 2 Chemical structures of a second-generation polyphenylene [1], poly(propylene imine) [2], and polyamidoamine [3] dendrimers (Adopted from [26]). The shaded area represents the core of the dendrimers

methacrylamide (pHPMA), poly(2-oxazolines) and polyphenylene [25–29]. PAMAM dendrimers are most commonly employed therapeutically [25] and the dendrimer core is mostly made of ethylenediamine but can also be made of more lipophilic moieties such as diaminododecane, diaminohexane, and diaminobutane [25, 30–32]. Examples of dendrimer based carrier systems that have been investigated for various indications are listed in Table 2. Recent development in the area include targeting triple negative breast cancer with a bifunctional poly(amidoamine) dendrimer [33]. Liu et al. 2019 have reported doxorubicin (DOX) encapsulating delivery system consisting of EGFR-binding peptide 1 and the cell penetrating peptide obtained from trans-activating transcriptional activator conjugated to PAMAM dendrimer. It is a dual-functional dendrimer delivery system with enhanced efficacy compared to free DOX in cell lines and nude mice [33]. However, dendrimers as drug delivery systems are still in its infancy with only one product currently in the market, VivaGel® by Starpharma.

2.3 Liposomes

Liposomes are lipid-based vesicles that are synthesized by the hydration of dry phospholipids and are the most advanced nanoformulations with few approved products in the market and those undergoing clinical trials. Liposomes contain a hydrophilic core and hydrophobic corona made up of phospholipids [40–43]. Phospholipids can be made of phosphatidyl-choline (PC), phosphatidyl ethanolamine (PE), or 1,2-dioleoyl-3-trimethylammonium-propane [44]. Liposomes can be classified as unilamellar or multilamellar depending on the number of lipid bilayers. Figure 3 shows the schematic representation of liposome self-assembly. Unilamellar vesicles consist of a single lipid bilayer with an aqueous core, while multilamellar vesicles consist of several lipid bilayers separated by aqueous spaces between them. Hydrophilic drugs are entrapped in the aqueous core/spaces and hydrophobic drugs are entrapped in the lipid bilayer. The lipid bilayer can further be decorated with ligands for targeted drug delivery.

Liposomes can also be classified based on their functionality; such as conventional, stealth ligand-targeted, long-release, triggered-release, and multi-functional liposomes [40]. Shorter circulation time and formulation instability are common

Table 2 Examples of dendrimer based carrier systems for various indications

Polymer	Generation	Payload	Application	Reference
PAMAM	G2	Doxorubicin + siBCL-2	B-cell lymphoma	34
	G2	Paclitaxel + siTR3	Pancreatic cancer	35
	G3	Doxorubicin + shMMP-9	Breast cancer	36
	G4	Doxorubicin + siGFP	–	37
	G5	Doxorubicin + siMVP	Breast cancer	38
PPI	G5	Paclitaxel + siCD44	Ovarian cancer	39

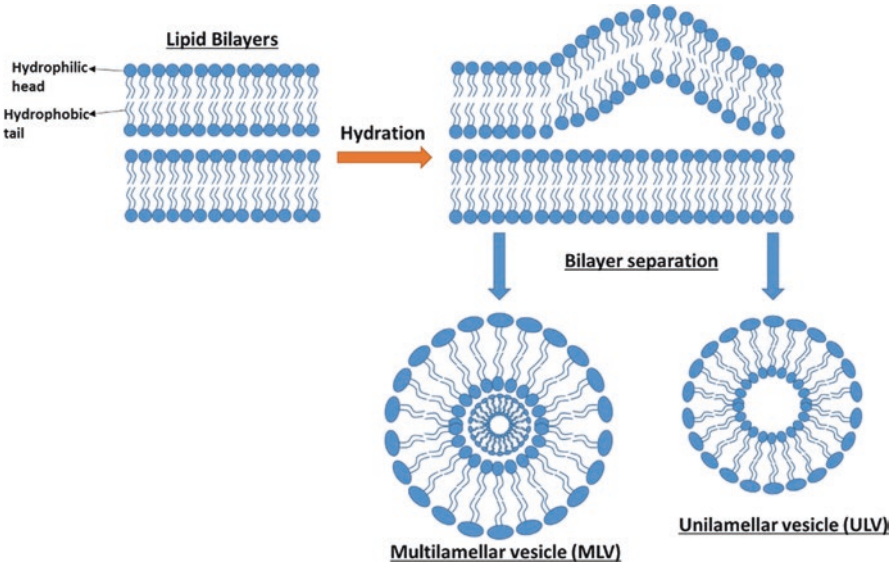

Fig. 3 Schematic representation of liposome self-assembly (Adapted from [40])

drawbacks associated with liposomes; several efforts have been made to increase the circulation time of liposomes and has led to the development of surface modified liposomes [42], such as long-circulation stealth liposomes, immuno liposomes, magneto liposomes, cationic liposomes, pH sensitive lipososmes [42, 44]. Parenterally administered liposomes are readily cleared by liver and spleen through the reticuloendothelial system (RES) [45]. Surface modification with hydrophilic polymer such as PEG has been extensively evaluated and is effective in avoiding clearance by the RES [45]. An example of surface modified liposomes is the PEGylated liposomes of Doxorubicin (Doxil®), which has shown potent beneficial pharmacological effect with minimal side effects [45]. Yang et al., 2007, has reported PEGylated freeze-dried liposomes of paclitaxel (PTX) containing tween 80 (3% (v/v)) and sucrose as a cryoprotectant [46]. The resulting formulation showed an increase in solubility from 1.6 μg/mL to 3.39 mg/mL [46]. Further, PEGylation of the liposomes increased the blood circulation half-life and thereby made it a more potent tumor growth suppressor [46]. Similarly, use of cationic liposomes for targeted tumor delivery of anti-angiogenic agent has gathered significant attention and can be attributed to distribution of angiogenic blood vessels [47]. Monpara et al. 2018 has reported PTX encapsulated cationic liposomes based on a derivative of cholesterol, Cholesteryl Arginine Ethylester (CAE) [47]. Molecular dynamic simulation and *in vitro* testing was used for understanding the interaction between PTX and CAE [47]. PTX encapsulated CAE liposomes showed improved membrane stability and drug loading, compared to PTX encapsulated cholesterol-based liposomes [47]. The improved stability could be attributed to hydrogen binding between CAE and PTX [47]. Further, in comparison to cholesterol-based liposomes, the

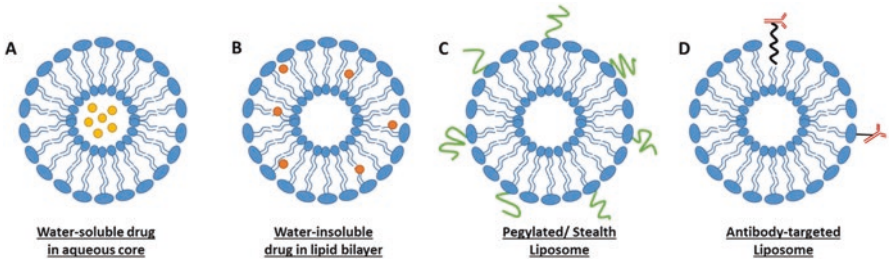

Fig. 4 Different types and modifications of liposomes; (**A**): Conventional phospholipid liposome with water-soluble drug entrapped in the hydrophilic center, (**B**): Conventional phospholipid liposome with hydrophobic drug entrapped in the lipid bilayer, (**C**): Long-circulating/ Stealth liposomes (PEGylated), and (**D**): Long-circulating immunoliposomes with grafted antibody. (Adapted from [42])

CAE-based liposomes showed lower cytotoxicity profile and improved endothelial cell migration inhibition [47].

Figure 4 shows liposomes with different modifications. Liposomes can vary from nanometers to micrometers in size depending on the intended use; generally liposomes with ~100 nm size are utilized in medical applications [41]. Clinical uses and advantage of liposomes are well known; biocompatibility, amphiphilic nature, protecting the drug molecule from degradation, lower side effects, targeted drug delivery, easy modification of particle size and surface potential makes them a suitable candidate for drug delivery [42, 43].

Several methods have been used for liposome preparation and can be classified broadly into two types; bulk methods, involving movement of lipids from non-polar to polar phase and film methods, involving forming a thin film onto a substrate and its subsequent hydration [40]. Some of the methods used for the preparation of uni- and multi-lamellar vesicles include; thin film hydration, solvent spherule method, hydration of pro-liposomes (pre-made granules of phospholipids and drug), reverse phase evaporation, injection method, surfactant dialysis, size reduction techniques, supercritical fluids, freeze drying of double emulsion, and microfluidic methods [40]. Each method has its own advantages and disadvantages and are discussed in Chap. 2. Table 3 lists liposomal formulations approved for the treatment of various ailments or are currently undergoing clinical trials.

2.4 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are colloidal particles made of lipids, which can be solid at both ambient and physiological temperatures [8, 49]. SLNs were proposed to overcome the limitations and disadvantages associated with liposomes and other nanocarrier-based systems such as stability and shorter circulation time [49, 50]. Diameter of SLNs can vary from 40 to 1000 nm [8, 51]. Therefore, SLNs offer

Table 3 Nanoliposomal formulations approved for treatment or undergoing clinical trials [48]

Product name (Approval year)	Route of administration	Active ingredient	Lipid (Lipid: Drug molar ratio)	Indication	Company
Doxil® (1995)	Intravenous	Doxorubicin	HSPC:Cholesterol:PEG 2000-DSPE (56:39:5 molar ratio)	Ovarian, breast cancer, Kaposi's sarcoma	Sequus pharmaceuticals
Myocet® (2000)	Intravenous	Doxorubicin	EPC:Cholesterol (55:45 molar ratio)	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan pharmaceuticals
Mepact® (2004)	Intravenous	Mifamurtide	DOPS:POPC (3:7 molar ratio)	High-grade, resectable, non-metastatic osteosarcoma	Takeda pharmaceutical limited.
Marqibo® (2012)	Intravenous	Vincristine	SM:Cholesterol (60:40 molar ratio)	Acute lymphoblastic leukemia	Talon therapeutics, Inc
OnivydeTM (2015)	Intravenous	Irinotecan	DSPC:MPEG-2000:DSPE (3:2:0.015 molar ratio)	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack pharmaceuticals Inc.
Ambisome®(1997)	Intravenous	Amphotericin B	HSPC:DSPG:Cholesterol:Amphotericin B (2:0.8:1:0.4 molar ratio)	Presumed fungal infections	Astellas Pharma
Visudyne® (2000)	Intravenous	Verteporphin	Verteporphin:DMPC and EPG (1:8 molar ratio)	Choroidal neovascularisation	Novartis
DepoDurTM (2004)	Epidural	Morphine sulfate	DOPC, DPPG, Cholesterol and Triolein	Pain management	SkyPharma Inc.
Exparel® (2011)	Intravenous	Bupivacaine	DEPC, DPPG, Cholesterol and Tricaprylin	Pain management	Pacira pharmaceuticals, Inc.
Arikayce (2018)	Aerosol	Amikacin	DPPC and cholesterol	Lung infections	Transave Inc.
Stimuvax	Subcutaneous	Tecemotide	Cholesterol, DMPG, DPPC	Non-small cell lung cancer	Oncothyreon Inc.

(continued)

Table 3 (continued)

Product name (Approval year)	Route of administration	Active ingredient	Lipid (Lipid: Drug molar ratio)	Indication	Company
T4 N5 liposomal lotion	Topical	T4 endonuclease V	Egg lecithin	Xeroderma pigmentosum	AGI Dermatics Inc.
Liprostin	Intravenous	Prostaglandin E-1 (PGE-1)	Unknown	Restenosis after angioplasty	Endovasc Inc.
ThermoDox	Intravenous	Doxorubicin	DPPC, Myristoyl stearyl phosphatidylcholine and DSPE-*N*-[amino(polyethylene glycol)-2000]	Hepatocellular carcinoma and also recurring chest wall breast cancer	Celsion
Lipoplatin	Intravenous	Cisplatin	DPPG, soy phosphatidyl choline, mPEG-distearoyl phosphatidylethanolamine lipid conjugate and cholesterol	Non-small cell lung cancer	Regulon Inc.

advantages such as; stability, protection of encapsulated material from degradation, reduced toxicity (depending on the material of construction), controlled release, and easy scale-up [8, 49, 51, 52]. However, SLNs have a few disadvantages including reduced loading capacity, drug discharge after polymorphic transition upon storage and requiring to disperse in high water content (70–99.9%) [49, 51]. Variety of solid lipids can be employed for preparation of SLNs such as mono-, di- and tri-glycerides, fatty acids, waxes, steroids, surfactants providing stability, phospholipids, poloxamers, and polysorbates [8]. In case of SLNs, drug can be loaded either in the core, shell, or dispersed in the lipid matrix (Fig. 5) [8, 52–56]. Shell of SLNs can be modified with various compounds for improved targeting such as; proteins, oligosaccharides, ligands for receptors or antibodies [8, 57–60].

Numerous techniques have been developed for preparing SLNs such as; high pressure homogenization, microemulsification, solvent emulsification-evaporation

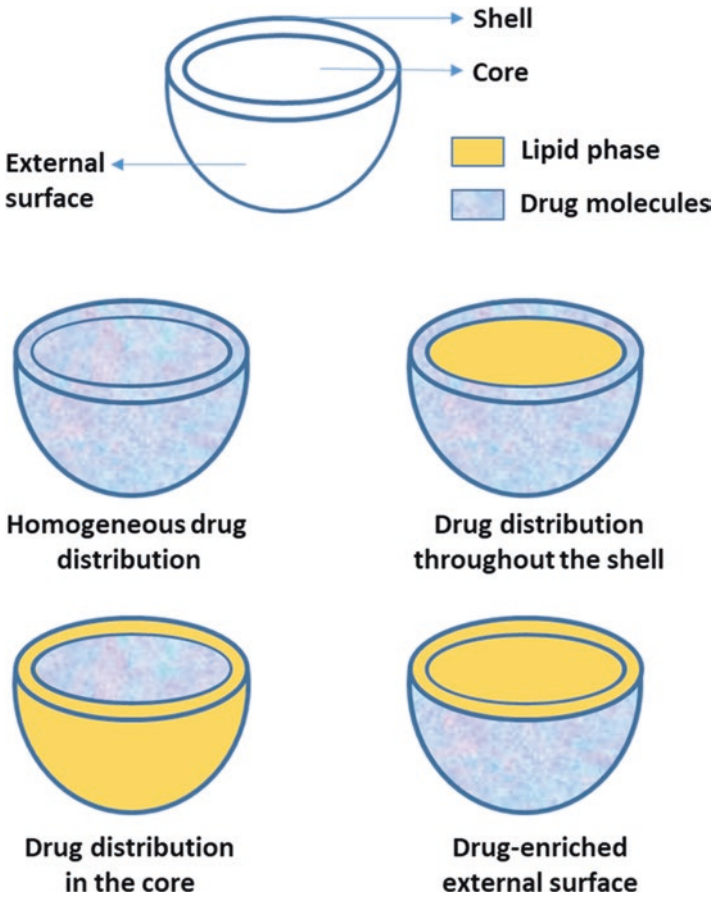

Fig. 5 Different loading patterns of SLNs (Adapted from [8])

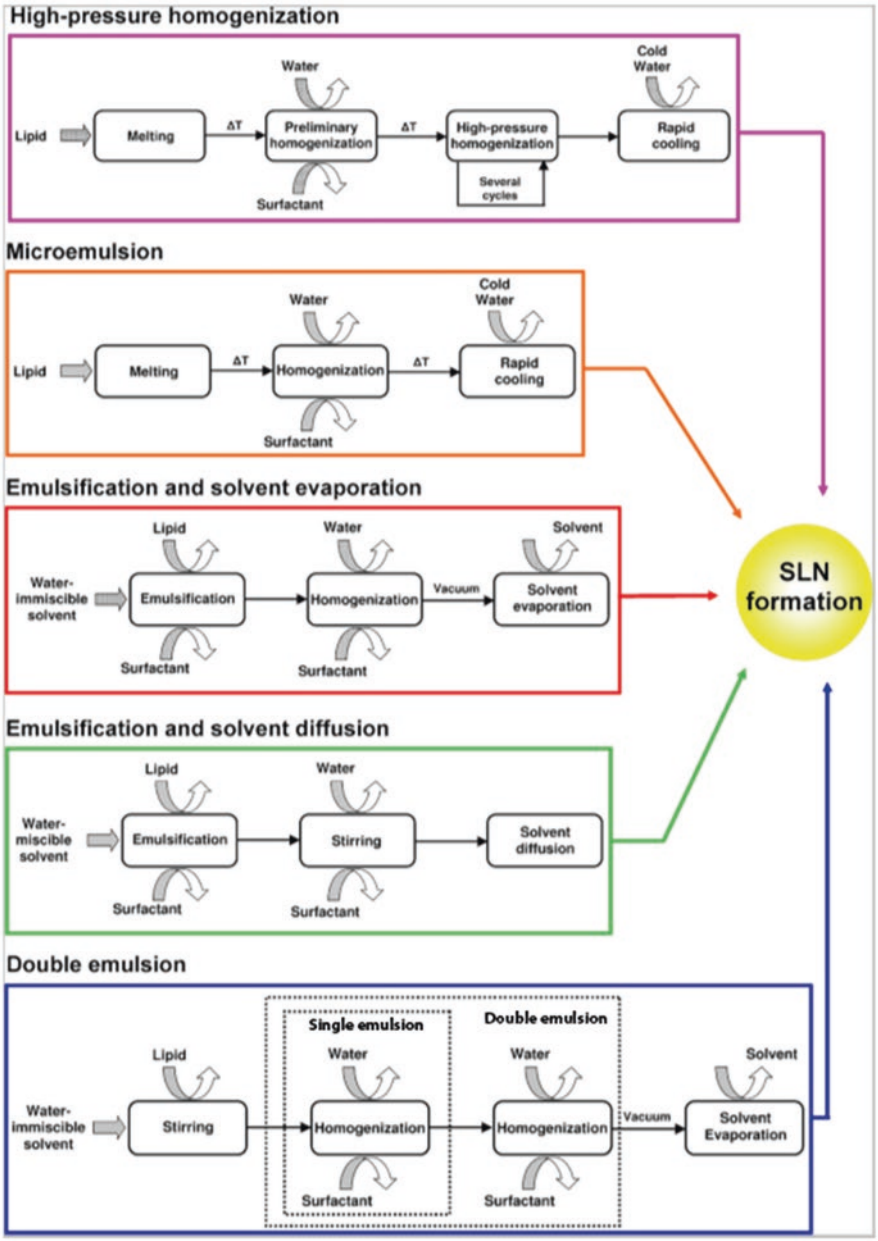

Fig. 6 Schematic representation of solid lipid nanoparticle (SLN) preparation techniques [8]

or diffusion, and double emulsion technique [8, 49, 52]. Figure 6 shows the schematic representation of these techniques.

The SLNs have been used for targeting vast variety of human malignancies, such as cancer and ocular drug delivery [61–63]. SLNs are taken up by endocytosis pathway followed by subcellular distribution which is essential for the biological effect of the biomolecule [63]. Arana et al. 2019 have reported enhanced antitumor activity of all-trans retinoic acid after encapsulation in phosphatidylethanolamine polyethylene glycol (PE-PEG) containing SLNs of stearic acid, Epikuron 200, and sodium taurodeoxycholate. In an oral adenocarcinoma cell line, these SLNs had improved active cell internalization and reduced non-specific internalization mechanisms [63]. Encapsulation of model drug all-trans retinoic acid into PE-PEG coated SLNs showed superior chemotoxicity compared to non-coated SLNs [63]. In another study by Eid et al. 2019, SLNs with stearic acid were formulated with ofloxacin for the treatment of local eye infections [61]. These SLNs were coated with chitosan (CTS) and polyethylene glycol (PEG) to improve corneal retention time and transcorneal bioavailability. Results showed that, compared to Oflox® drops (ofloxacin solution), CTS-PEG coated SLN encapsulated ofloxacin, prepared by modified emulsion/solvent evaporation technique, displayed better tolerability and two-to-three fold higher concentration at the site of action i.e. the eyes of rabbits [61]. Another example of coated SLNs is polysorbate 80 coated SLNs for the treatment of brain cancer [62]. Jain et al. 2019, have reported novel drug carrier system for delivery of doxorubicin (DOX) by encapsulating it into polysorbate 80 coated SLNs [62]. The polysorbate 80 coated DOX-loaded SLNs showed higher cytotoxicity and uptake in U87MG cell line (brain cancer cell line) compared to plain DOX. The coated SLNs could protect the incorporated DOX from RES uptake and P-gp efflux [62]. From the above studies, it can be inferred that surface coating of SLNs has the potential to improve its efficiency as a drug delivery vehicle [61–63].

2.5 Polymeric Nanoparticles

Polymeric nanoparticles (PNPs) are solid, colloidal particles with a size range of 10–1000 nm [64]. PNP is a communal term attributed to polymeric nanoparticles, but explicitly for nanospheres and nanocapsules [64, 65]. Nanospheres are matrix particles, which are completely solid and are generally spherical in shape and the drug can be either encapsulated or dispersed within the polymer matrix [64–66]. Nanocapsules are vesicular reservoir system, which acts as reservoir and wherein the drug is dispersed or dissolved in a liquid core (oil/water) that is enclosed by a polymer [64, 65, 67] (Fig. 7). Polymeric nanoparticles have many advantages including protection of the active substance by encapsulation, localization to specific tissues, controlled drug release, enhanced stability, and ease of surface modification.

Intended use of PNPs decides it's properties and the appropriate method of formulation [64]. Ideal PNP properties include desired targeting and controlled drug

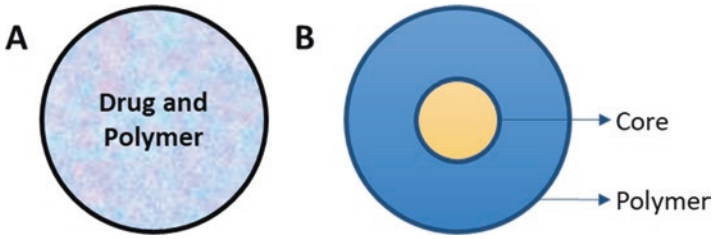

Fig. 7 Types of polymer nanoparticles, (**A**) Nanospheres, wherein the drug is distributed within the polymer matrix, and (**B**) Nanocapsules wherein the drug is dispersed or dissolved in the liquid core

release, and can be obtained by regulating features of PNPs such as size, solubility, improved flexibility, or/ and controlled release [65, 68]. Major component for PNP formulation is the polymer, there are two major types of polymers utilized for PNP preparation based on their origin; natural and synthetic [65]. Natural polymers commonly employed include; sodium alginate, albumin, chitosan, and gelatin [69–72]. Synthetic polymers utilized are; PLA [73–75], PLGA [76, 77], polyglycolides [78], polyanhydrides [79], polyorthoesters [80], polycyanoacrylates [81], PCL [82], poly (malic acid) [83], polyglutamic acid [83], poly (methyl methacrylate) [84, 85], poly (N-vinyl pyrrolidone) (PVP) [86], PVA [87], polyacrylamide [88], polyethylene glycol (PEG) [89, 90], polyacrylic acid (PAA or Carbomer) [91, 92], and poly (methacrylic acid) [93]. PNPs loaded with the drug are commonly prepared by solubilizing the drug and polymer into water immiscible solvent to produce a nanoemulsion and probe sonicated to generate the appropriate particle size range [68]. Rotary evaporation may be used for removing solvent followed by washing of nanoparticles to remove any residual solvent and particle collection by centrifugation [68]. However, nanoparticles can be prepared in various ways; an overview of different preparation techniques are presented in Chap. 2.

PNPs have been employed in delivering small molecules and macromolecules (e.g. proteins, genetic material, and antibodies). Al-Nemrawi et al. 2018 developed tobramycin encapsulated PLGA NPs for the treatment of *Pseudomonas aeruginosa* infections for cystic fibrosis [94]. Further, to increase the mucoadhesive properties of the PLGA-NPs, they coated the NPs with low molecular weight chitosan (LMWC). The LMWC coated tobramycin PLGA-NPs delayed the release of tobramycin over two days and displayed antimicrobial activity that increased with higher LMWC concentration [94]. In another study, Deacon et al. 2015 prepared tobramycin alginate/chitosan NPs; a lethal inoculum of *P. aeruginosa* in an animal model was cleared by the tobramycin NPs in a dose dependent manner. Further, to improve the NP penetration of CF sputum they functionalized the tobramycin NPs with dornase afla that exhibited anti-pseudomonal effects [95]. Polymeric nanoparticles have also proven to be effective in vaccine drug delivery. Muttil et al. 2010 successfully prepared dry powder formulation of recombinant hepatitis B surface antigen (rHBsAg) encapsulated PLGA/PEG NPs and demonstrated that high IgA titers can be achieved after immunizing guinea pigs by administering the dry powder to the

lungs [96]. Pulmonary route has also proven to be effective in diphtheria immunization; studies conducted by Muttil et al., 2010 demonstrated improved local and systemic immune response in guinea pigs with diphtheria CRM-197 antigen delivered via pulmonary route as dry powder [97].

Khademi et al. 2019 formulated cationic lipid-modified PLGA NPs for *Mycobacterium tuberculosis* HspX/EsxS fusion protein delivery with encapsulation rate up to 90% [98]. In another study, Kunda et al. 2015 reported the preparation of bovine serum albumin (BSA) adsorbed poly(glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL polymeric NPs for pulmonary vaccine delivery [99]. The BSA adsorbed NPs were spray-dried using L-leucine and displayed excellent aerosolization properties with the NPs facilitating enhanced uptake by dendritic cells to initiate a robust immune response [99]. Polymeric nanoparticles have also been used for the delivery of genetic material. For example, PLGA and PGA-co-PDL based nanoparticles have been reported for the delivery of miRNA [100]. The use of miRNA for targeting inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) is restricted due to the inability of neat negatively charged miRNA to cross anionic cell membranes and potential degradation by nucleases [100]. Mohamed et al. 2019 reported the preparation of positively charged PNPs using cationic lipid dioleoyltrimethylammonium propane (DOTAP). The authors adsorbed the negatively charged miRNA onto the positively charged PGA-co-PDL polymeric NPs. The positively charged PNPs increased miRNA adsorption compared to neutral and negatively charged PNPs; this led to an increased cellular uptake of PNPs by A549 cells, and also reduced the target gene IRAK1 expression. This data suggests that miRNA retained its biological activity after formulation and that PNPs can be a potential treatment option for COPD [100]. With these recent studies, it can be concluded that polymeric nanoparticles are an effective delivery tool for drugs, vaccines and genetic materials and should be pursued for early human trials.

3 Inorganic Nanoparticles

Inorganic nanoparticle-based drug delivery systems are finding greater application in simultaneous diagnosis and therapy for various diseases due to their easy modification and detection, high drug loading capacity, and stability. Modification of the particles is usually performed to enhance the interaction with the biological membranes [101]. Inorganic nanoparticles find use in molecular imaging techniques such as optical imaging, positron emission tomography, computed tomography, magnetic resonance imaging, and ultrasound [102]. In particular, gold and silver nanoparticles are used in the biomedical field owing to their inertness and high electron conductivity [103]; this chapter will briefly discuss these two types of inorganic nanoparticles and their use in drug delivery.

3.1 Gold Nanoparticles

Gold nanoparticles (Au-NPs) are inert, biocompatible, have high surface-to-volume ratio, can be functionalized with several molecules, and are the most stable metal nanoparticles. In addition, Au-NPs can be synthesized in various shapes including spheres, rod-like, core-shell and others. They are also capable of penetrating blood vessels and tissue barriers including the blood-brain barrier and can be targeted to specific cells using targeting ligands [104–107].

Gold has a high atomic number which allows high absorption and enhancement of ionizing radiation and is an excellent photon absorber for imaging applications. Gold radioisotope (Au198) has found great use in cancer radiotherapy. Further, Au NPs small size enables wide biodistribution and preferential accumulation at the tumor sites due to enhanced permeation and retention effect [105]. For example, Au-NPs functionalized with cetuximab, an immunotherapeutic agent, compared to cetuximab alone, had higher endocytosis, suppressed tumor cell proliferation and migration in the A549 lung cancer cell line [108]. Ramalingam et al. developed a delivery system by conjugating doxorubicin (dox) on the surface of AuNPs with polyvinylpyrrolidone (Dox@PVP-AuNPs). Dox@PVP-AuNPs increased ROS generation, sensitized mitochondrial membrane potential, upregulated the expression of tumor suppressor genes, and induced both early and late apoptosis in lung cancer cells (A549 human adenocarcinoma lung cancer cells, H460 human large-cell lung carcinoma cells, and H520 human squamous cell carcinoma cells). These results suggest that Dox@PVP-AuNPs showed enhanced inhibition of lung cancer cells growth compared to free doxorubicin and PVP-AuNPs [109].

Au-NPs are increasingly finding greater application in the field of vaccines with the shape determining the type of immune response generated [110]. Research by Niikura et al. showed that gold nanospheres induced enhanced secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6, IL-12, and granulocyte macrophage colony-stimulating factor (GM-CSF) whereas gold nanorods induced higher secretion of inflammasome-related cytokines, such as IL-1β and IL-18 [110].

Most Au-NPs synthesis strategies utilize Faraday's reaction wherein a solvated gold salt is chemically or electrochemically reduced in the presence of surface capping ligands. All Au-NPs synthesis require a reducing and stabilizing agent, and the major differences between methods are the types of chemicals used and the ratio between them. The most popular methods are the reduction of chloroauric acid and seeding growth procedure. Other physical methods include photochemistry, sonochemistry, radiolysis, and thermolysis [101, 105]. Table 4 discusses different physical, chemical, and biological methods used for the preparation of gold and silver nanoparticles. A more detailed description of these methods is available in a recent review by De Matteis et al. (2018) [111].

Table 4 Types of methods for synthesis of gold and silver nanoparticles [111, 113, 118]

Methods	Description	Advantages	Disadvantages
Physical methods	Types include evaporation-condensation, spark discharging, ultrasonic spray pyrolysis, thermal decomposition, and laser ablation	Speed, no hazardous chemicals used, radiation as reducing agent	Lack of uniform distribution, high energy consumption, low yield, solvent contamination
Chemical methods	Uses water or organic solvents to prepare the Ag-NPs. Mainly involves three components: (1) metal precursors, (2) reducing agents, and (3) stabilizing agents. Types include microemulsion, sol-gel, chemical reduction, sonochemical, and electrochemical synthetic method	Ease of production, low cost, high yield	Low purity, presence of toxic and hazardous chemicals used in the process
Biologic methods	Synthesis involves three main factors: (1) the solvent, (2) reducing agent, and (3) non-toxic material. Use of bacterial proteins and plant extracts to control the size, shape, and monodispersity	Simple, cost-effective, environment friendly, high yield, well defined size, solubility in water	Length of production

3.2 Silver Nanoparticles

Silver nanoparticles (Ag-NPs) are the most commonly used metallic nanoparticles in the biomedical field. They find applications in food and cosmetics, health care, medical, and industrial purposes due to their optical, electrical, thermal, and biological properties. Ag-NPs are used as antibacterial agents and anticancer agents [112, 113]. Ag-NPs upon exposure to water and oxygen release silver ions that are cytotoxic to microorganisms. In addition, factors such as surface chemistry, size, size distribution, shape, surface morphology, particle composition, coating, agglomeration, dissolution rate, particle reactivity in solution, and cell type that the Ag NPs interact with, all could determine the biological activity of Ag NPs [113, 114]. Therefore, development of Ag-NPs with defined properties are essential for demonstrating consistent results and their wider use in the biomedical field.

Ag-NPs are prepared by biological, physical, or chemical methods. The physical and chemical methods are hazardous and very expensive whereas the biological method is simple, rapid, non-toxic, and dependable with Ag-NPs that have high stability [113, 115]. He et al. demonstrated the cytotoxicity of green-synthesized Ag-NPs to human lung cancer H1299 cells *in vitro* and *in vivo*. The effects of Ag-NPs on H1299 cells were correlated with the inhibition of NF-κB activity, a decrease in bcl-2, and an increase in caspase-3 and survivin (apoptosis inhibitor) expression. Further, the H1299 tumor growth in a xenograft severe combined immunodeficient (SCID) mouse model was significantly suppressed after treatment with Ag-NPs [116]. In another study, Yang et al. prepared uniform and stable curcumin modified Ag-NPs (cAgNPs), where the source of curcumin, *Curcuma longa*, acted

as a reducing and capping agent. The cAgNPs showed a higher inhibition effect of respiratory syncytial virus (RSV) infection with two orders of magnitude decrease in viral titers at a concentration that was non-toxic to host cells [117].

4 Conclusion

The application of nanotechnology for medical diagnosis and treatment in the biomedical and pharmaceutical industry has significantly increased over the last few years; this is reflected by the increasing number of clinical trials being conducted on nanopharmaceutical products, and their subsequent approval for human use. As discussed in this chapter, as well as in subsequent chapters in the book, extensive research in the field of nano-sized particles has led to the development of novel drug delivery systems based on polymeric nanoparticles, liposomes, dendrimers, solid lipid nanoparticles, silver and gold nanoparticles; these delivery systems are finding important applications in human diagnostics and therapeutics. However, with the advancements in complex nano-delivery systems that are capable of being multifunctional, there are new challenges that need to be addressed before nanoparticles can be widely accepted by the medical community. Some of these challenges include particle toxicity, uncontrolled drug release, unconventional distribution and behavior inside the body, to name a few. In the near future, we will hopefully see the tremendous potential of nanomedicine based therapeutic and diagnostic agents in the clinic as these challenges are addressed by the research community.

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Methods to Characterize Nanoparticles for Mucosal Drug Delivery

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Abstract Physicochemical properties of the nanoparticles are associated to their *in-vivo* behavior including pharmacokinetic, bio-distribution, efficacy, and toxicity profiles. It is imperative to gain a comprehensive understanding of the nanoparticle properties through their characterization. Characterization of nanomaterials depends upon their unique physical and chemical properties with different level of complexity at molecular levels. Distinct properties of nanoparticles often hinder when standard methods of characterization of particles are used, which compromise the reliability and reproducibility of the outcome. Nano-therapeutics characterization depends on various aspects, including the encapsulated drug, delivery vehicles, disease, route of administration, dosing amount and its application. The precise control over nanoparticle properties need robust and advanced characterization techniques. Generally, characterization of nanoparticles is based on the composition, size- distribution, morphology, surface charge, purity and stability, using sophisticated techniques such as dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) etc. Mean particle size, morphology and surface charge of nanoparticles affect their physical stability, re-dispersibility and *in-vivo* biodistribution. This chapter summarizes the basic principles, associated challenges and practical concerns in standard and promising physicochemical techniques used for characterization of nanoparticles.

Keywords Nano particles · Mucosal delivery · Characterization · Composition · Morphology · Size · Charge · Interaction · Stability

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1 Introduction

Nanotechnology has several conceivable benefits to pharmaceutical research by making medicines more effective and minimizing their side effects. Nanoparticles have wide ranging implications in diverse field of science and technology including medicine, biotechnology, material science, etc. [1]. Designing precise drug delivery systems has long been a major challenge for pharmaceutical researchers. Further, drug delivery via mucosal sites offers several advantages over the traditional parenteral administration. However, it is challenging for several nanoparticulate formulations to cross the mucosal barrier and reach their target site in the deeper tissues or after subsequent absorption to the systemic circulation. Thus, evolving nanoformulation technologies explore several strategies that would enhance interaction with the mucus surface and the epithelial cell layer in order to achieve high drug levels at the target site [2]. Therefore, comprehensive knowledge about nanoparticle characterization is essential in order to improve current approaches and to develop new delivery systems to lower the barrier for improving mucosal delivery. In order to overcome these barriers, nanoformulations need to have several features like mucoadhesive properties, membrane permeation, cellular uptake augmenting, and drug release governing properties.

In order to gain a comprehensive understanding of the composition and nature of the nanoparticles, researchers first need to have enough information on the available nanoparticles characterization techniques. Characterization of nanoparticle proposed for therapeutic or diagnostic applications is complicated due to the wide variability of materials used for their preparation. Further, the multi-functionality and distinctive surface properties of nanoparticles makes characterization even more difficult by standardized methods. Several nanoparticles like colloidal gold-particles, silver–particles, quantum-dots etc. have optical properties which interferes with calorimetric assays and potentially give false positive results [3]. Some nanoparticles like dendrimers, silicon, cadmium selenide, titanium dioxide etc. can have catalytic properties that interfere with enzymatic testing of nanoparticles. Polymeric nano-formulations may contain variable concentrations of surfactants to improve stability and dispersibility in liquid. The surfactant alters the surface tension of the medium and hence interferes in their characterization. Nanoparticles readily absorbs impurities from the medium due to their large surface area and surface charge, which gives inconsistent analytical results. Selectively delivering drugs to targeted disease site using nanoparticles can help to enhance the therapeutic effectiveness and reducing adverse effects in normal tissues. Several advanced nanoparticles are functionalized by specific targeting moieties that facilitate their precise recognition and effective delivery to target cells. These targeting moieties includes proteins, antibodies, peptides (arginine-glycine-aspartate; RGD), aptamers, polysaccharides, glycoproteins, folate etc. which are broadly used in developing multifunctional nanoparticles. However, the complexation of nanoparticles with targeting moieties further increase the complexity of delivery system and make their

Table 1 Different methods to characterize nanoparticles

Parameters	Techniques
Particle size distribution	Dynamic light scattering (DLS), photon correlation spectroscopy (PCS), scanning electron microscopy, transmission electron microscopy, atomic force microscopy
Surface charge (zeta potential)	Laser doppler anemometry, Zeta potentiometer
Shape	Scanning electron microscopy, transmission electron microscopy, Atomic force microscopy, Coulter counter
Surface hydrophobicity	X-ray photoelectron spectroscopy, water contact angle measurement Hydrophobic interaction chromatography
Surface properties	Static secondary-ion mass spectrometry
Surface area	Brunauer–Emmett–teller (BET) Analyzer
Crystallinity	X-ray diffraction, Small angle X-ray scattering (SAXS), differential scanning calorimetry thermal analysis (DSC-TGA)
Density	Helium compression pycnometry
Drug loading and release	Dialysis membrane *in-vitro* drug release assay using analytical techniques, such as HPLC, UV, *etc*

characterization more challenging. These complications therefore tend to impede the development of standard characterization methods for nanoparticles.

For nanomaterials, unfortunately there is a lack of known regulatory protocol and standardized set of detection and characterization methods [4]. Nanoparticle researchers usually establish their own characterization and quality control methods for their nanoparticles. Regulatory authorities therefore face various issues in interpreting and evaluation of characterization data without any substantial reference to published literature. These issues further complicate the approval process for nanoformulation for diagnostics and therapeutics.

Physicochemical characterization of nanoparticles such as shape, size, surface area, chemical composition, aggregation, surface functionality, stability etc. can provide better understanding of structure–activity relationships and biological activity *in vivo*. In this chapter, we will provide a comprehensive description of various nanoparticle characterization technique and are out lined in Table 1 [2, 5, 6].

2 Nanoparticle Characterization Parameters

The basic of nanotechnology lies in the fact that properties of materials change significantly when particle size is reduced to nanoscale range. The behavior of nanoparticles is fundamentally different from their bulk counterparts due to the change in surface-to-volume ratio and enhancement of quantum properties. However, measuring this aspect is difficult and pose challenges to researchers. Physicochemical properties, like particle size, surface zeta potential, shape, surface

properties, adsorption potential, molecular weight, composition, solubility, stability, purity, identity, aggregation, wettability, porosity, distribution of conjugated moieties, and impurities are critically relevant to physiological interaction in biological systems. It is therefore crucial to understand various nanoparticle properties based on their different features by reliable and robust characterization techniques: as *in-vivo* physiological interaction behavior of particles may influence the therapeutic efficacy and diagnostic accuracy in medical applications.

2.1 Particle Size Distribution

Most of the unique properties of nanoparticles are size-dependent which do not exist unless the size of the particle is decreased to nano-dimensions. The surface-to-volume ratio along with quantum effects of nanomaterials exhibits several size-dependent phenomena like optical, electronic, magnetic and mechanical properties. The particle size plays an important role in inherent nanoparticle properties and hence it is an essential task in the characterization of nanoparticles. The particle size distribution (PSD) of the nanoparticle governs biological fate that could affect physiological processes including deposition, distribution, targeting, metabolism and toxicity. For the development of drug delivery systems, the size of nanoparticles plays a major role as particle has to navigate from the administration site to the targeted site via various biological barriers. Subsequently, after administration of the nanoparticle formulation, it undergoes a biodistribution step and reaches the different organs or target site according to their size. To achieve better biodistribution of drug encapsulated in nanoparticles via crossing epithelial barriers to the target site, nanoparticles <1 μm are preferred. Depending upon their size, nanoparticles could escape from the systemic circulation through openings of endothelial barrier called as fenestrations. It has also been demonstrated that nanoparticles 200 nm or larger could activate the lymphatic system and are cleared from circulation faster. Micron sized particles (1–5 μm) are majorly cleared through mononuclear phagocytic system cells, whereas 150–300 nm sized particles could be found in liver and spleen. Nanoparticles of 30–150 nm may also accumulate in the heart, kidney, and bone marrow. Smaller nanoparticles (5–10 nm) are rapidly cleared from systemic circulation while 10–70 nm diameter nanoparticles may penetrate capillary walls throughout the body [7]. In order to cross the endothelial barrier, nanoparticles should have size smaller than 150 nm. Desai et al. 1997 reported that nanoparticles of size 100 nm demonstrated a 2.5-fold more cellular uptake as compared to a 1 μm particle, and 6-times greater uptake than a 10 μm particles [8]. In various pathological conditions, vasculature structure undergoes several modifications, as in tumor grows where neo-vascularisation occurs and endothelium structure becomes discontinuous resulting in passage of larger nanoparticles (200–780 nm) accross the barrier [9, 10]. Therefore the nanoparticles need to have an optimum size which can deliver sufficient drug and further evade the immediate clearance by the lymphatic system.

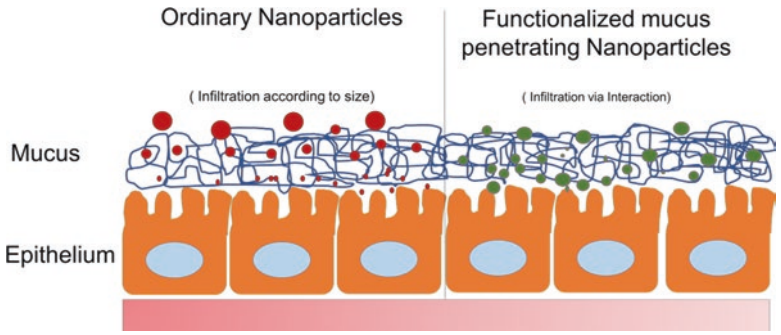

Fig. 1 Translocation permeability of nanoparticulate drug delivery systems across mucosal barriers

For mucosal delivery, particulate delivery systems could be trapped in mucus layers (lung airways, gastrointestinal tract, vagina, eye etc) by steric barrier or adhesion. The thickness of the mucus layer in humans could vary depending on its site: for instance stomach has 50–600 μm and 15–450 μm in intestine [11]. These particles are normally removed quickly from the mucosal tissue and hence prevent delivery of drugs in these areas. Mucus is a dense porous structure composed of cross linked mucin fibers by hydrophobic interactions. Mucus show diverse pore size ranging from ~10 to 1800 nm depending on its site in the body, for example, the mean mesh pore size of human intestinal mucus pore size ~200 nm, human vaginal mucus ~350 nm, cystic fibrosis lung mucus ~140 nm, bovine vitreous ~500 nm. Hence, in order to penetrate and infiltrate mucus, particles need to have an optimum size to evade any obstruction [5, 12] (Fig. 1).

2.2 Surface Area

Surface area is another important parameter for therapeutic nanoparticles as it affects reactivity and surface interactions with ligands. A decrease in particle-size leads to an exponential increase in surface area, and an increase in the availability of reactive surface. The high surface area of drug delivery systems can be achieved either by making small particles where the surface-to-volume ratio of particle is high or by developing materials with large number of voids compared to bulk material. Nanoparticles having high surface-to-volume ratio results in augmented surface reactivity, enhanced rate of dissolution, improved bioavailability and altered pharmacokinetic and toxicity profile. The interactions of nanoparticles with cells or microorganisms generally take place at the particle's surface, so the surface area is a major factor for possible therapeutic effects of nanoparticles. The larger surface area of nanoparticles dramatically enhances the equilibrium solubility, the rate of dissolution and generation of reactive oxygen species [13]. Further, the large surface

area allows for outer surface functionalization and can be done specifically for a particular receptor.

2.3 Shape

The shape of the nanoparticles is also an important design parameter that affects various biological phenomenon like movement of particles in systemic circulation, internalization by cells, physiological efficacy and degradation. Furthermore, the *in-vivo* circulation of nanomedicine can be altered by modifying the shape of drug loaded nanoparticles. Most of the nanoformulations are prepared in spherical shapes but advanced fabrication approaches have permitted the manufacture of wide-ranging shapes of nanocarriers with high precision. These shapes include rods, cubes, disks, ellipsoids, cylinders, hemispheres, cones, chains, biconcave discoids, dendrites etc. Irregular shapes show noteworthy influence on their transport through systemic circulation, their half-lives, cellular uptake and following intracellular targeting. Recent literature demonstrates the significance of shape of nanocarrier on many biological processes. Though, spherical structure is the most common shape in use, asymmetrical shapes could also be favorable in several occasion to enhance circulation time of the particles in vessels with decreased collisions against the vessel walls [14]. Asymmetric nanoparticles show different hydrodynamic behavior compared to spherical particles and are less susceptible to phagocytic clearance by the macrophages, which ease better sustained delivery of drugs [14].

Non-spherical systems demostrated varied biodistribution profiles compared to their spherical counterpart, providing a different approach for targeting specific sites. Yu et al. 2016 demonstrated that the nanoparticle shape can have significant role in the mucus-penetrating abilities. They showed nanorods penetrates faster in GIT mucus of rat as compared to spherical nanoparticles of equivalent size. This phenomenon was attributed to rotational movement enabled by the flow and the mesh size of mucus [15].

2.4 Zeta Potential

Dispersed nanoparticles in solution are located at different locations in the diffuse layer of liquid due to the electric potential difference, so the movement of particles in this layer of liquid is called slipping and shear plane. The measurement of potential at this plane is called zeta potential [16]. The zeta potential of the nanoparticles is an important factor as it affects the particle dispersion characteristics and influences the adsorption of ions and biomolecules. The surface charge of nanoparticles is approximated by zeta potential measurements which is the function of surface charge of nanoparticle, adsorbed molecules on its surface, and the ionic strength and composition of the surrounding solutions. Therefore, the storage stability of

colloidal nanoparticles dispersion is mainly dependent on its zeta potential. Electrostatic interaction of nanocarriers is controlled by modification in their surface charges, that is analyzed by measuring the zeta potential of nanoparticles [17]. To achieve higher stability and to avoid aggregation of nanoparticles, higher value of zeta potential (either negative or positive) is preferred. The measured value of zeta potential indicates the surface hydrophobicity, the nature of the material encapsulated in nanoparticles and coating properties of the surface [13, 18]. Surface of nanocarrier is an important concern in targeting drug delivery systems. Un-functionalized nanoparticles having neutral or negative charge are swiftly opsonized and cleared by macrophages. Surface functionalization of nano-formulations is a common practice to evade the opsonization phenomenon and to maintain sustained drug delivery in systemic circulation. Appropriate zeta potential can enhance the drug targeting, its release profiles and stability of the nano-particulate formulations. It is demonstrated that nanoparticle with high surface charge and large particle size are engulfed more efficiently by macrophage. Small difference in surface charge and size has significant effect on cellular uptake. Nanoparticles with adequate hydrophilicity and uncharged surface can efficiently diminish the positive interactions between mucus and particles by decreasing electrostatic interactions.

2.5 Surface Properties

The surface properties of nanoparticles are functions of molecular or atomic compositions, charge and functional groups present on the surface which are responsible for the interaction with the surrounding environment. The surface properties of nanoparticles are intrinsically relevant to the superficial layer, but not to the overall bulk material. Surface properties of nanoparticles have potential effects on physiological barrier penetration, receptor binding, dispersion stability and aggregation. The manipulation of surface properties of nanoparticles is another prospect to design superior nanocarrier systems. To design an ideal nanocarrier system, the functionalization of particle surface with suitable targeting moieties, modification of surface hydrophobicity and reactivity can be useful to address aggregation, stability, and receptor binding. In multifunctional nanoparticles, functional moieties are conjugated with the surface to bind to target receptor in specific tissues/organs. The selection of materials and their surface properties like hydrophobicity or cross-linking density can be important factors for the designing of a mucus penetrating particles. Pharmaceutical scientists have developed several surface engineering strategies to generate hydrophilic coating and lessen the particle adhesion with mucus. Despite having negative charge on ther surface, hydrophobic nanoparticles like polystyrene particles diffuse into mucin hydrogel by hydrophobic interactions. Researchers have proposed various active targeting strategies to increase nanoparticle penetration through mucus, mucoadhesion and cellular uptake by covalently functionalizing specific targeting moieties on the surface of the nanocarriers [2, 12]. Lectins are commonly used ligands for enhanced mucoadhesion and cell

internalization via specific cell interactions [19]. Another promising strategy to increase nanoparticle penetration through mucus is the incorporation of mucolytic-enzymes on the surface of the particles [20]. The characteristic ability of multifunctional nanoparticles to combine therapeutic, targeting and imaging modalities is a key aspect of their versatility and anticipated specific clinical impact [15].

2.6 Composition and Purity

A vast variety of nanomaterials are being used to design and develop nanoformulations for therapeutic purpose. These nanomaterials includes polymers, lipids, proteins, DNA, metal and metal oxides, inorganic minerals or other organic compounds. The composition and purity of nanoparticles majorly influences the transport, delivery, pharmacokinetics and biodistribution. In therapeutic applications of nanoparticles, it is common to combine two or more types of materials to form a complex or conjugant. Each ingredient exhibits their own inherent physicochemical properties including solubility, size, shape, surface charge, hydrophobicity and aggregation tendencies which are designed for different therapeutic response. The potential interaction of these materials with biological systems like cell and tissues is relatively different from each other depending upon the nature of material. Hence efficacy and toxicity significantly depends on the actual composition of the nanoparticle formulation. The measurement of chemical composition of multifunctional nanocarriers is more complicated compared to single entities. The occurrence of impurities in nanoformulations may considerably affect efficacy or introduce adverse effects. In accordance, the purity analysis of nanoparticles through their chemical composition is necessary. Prior to nanoparticle formulation synthesis, proper purification processes must be performed to remove side products, residual manufacturing components and endotoxin contamination. The purity analysis of nanoparticles must be carried out to check the presence of solvents, chelates and free metals, precursors, dimers, unconjugated therapeutics or other agents. Appropriate methods and techniques to detect the presence of such unwanted entities are required to ensure the quality and purity of the nanoparticle preparations [4, 10].

2.7 Stability

Pharmaceutical stability refers to the retaining of the bio-physico-chemical properties for a stated period of time after its manufacturing. Various factors affecting the conventional single molecule pharmaceuticals stability are similar for the conventional nanoparticles, including moisture, temperature, particle/molecular size, pH, solvents, enzymatic degradation, exposure to different types of ionizing and non-ionizing radiation, and presence of other excipients and impurities [4]. The multifunctional nanoparticles have complex compositions, so the stability of all the

components included in nanoparticles is essential to achieve its potential biological function. If any of the components of the complex will prematurely release, then it will compromise the efficiency of the nanoparticles activity. Therefore, it is necessary to evaluate the *in vitro* stability of functional components in various physiological conditions including pH, temperature and ionic strength. Further, stability evaluation in non-physiological conditions is also important to check the effects of short term and long term storage, ultrafiltration, lyophilization, freeze-thawing, pH variation, thermal and light exposure [10].

2.8 Drug Release

Drug loading is the amount of drug bound or encapsulated per total mass of polymer. Drug loading capacity of nanoparticles and its release influences the dose of the drug. The drug release from the nano-formulation depends upon several aspects of the material including matrix, porosity, matrix degradation, pH, temperature etc. Further, drug release rate from nano-formulations depends on the solubility of drug, desorption of the adsorbed drug, diffusion via matrix, degradation and diffusion process. Determination of extent of drug release from nanoparticles and subsequent biodegradation of the matrix is very essential for development of successful nano-formulations. The amount of drug is quantified by the use of UV-Vis spectroscopy, high performance liquid chromatography (HPLC), or liquid chromatography mass spectrometry (LC-MS) [18]. The *in vitro* drug release from nanoparticles is determined by various methods like dialysis bag diffusion technique, side-by-side diffusion cells with biological or artificial membranes, reverse dialysis sac technique, ultrafiltration, ultracentrifugation, and centrifugal ultrafiltration. The release profile of drugs from nanoparticles depends upon the nature and type of the drug delivery system [21, 22]. When the drug is uniformly dissolved or distributed in the polymer matrix (e.g nano-spheres), the release majorly occurs by erosion or diffusion of the matrix. The adsorbed or weakly bound drugs on the large surface area of nanoparticles are rapidly released (burst released) compared to the incorporated drug. In heterogeneous systems (e.g nanocapsules), the release of drug is mainly governed by diffusion over the polymeric degradation mechanism. The rate of diffusion of drug is faster as compared to matrix degradation, and is less dependent on the type of polymer.

3 Methods of Characterization of Nanoparticles

The characterization of nanoparticles is mainly performed for the measurement of their size distribution, shape, morphology, average particle diameter, charge affects, surface and elemental analysis, thermal stability and optical properties. Size and surface morphology are determined by techniques such as transmission electron

microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM) and dynamic light scattering (DLS). Stability and shelf-life of nanomedicine depends upon surface properties, surface charge, composition and storage conditions. Other techniques like ultraviolet-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), small angle X-ray scattering (SAXS), fourier transform infrared spectroscopy (FTIR), mass spectrometry (MS), differential scanning calorimetry (DSC), thermo-gravimetric analysis (TGA), etc. will also be discussed. On the basis of characterization techniques and instrumentation, nanoparticle analysis can be categorized as described in the below sections.

3.1 Particle Size and Morphology Analysis

3.1.1 Dynamic Light Scattering (DLS) or Photon-Correlation Spectroscopy (PCS)

The therapeutic properties of nanoparticles are highly dependent on their size and their tendency to agglomerate. Various techniques like sieve analysis, electro resistance counting methods, optical counting methods, sedimentation techniques, acoustic spectroscopy, dynamic light scattering, laser diffraction methods, etc. are all useful for particle size determination. Amongst them dynamic light scattering (DLS) is most popular and frequently used technique for obtaining size distribution of nanoparticles [23]. DLS can measure particle size distribution of small particles or polymers at the submicron or nanometer scale in suspension, and emulsion form by using a monochromatic light source, *e. g.* laser. Nanoparticles in the liquid phase experience brownian motion which is inducted by the bombardment of solvent molecules. Monochromatic light exposure hits the moving nanoparticles in solution which leads to a shift in incident light wavelength (at a fixed scattering angle) and the extent of the shifting in wavelength is due to interferences of the scattered light that measures the size of the particle (Fig. 2A). When moving particles are illuminated with laser, the intensity of light fluctuates which depends upon the size of the particle. This random motion of the particle is modeled by the Stokes-Einstein equation which that links diffusion-coefficient measured by dynamic light scattering to particle size. This formula is most often used for particle size analysis.

$$D_h = \frac{k_B T}{3\pi\eta D_t}$$

D_h: Hydrodynamic diameter
D_t: Diffusion coefficient.
k_B: Boltzmann's constant
T: Temperature.
η: Solvent viscosity.

The major advantage of DLS is its short experimental time duration, ability to characterize diluted samples, accuracy in measurement of the hydrodynamic diameter of the monodisperse/polydisperse sample (Fig. 2B), lower apparatus cost and reproducible results and analyzing samples in a wide range of concentrations. However, DLS has limited utility for investigation of heterogeneous size samples and resolving the dimension measurements of a mixed sample population. It is also not suitable for accurately measuring the sizes of non-spherical nanomaterials. Griffiths et al. evaluated the interaction between nanoparticles and mucin, where they demonstrated that negatively charged and hydrophilic nanoparticles did not display any interaction with mucin while positively charged and hydrophobic nanoparticles illustrated a strong interaction. This study showed that the DLS technique is a potential screening tool to nanoparticle-mucin interactions [24].

3.1.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy determines the size, shape, surface morphology of agglomerated/ dispersed nanoparticles, and surface functionalization with direct visualization of the nanoparticles [18]. It shows detailed three-dimensional images

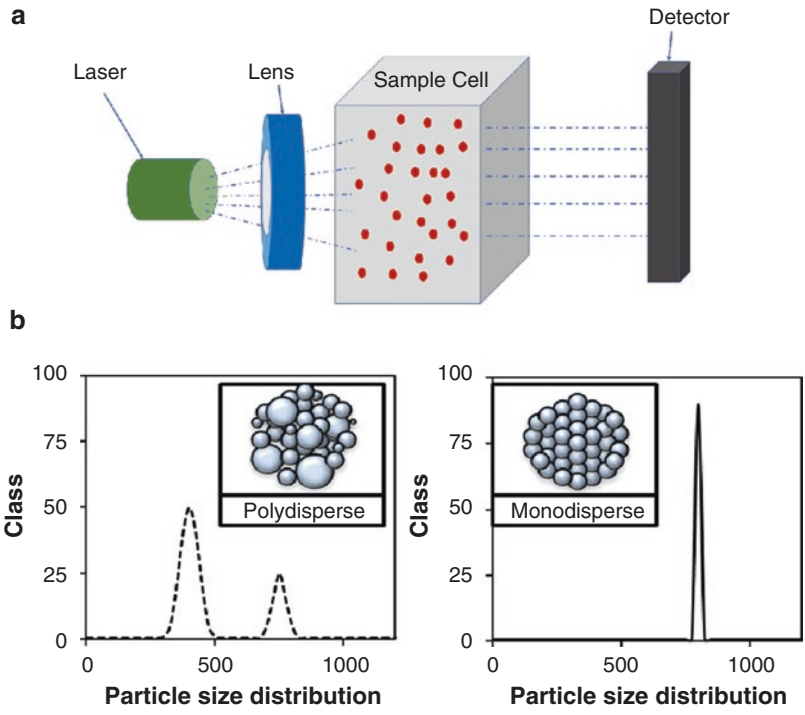

Fig. 2 **(A)** A schematic diagram of a dynamic light scattering instrument (**B**) Graph of two nanoparticle batches of a bimodal polydisperse population and a monodisperse population obtained after analysis by light scattering

of high magnification particles (upto 300,000X). The mean particle size obtained by SEM is comparable with the size obtained by dynamic light scattering. In contrast to optical microscopy, that uses a light source and lenses to observe the samples and to generate magnified images, SEM uses beams of accelerated electrons to generate higher magnification images. For SEM analysis, suspension of particles is mounted on a sample holder and dried, followed by coating of conductive metal (e.g. gold) using a sputter coater under vacuum. Nanoparticles are evaluated by scanning with a beam of electrons, and the secondary electrons/backscattered electron/characteristic X-rays generated from the specimen surface reflects the atomic composition and topographical information of the particles [25] (Fig. 3).

X-rays are the second most common imaging mode for SEM analysis, which gives information of element composition of nanoparticles. The specific technique is known as Energy Dispersive X-Rays (EDX) SEM. Back scattered electron generated from samples are also occasionally in SEM analysis for elements. The image that is displayed on the monitor is the distribution map of the intensity of the signals emitted from the scanned part of the sample. The major limitation of SEM is that it requires conductive surface of the sample to scan the surface by an electron beam. Many biological molecules and polymers have nonconductive surfaces that may acquire a static electric charge and insufficiently deflect the electron beam which

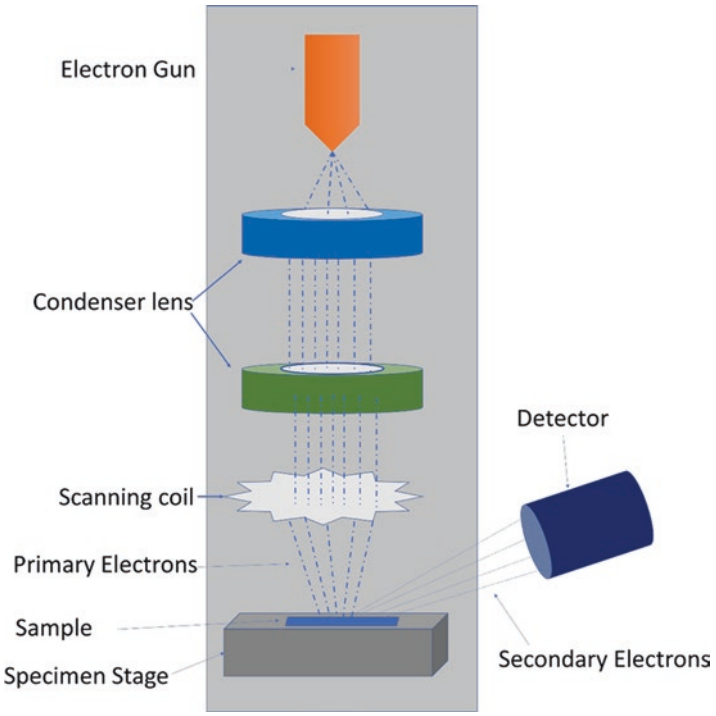

Fig. 3 Schematic of Scanning electron Microscope (SEM)

tends to generates artefacts or imaging faults. Therefore, nonconductive samples are coated with an ultrathin layer of electrically conductive material under high vacuum evaporation or low vacuum sputter coating.

Nonconductive samples may also be imaged by specialized "Environmental SEM"(ESEM) or in field emission gun SEM which is operated at high vacuum, low voltage or at high voltage, low vacuum [4]. Sometimes, electron beam can damage the nanoparticles or the biological samples. The process of drying and contrasting of nanoparticles may also cause shrinkage of the sample and therefore change the characteristics of the nanoparticles. More sophisticated instruments such as, field emission SEM uses narrower probing beams at high and low electron energy which gives better spatial resolution while reducing the sample destruction [25, 26]. However, this method is time consuming, expensive and often needs additional information about size distribution.

3.1.3 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a frequently used technique for the characterization of nanoparticles. It provides direct images and elemental information of nanoparticles at a spatial resolution down to the level of sub-nanometer/atomic dimensions. In the conventional TEM mode, an electron beam emitted from a source is accelerated at high voltage potential and transmitted through an ultrathin foil specimen. The incident electrons interact with the sample and transform to either unscattered electrons or elastically/inelastically scattered electrons [27]. The scattered or unscattered electrons are focused through various electromagnetic lenses and then projected on a screen to obtain electron diffraction pattern, which forms a phase contrast image, amplitude contrast image or a shadow image according to the density of unscattered electrons. The image is magnified and focused by adjusting the ratio of the distance between the specimen and objective lens (Fig. 4). Newer TEM are specifically equipped with the specimen holder which allows tilting the specimen at different angles in order to get specific diffraction patterns [28]. A range of analytical techniques can be coupled with TEM for different type of applications, for example (i) electronic structure of the nanoparticles that can be quantitatively investigated by chemical analyses of electron energy loss spectroscopy and (ii) chemical composition of the nanoparticles can be quantitatively investigated by energy dispersive X-ray spectroscopy [25, 28]. The TEM is widely applicable in biological sciences, material sciences and metallurgy. However, there are certain drawbacks of TEM, it requires thin layer samples (to transmit sufficient electrons to produce images) and high vacuum conditions which may lead to sample destruction. The preparation of extensive thin specimen enhances the chances of altering the structure and makes analysis a time consuming process.

The nanoparticles dispersion is deposited onto the support grids or films for characterization. TEM is useful in the measurement of particle size, aggregation/agglomeration, and dispersion, of nanoparticles (Fig. 5).

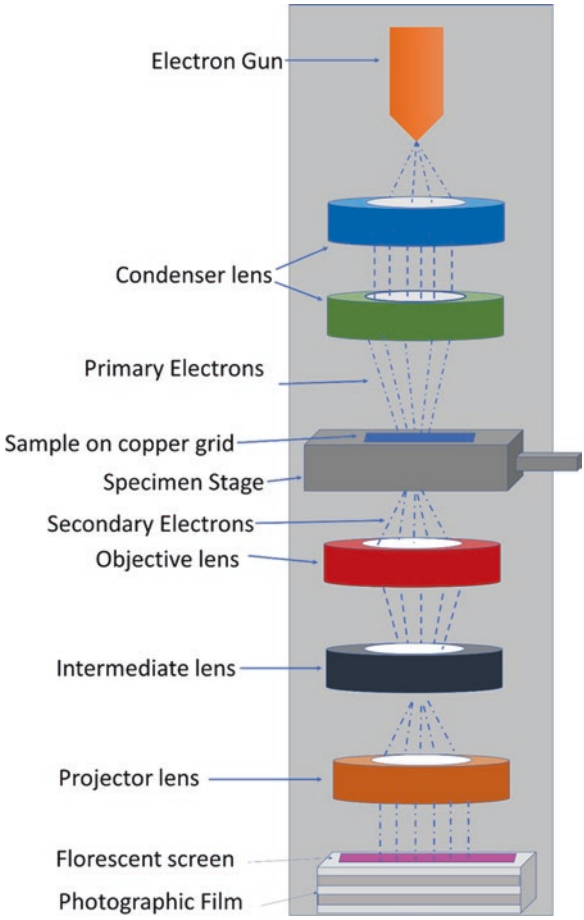

Fig. 4 Schematic diagram of transmission Electron Microscopy (TEM)

3.1.4 Atomic Force Microscopy (AFM)

In contrast to electron microscopy technique, AFM is a scanning probe technique/scanning force microscopy, which can divulge a range of information regarding the nanoparticles or biomolecules and its interaction on a single particle basis. The reported resolution of fractions of nanometer that is more than 100 times better than to optical diffraction limit. AFM is ideal for quantitative measurement of surface roughness and visualizing surface nano-texture of nanoparticles. It also helps in the determination of the size, shape, structure, aggregation and dispersion of nanoparticles. Due to its non-destructive analysis and high 3D spatial resolutions, it is a very useful tool in the analysis of conductive/nonconductive, dry/wet, soft/hard, or any other type of material in physiological conditions [25, 29]. AFM consist of a silicon/silicon nitride micro machined cantilever with a sharp tip (radius ~ 10 nm) attached at one end to detect whether the deflection of the cantilever tip occurred due to van-

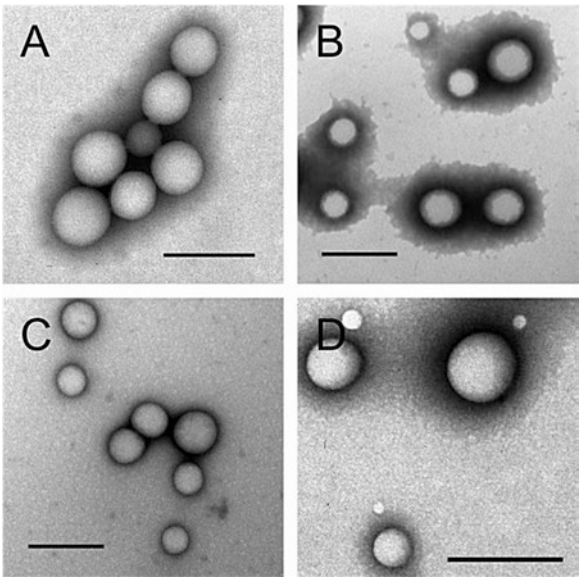

Fig. 5 PLGA nanoparticles observed by TEM: (**A**) stabilizer-free PLGA, (**B**) PLGA/PVA, (**C**) PLGA/Chitosan and (**D**) PLGA/PF68 (bar = 500 nm) (Reprinted with permission from ref. [26])

der Waals repulsion, electrostatic interaction and attraction between atoms at the tip and sample surface. The oscillating cantilever scans peaks and valleys over the sample surface in a vertical position and generates a topographical image of up to around 0.5 nm in vertical resolution [4]. During scanning of sample surface, the tip oscillates vertically and contacts the surface alternatively and lifts off, usually at a frequency of 50,000-5,00,000 cycles/s [1].

AFM has different scanning modes, includes dynamic/tapping (contact and intermittent contact with the sample) and static (noncontact) which provides details of various sample parameters like morphological information (size and shape) and elasticity parameters (Young's modulus, adhesion, and stretching) [29] (Fig. 6). However, the major limitation associated with AFM analysis is that the size of the cantilever tip and its geometry is larger than the dimensions of nanoparticles which leads to the widening of the lateral dimensions, which may leads to overestimation of size. AFM has several advantages over the SEM/TEM, which provides a two-dimensional projection of a sample; the AFM provides a three-dimensional surface profile. AFM is capable of producing a three-dimensional topography using just a single scan. In addition, samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would damage the nanomaterials. AFM also provides a greater level of detail for particle surfaces, as SEM is not as efficient in resolving the subtle changes on a highly smooth surface. As shown in Fig. 7, Cetin et al. synthesized Eudragit® L100/poly(lactic-co-glycolic acid) (PLGA) based nanoparticles and determined its size and morphology by AFM [30].

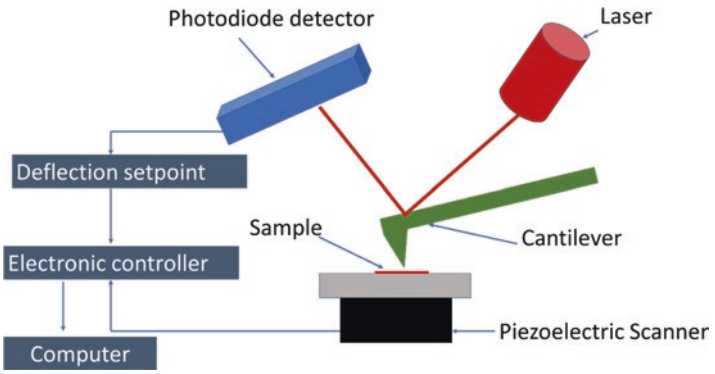

Fig. 6 Schematc diagram of Atomic Force Microscope (AFM)

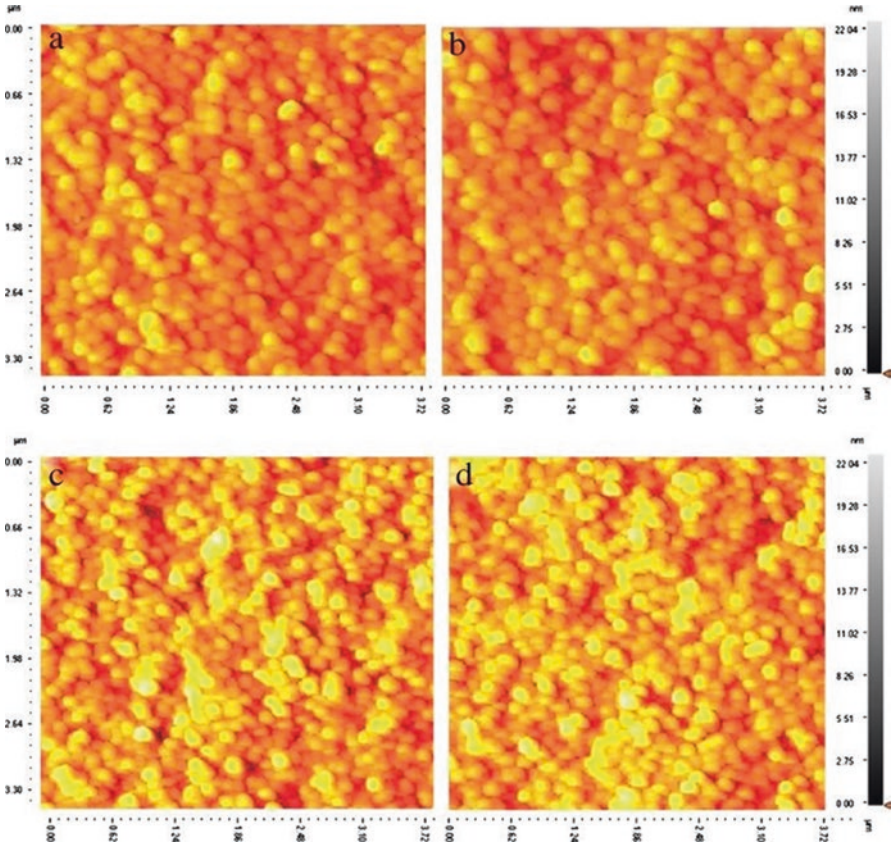

Fig. 7 AFM images of nanoparticles having various polymer ratio of Eudragit and PLGA (**a**) 20:80, (**b**) 30:70, (**c**) 50:50, and (**d**) Pure Eudragit (Reprinted with permission from ref. [30])

3.2 Surface Charge Analysis

3.2.1 Zeta Potential Measurement

As we have discussed previously, measurement of zeta potential is essential to know the stability of nanoparticles. It can be measured by various techniques, like electrophoretic light scattering, acoustic and electro-acoustic. Among these techniques, electrophoretic light scattering is frequently used because of its accuracy, sensitivity and versatility. Moreover, it can simultaneously determine the velocities of many charged species in the sample. The classical electrophoretic light scattering transmits light and receives at a small scattering angle (typically 8–30°). It is generally determined by measuring the velocity of the charged molecules towards the electrode in the sample solution due to the presence of an external electric field, which is proportional to the ζ potential, and the electrophoretic mobility of nanoparticles is measured by laser Doppler velocimetry (LDV). However, this technique is not suitable for turbid samples because the incident light cannot penetrate the samples. Zeta potential is not only dependent on the surface charge of the nanoparticles but also affected by their surrounding environment like ionic strength, temperature, pH, radiation, nature of the surface ligands and types of ions in the suspension. Therefore, in some cases, when we measure the zeta potential of suspended particles after dilution to produce high resolution and accurate results, it may differ greatly from their original values in that particular environment and may mislead the user [16, 31, 32]. Usually, higher zeta potential value > ± 30 mV (strongly anionic or cationic) is chosen to infer the particle is stable, whereas a lower value of zeta potential < ± 30 mV indicates a condition towards aggregation, instability, coagulation or flocculation. Nanoparticles with a zeta potential of – 10 mV to +10 mV are considered as neutral [17, 31]. Ngo et al. synthesized gold nanoparticle using of citrate as reducing and stabilising agent and obtained the higher negative zeta potential value −23.9 mV which shows the higher stability of gold nanoparticles [33] (Fig. 8).

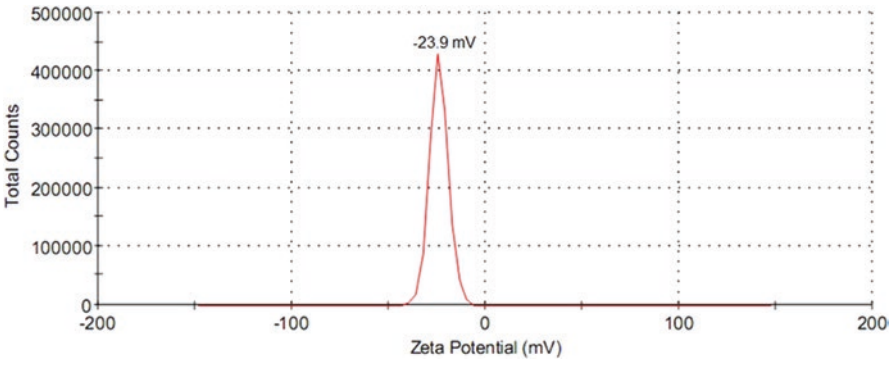

Fig. 8 Zeta potential graph of synthesized gold nanoparticles (Reprinted with permission from ref. [33])

3.3 Surface Area and Porosity

3.3.1 Brunauer, Emmett, and Teller (BET) Analysis

Surface area and porosity are two important physical properties that influence the quality of nanomedicine. The specific surface area of the nanoparticles is the summation of the exposed areas per unit mass. Particle size has inverse relationship with surface area. Several unique properties of nanoparticles are due to their large surface-to-volume ratio. Surface area and porosity properties are also relatable to efficacy and toxicity of nanoparticles. It is therefore important to precisely measure surface area for nano-material characterization. The method of Brunauer, Emmett, and Teller (BET) is used to evaluate the total surface area of nanomaterials. The BET theory assesses the gas-adsorption data and creates aspecific-surface area results which are then expressed in units of area per mass of sample (m^2/g). The actual surface area including surface pores cannot be estimated from particle size and shape information. Rather, it is determined at the atomic level by the adsorption of an inert gas. Nitrogen adsorption is commonly used to measure the specific surface area of particles. Amount of nitrogen adsorbed not only depends upon exposed surface but also on the temperature, gas pressure and strength of interaction between the gas and solid. Generally, interaction between gas and solid surface is low, the surface need to be cooled using liquid N_2 to have sufficient detectable quantities of adsorption. As adsorption layers are formed, the sample is taken out from the nitrogen atmosphere and heated to release the adsorbed nitrogen from the nanomaterial and quantified. The data is presented in the form of BET isotherms, which plots the quantity of gas adsorbed on material vs relative pressure. These isotherms may be of different shapes depending upon adsorbent, adsorbate and their interaction. Generally, five types of adsorption-isotherms are used. Type I shows monolayer adsorption and easily explained using Langmuir adsorption theory. This type of isotherm is characterstics of microporous material (pore diameters less than 2 nm) having relatively small external surfaces. Materials like charcoal, molecular sieve zeolites, Metallic Organic Framework (MOFs) and some porous oxides exhibits this type of isotherm. Type II isotherm represents unrestricted monolayer-multilayer adsorption which is charactersics of non-porous or macroporous material like Iron (Fe) catalyst and silica gel. The midway flat region of the isotherm represent the monolayer formation. Type III isotherm explains the formation of unrestricted multilayer. Here lateral interactions between gas molecules are strong compared the interactions between the material surface and adsorbate. Such materials including iodine, bromine, etc. Adsorption on mesoporous materials continues with multilayer adsorption followed by capillary condensation. Mesoporous materials with pore size ranging 2–50 nm, gives type-IV of isotherm. It displays the formation of a monolayer after development of multilayers. Type V isotherms are very similar to type IV isotherms but have relatively weak adsorbate-adsorbent interaction [34] (Fig. 9).

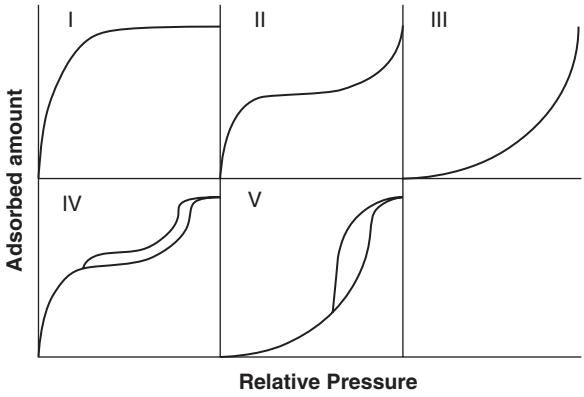

Fig. 9 Graphical Forms of different BET Isotherms

3.4 Chemical Composition and Crystal Structure Analysis

3.4.1 Mass Spectrometry (MS)

Mass spectrometry (MS) is one of the major analytical method which can analyze the samples based on their mass to charge ratio and provides information like mass, chemical composition and elemental composition of a particle or a molecule. MS has high detection sensitivity (10^{-9} to 10^{-21} mol of sample requires) with high degree of precision and accuracy in determination of molecular weight. Various physicochemical characteristics of nanoparticles, such as mass, structure and composition can be examined by using different MS procedures and differentiated by their ion sources, separation techniques and detector systems. Among the ionization methods coupled with MS analyzers, electrospray ionization (ESI, usually in conjunction with HPLC/UPLC) and matrix assisted laser desorption/ionization (MALDI) are most frequently used to ionize and volatilize temperature sensitive biomolecules instead of introducing significant decomposition or fragmentation of the molecules. In ESI mode of MS, ions are formed through electrospray by applying a voltage (positive or negative) to liquid flow, which nebulizes the liquid into fine droplets. The droplets travel in high pressure and temperature through the ion source of MS which desolvate droplets and finally release the ions into the gas phase. MALDI-coupled with time of flight-MS (MALDI-TOF)-MS is a highly sensitive and powerful soft ionization technique which is suitable for analysis of complex molecules, like functionalized nanoparticles and proteins [35]. It is a solid phase ionization technique, in which sample and matrix co-crystallized on a solid support. The irradiation from nitrogen laser at 337 nm sublimates the sample/matrix mixture to gas phase where ionization of the sample occurs and proton transfer takes place. The data can be characterized by relatively simple spectra with a pseudomolecular ion, $[M + H]^+$ for singly charged ions and $[2\ M + H]^+$ dimer for doubly charged +2 ion (31). Fig. 10 represents the (MALDI-TOF)-MS spectrum with predictabale molecular weight and purity of the synthetic Magainin-I analog peptide (MIAP) [36]. (MALDI-TOF)-MS is useful in the characterization of nanomaterial bioconjugates,

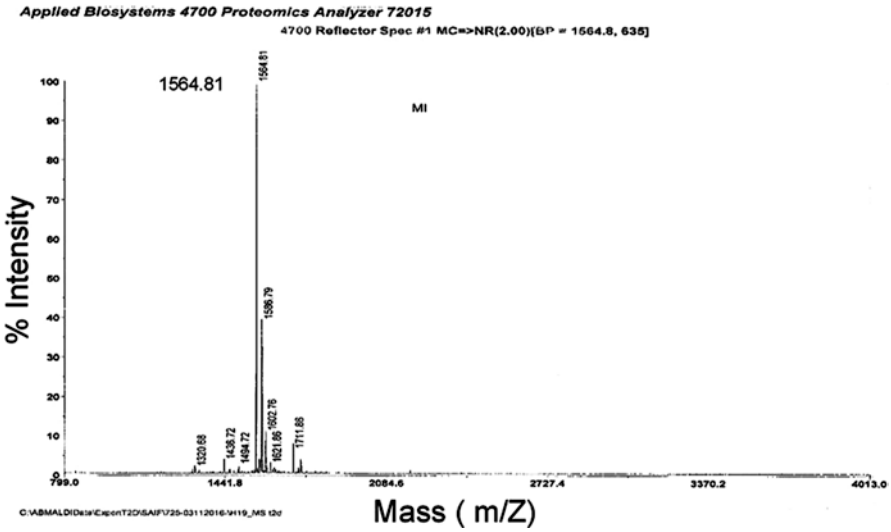

Fig. 10 MALDI-TOF mass spectrum of the synthetic MIAP peptide (Reprinted with permission from ref. [36])

especially in protein based nanoparticles, such as viral nanoparticles, in that mass increases in the viral coat protein because of the addition of biotin or fluorophore species. It is also useful for determination of size/size distributions of nanomaterials, molecular weights of macromolecules, dendrimers and polymers, as well as to illustrate proteins binding to nanoparticles (4, 27). On the other hand, inductively coupled plasma (ICP) ionization MS (ICP- MS) is mainly useful in the analysis of metal containing nanoparticles. It is implemented to validate the conjugation reaction between functionalized nanoparticles and modified contrast agent, where the secondary ion MS provides the molecular and elemental properties of the top layer of nanoparticles, as well as to determine biomaterial surface properties in physiological conditions. However, the application of MS techniques have some limitation in nanomaterials- bioconjugate characterization which may in part because of the relative cost of the instrumentation, the destruction of the sample during measurement, and the required level of expertise needed to run analysis (4, 27).

3.4.2 X-Ray Diffraction (XRD)

X-ray diffraction (XRD) is a common and effective technique for the study of nanomaterials. The wavelength of X-rays is in atomic scale, hence XRD is an important method for investigating structure of nanoparticles. It helps in completely deciding the tertiary structures of crystalline materials at the atomic scale. Crystalline phases are identified by comparing the interplanar distance values obtained from data. X-rays are electromagnetic radiation with a very short wavelength (few Angstrom)

which is produced when the electrically charged particles with sufficient energy are decelerated. X - rays, generated from cathode ray tube converge as monochromatic collimated radiation and directed towards the sample. The X – rays interfere constructively and destructively producing a diffraction pattern on the detector. Crystalline and semi crystalline materials like polymers, metal, metal oxides nanoparticles have a characteristic atomic structure which diffracts X-rays in a unique diffraction order or pattern. The X-ray diffraction data of polymers or nanomaterials gives information regarding crystalinity, orientation of the crystallites, lattice strain, thermal expansion, grain size, internal stress of small crystalline regions, order-disorder transformation, phase composition in semi crystalline polymers and thickness of thin films.

It is also used to determine nanosized components embedded in biological matrix or nanobioconjugate layered materials like nano-hybrids where the analysis of d-spacing alters upon bioconjugation between layers of the nanoparticles. This technique is also helpful to assess the polymorph stability of solid lipid nanoparticles and PEG content on the self assembly of peptide fibril nanostructures [31, 32]. When the pure drug is incorporated in polymer. matrix, change in its crystal property can be measured by XRD. On the basis of different diffraction pattern of pure drug, pure polymer and drug loaded nanoparticles (Fig. 11), incorporation of drug in polymer matrix can be easily distinguished. The major limitation of XRD is its very low diffraction intensity, especially for low atomic number molecules. A recent XRD study shows a novel approach by use of femtosecond pulses generated from a hard X-ray free electron laser for determination of structure of macromolecules which do not have sufficient crystal size [4].

3.4.3 Small Angle X-Ray Scattering (SAXS)

In comparison to X-ray diffraction, where applications are confined to crystalline materials, small angle X-ray (SAXS) scattering gives minute detail of different characteristics by determining either amorphous or crystalline materials from polymers, protein to nanoparticles [4]. The principles of SAXS is that, a collision between an incident X-ray beam and a surface particle results elastical-scattering from the sample and forms pattern on a 2D flat X-ray detector which is perpendicular to the direction of the incoming X-ray beam. The reflected waves interfere with each other by constructive interferences at a certain angle and form a peak. The incident X-ray beam interacts with the surface of particles electron clouds and forms scattering pattern according to inhomogeneity in the electron density [4, 26]. By examining the intensity of the scattered X-ray obtained within the scattering angle from 0.1 to 3°, SAXS can determine the size/size distribution, orientation, shape, morphology, structure, and characteristic intra-assembly of a various polymers and nanoparticles in solid/solution form [31, 32]. The periodic varions in the intensity profile are inversely proportional to the particle size as well as the intensity profile of monodisperse particles captures the intensity maxima towards the largest

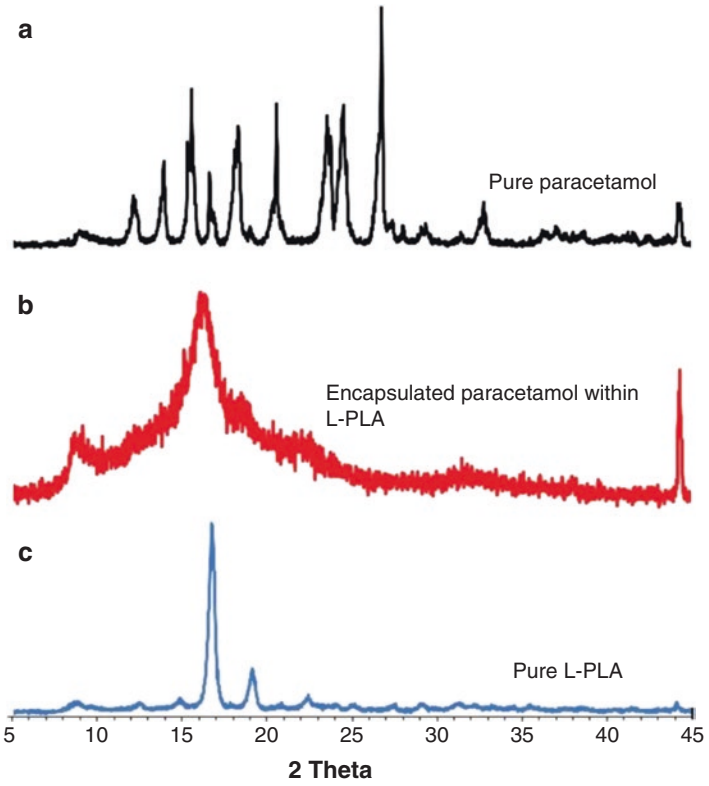

Fig. 11 XRD pattern of pure paracetamol, pure L-polylactic acid (L-PLA), and encapsulated paracetamol inside L-PLA (Reprinted with permission from ref. [33])

extent, whereas the smeared intensity minima indicates a modest polydispersity in SAXS data of particle size distribution of gold nanoparticles [37] (Fig. 12).

The recent advancement in SAXS can achieve higher resolution measurements through using synchrotron as the high energy X-ray source.

3.5 Drug-Polymer Interaction Studies

3.5.1 Differential Scanning. Calorimetry (DSC) & Thermogravimetric Analysis (TGA)

Thermal techniques are mainly important in determination of drug-polymer interaction and biomolecules conjugation with nanomaterials and thier thermal stability. DSC records the heat released by a chemical process (either a conformational alteration or a chemical reaction) from the test and control samples which are placed in separate chamber of a calorimeter. The heat of reaction (ΔrH) that defined as the

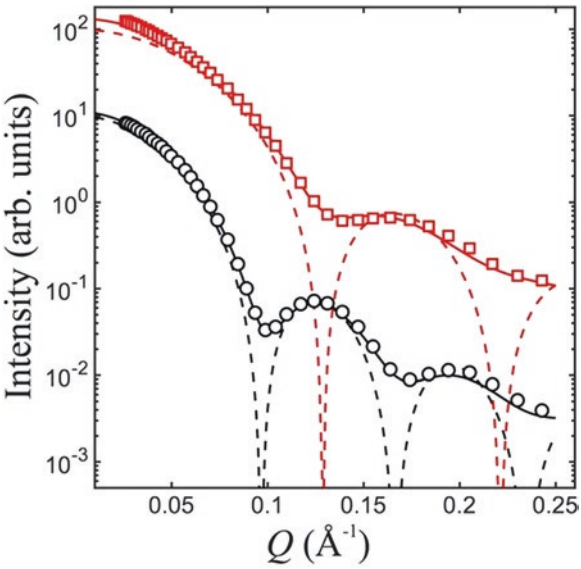

Fig. 12 1D SAXS data of suspended gold nanoparticles. Red squares and black circles represent the data from gold nanoparticles of diameter 5 and 10 nm, respectively. The dashed lines and solid lines are intensity profiles from monodisperse and polydisperse spheres, respectively (Reprinted with permission from ref. [34])

change in enthalpy associated with a chemical reaction is recorded by DSC. The positive value of ΔrH indicates endothermic reaction, whereas the negative value of ΔrH indicates an exothermic reaction. DSC is very useful for measurement of various material transitions including crystallization, melting, decomposition and glass. transition. Further analysis can show the state of the nanoparticles-bioconjugate including stability of the biomolecules, underlying crystalinity and interaction of each component with each other. It also helps to elucidate the stability and structure of surface coatings of the nanoparticles-bioconjugate as well as the state of their therapeutic payloads [31]. On the basis of surface area and intensity of endothermal and exothermal peak, the percentage of crystalinity in the drug and polymer can be differentiated. When the drug is incorporated in polymer, it forms molecular dispersion or solid solution in the polymer matrix (Fig. 13). DSC spectra of drug shows broad and weak endotherm that shows transformation of crystalline to amorphous [38].

Thermal gravimetric analysis (TGA) is also useful in characterization of thermal stability of compounds. It measures exothermic and endothermic weight loss upon heating and cooling of the nanoparticles and generates its thermal profile. It uses a high precision balance to measure changes in the weight of a sample relative to change in temperature. It characterizes various nanoparticles functionalized with biomolecules on the basis of its unique sequence from physicochemical reactions happening over particular temperature range. Isothermal titration calorimetry is another thermally based technique which can gives details about the nanoparticles-bioconjugation. It has potential to determine the affinity, enthalpy and stoichiometry of the nanoparticles-biomolecules interaction [31].

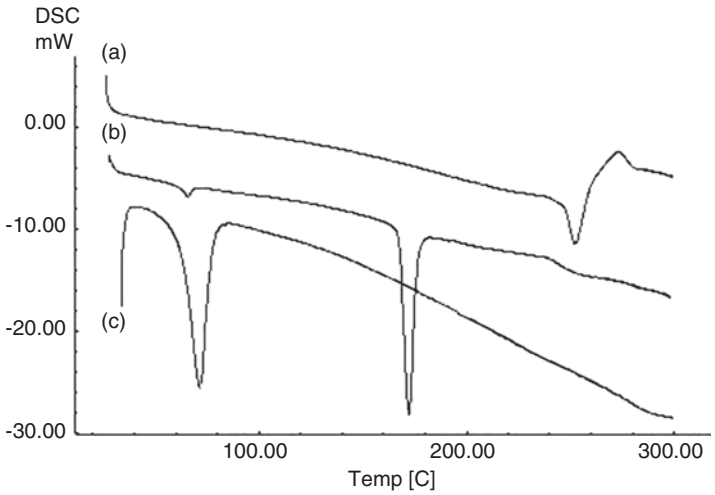

Fig. 13 DSC thermogram of (**A**) carboplatin, (**B**) carboplatin-loaded PCL nanoparticles and (**C**) PCL polymer (Reprinted with permission from ref. [38])

3.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measures the particular absorption of IR radiation, which occurs due to vibrational stretching, and bending of the sample molecules. If the molecules have time-variant dipole moment, their oscillating frequencies are same to the incident light frequency and absorb that frequency of IR radiation. When the molecule absorbs IR radiation, it transfers energy and induces corresponding covalent bond stretching, twisting and bending. Molecules without dipole moments are not absorbing IR radiation, like diatomic molecules of O2 and N2. Normally, vibration in molecules involves a variety of covalent bonds and coupled pairs of atoms and all of them must be considered as a combination of the normal modes, hence, the IR spectra illustrates the absorption or transmission versus incoming IR frequency. It is most frequently used for determination of conjugation between peptide or protein with nanoparticles. In globular proteins, stretching and bending vibrations in the amide region gives secondary structural information which is correspond to the conformational state of the bound protein [31]. The alteration in FTIR spectrum of pure DNA, gold nanoparticles without functionalizations and DNA-gold nanoparticles conjugates indicates the interaction between DNA and gold nanoparticles (Fig. 14). The recently developed attenuated total reflection-FTIR (ATR-FTIR) spectroscopy utilises the total reflection property in conjunction with IR spectroscopy to determine the structural information of adsorbed/deposited molecules at a solid/liquid or solid/air interface, by averting the drawbacks of spectral irreproducibility and sample preparation complexicity. ATR-FTIR spectroscopy can be implemented for the analysis of surface features of nanoparticles, although at nanometer scale, it is not a very sensitive surface analysis method as the penetration depth is in the same order of magnitude as the incident IR wavelength [4].

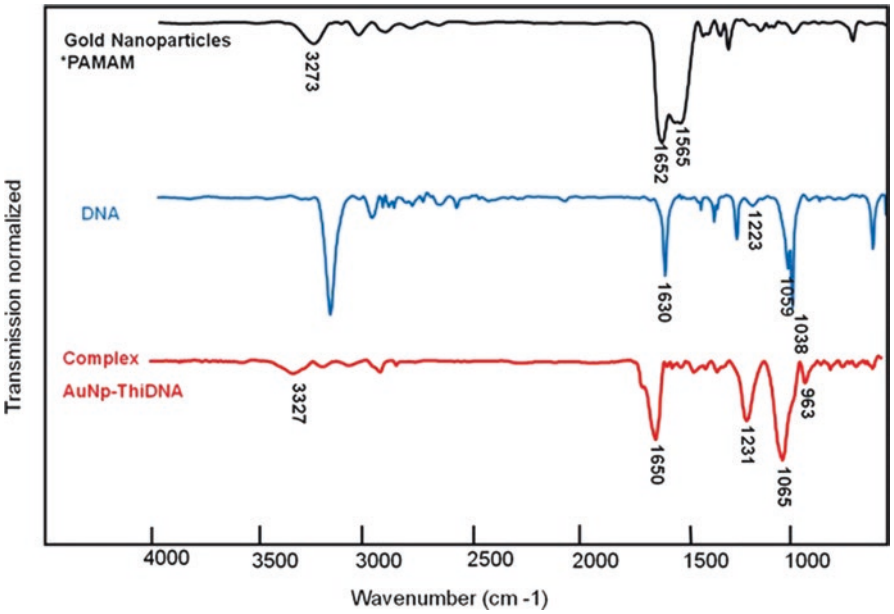

Fig. 14 FTIR spectra of pure DNA, gold nanoparticles without functionalization and AuNP-DNA nanocomplexes (Reprinted with permission from ref. [39])

3.5.3 Ultraviolet-Visible Spectroscopy (UV-Vis)

The UV-Vis absorbance of nanoparticles is useful in monitoring its pertinent properties, like size, concentration and aggregation state. Quantum dots have a size-dependent absorption profile which is helpful to characterize its size, composition, and purity. Metal nanoparticles like silver or gold with 40–100 nm size can scatter optical light with remarkable efficiency due to collective resonance of the conduction electrons and shows a strong absorption in the visible region, which is known as the surface plasmon resonance (SPR) band. The adsorption of peptides/protein on the surface of nanoparticles causes some alteration in the absorption spectrum, leading to broadening or shifting of the absorption peak. In case of metal nanoparticles, the alteration in plasmonic peak during peptide/protein adsorption can be monitored [40]. It is affected by various factors, such as size, shape, aggregation state, composition, and refractive index changes within the surface proximity. The wavelength of a light wave and its energy is inversely proportional, so as an increase of nanoparticles size, it absorbs radiation of lower energy.

When the size of gold nanoparticles rises from 10 to 100 nm, the absorption maxima increases from 400 to >560 nm with broadening of the peak. In case of silver nanoparticles, when the silver content increases, the absorption maxima shift towards higher wavelength. It also shows shape dependent peak shift in the spectrum, like pentagon form of particles appear green, the triangular shaped particles appear red, and the spherical particles appear blue. It also shows increase in the

UV-Vis extinction value when the particles size increases from 5–100 nm. Smaller nanospheres primarily absorb light and have peaks near 400 nm, while larger spheres exhibit increased scattering and have peaks that broaden and shift towards longer wavelengths (known as red-shifting). Shape dependent peak shift in the spectrum, like pentagon form of particles appear green, the triangular shaped particles appear red, and the spherical particles appear blue. It also shows increase in the UV-Vis extinction value (Fig. 15) when the particles size increases from 5–100 nm [41]. Smaller nanospheres primarily absorb light and have peaks near 400 nm, while larger spheres exhibit increased scattering and have peaks that broaden and shift towards longer wavelengths (known as red-shifting).

3.6 Stability of Drug Nanoparticles

The high surface area to volume ratio of nanoparticles may cause the reactive and colloidal instability as compared to their bulk. In general, nanoformulation stability is categorized in to physical, chemical and pharmaceutical stability. The common physical stability issues with nanoparticle formulations include agglomeration, sedimentation/ creaming, crystal growth and change of crystallinity state. The selection

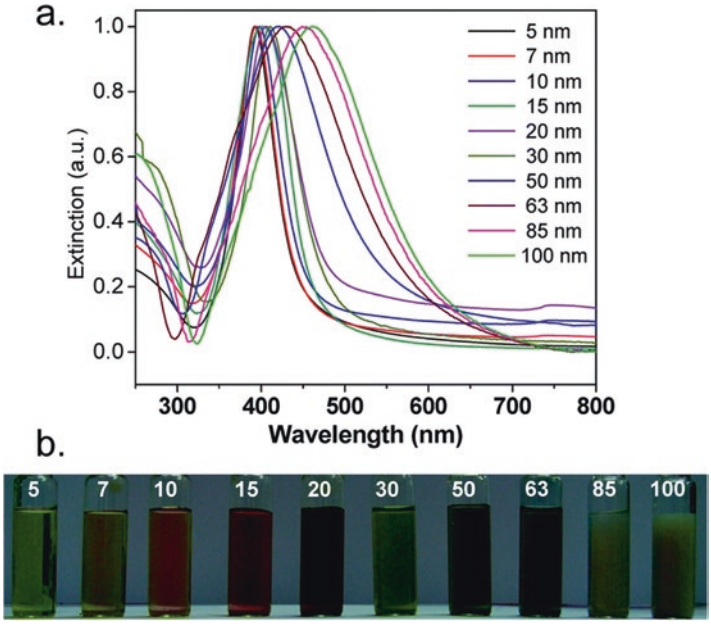

Fig. 15 (**a**) UV-Vis extinction spectrum and (**b**) the distinctive color of 5–100 nm sized silver nanoparticles (Reprinted with permission from ref. [41])

of characterization methodology for nanoparticles- stability is dependent on the kind of stability issues and formulations.

3.6.1 Sedimentation/ Creaming

The changes in nanoparticle size is usually used to predict the stability of most of the nanomedicine. The deviations from the average size range is an indication of nanoparticle association or dissociation or instability in that specific environment. Sometimes, nanoparticles can settle down in the medium depending on their density comparative to the medium. Decreasing particle size is the most common strategy used to reduce particle settling. Large particles (microscale or more) precipitate more easily due to gravitational force, whereas nanoscale particles below one micron do not settle due to Brownian motion. Conventional method of evaluation of sedimentation is visual observation over a span of time. The quantitative volume of sedimentation is evaluated by measuring settled volume relative to the total suspension volume in specific time. Using dynamic light scattering we illustrate that cluster size and fractal dimension which should be considered when evaluating the fate of aggregated nanomaterials.

3.6.2 Agglomeration

Aggregation of nanoparticles is serious issue which disrupt various properties of nanoformulations and leading to destabilization of colloidal systems. Aggregation depends on the type of nanomaterial, reagents or method used for nanoparticle synthesis. In this process, nanoparticles dispersed in the aqueous phase stick to each othe to form asymmetrical clusters, flocs, or aggregates. It modifies the physiochemical properties, activity, transport and biological interactions of nanoparticles. The unique properties of nanoparticles due to their size significantly changes due to aggregation. A quantitative measurement of nanoparticles aggregation would deliver a valuable assessment of colloidal stability. DLS is a very powerful characterization technique as it yields absolute values for an ensemble of particles. The variations of the intensity of light scattered by a nanoparticle dispersion are observed over a period of time and the analysis give yields information about the hydrodynamic radius (R) of the sample. Cryogenic transmission electron microscopy (cryo-TEM) is also used for the evaluation agglomeration of nanoparticles. Here sample is investigated under frozen–hydrated conditions which includes plunge freezing of aqueous sample. Another technique which is used to evaluate the agglomeration is Asymmetric flow field-flow fractionation (AF-FFF). This is a separation technique based on the theory of field flow fractionation (FFF) which is usually used for sample separation and size characterization of nanoparticles both in aqueous as well as organic solution. The separation is attained by cross-flow of suspension of nanoparticles in a narrow, ribbon-like channel which is built up by a spacer, between a porous and a nonporous plate. The porous plate is covered by a membrane allows

the liquid to pass the membrane, retain the nanoparticles. Extenstive characterization of nanoparticles and their aggregates is possioble by coupling AF-FFF with online detectors like UV, fluorescence detector etc.

3.6.3 Shelf Life

The self-life of the nano-medicine depends upon their chemistry, morphology and storage conditions. Depending upon the chemistry, polymer absorb moisture on storage which initiate degradation and a change in physicochemical properties, which in turn can alter their *in-vivo* performance. The presence of residual solvent, residual monomer or catalysts may weaken the storage stability and leading to degradation. The relative strength of water-polymer bonds and the process of crystallization also affects degradation of nanomedicine. The storage of nanomedicine is recommended in an inert environment to maintain physicochemical integrity of nanomedicine. Additionally, drug leakage, degradation and microbiological growth can be other issues that can cause degradation of nano-medicine. HPLC and LC-MS are the most common method used to assess the chemical stability which gives detailed quantitative analysis of degradation impurities. MS usually coupled with LC-MS or HPLC are used to ascertain the molecular structure of impurities. Some other techniques such as Fourier-transform infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) can also be used for chemical stability assessment Table 2.

4 Regulatory Requirement

Nano-medicines are complex products which are the result of difficult to control manufacturing processes. Detection and characterization of nanomaterials in complex matrices was considered an important issue by the regulatory community. Credible characterization methods for nanoparticles will significantly affect the uptake of these nanomaterial in commercial applications and allow the industry to comply with regulation. The identification of various critical points of nano-

Table 2 A few commonly used stability characterization techniques are listed

Parameters	Techniques
Sedimentation/creaming	Visual observation/laser backscattering/near infrared transmission
Agglomeration	DLS/Cryo-TEM/ Asymmetric flow field-flow fractionation
Chemical stability	HPLC/FTIR/NMR/MS
Shape	SEM/AFM/TEM

material products in existing legal framework to re-evaluate the changing characteristic properties is necessary requirement. There are several challenges in the characterization of nano-materials because of the interdisciplinary nature, the absence of suitable reference materials for the calibration, the difficulties linked to the sample preparation for analysis and the interpretation of the data. To correct for this, we need important standard methods to characterize available nanoscale reference materials (RMs) demonstrating their relevance for the characterization of nanomedicines. The United states-Nanotechnology Characterization Laboratory (US NCL) and the European-Nanotechnology Characterization Laboratory (EU-NCL) have developed and optimized protocols for the physicochemical and biological characterization of candidate nanomedicines. The International Organization for Standardization (ISO) and American Society for Testing and Materials (ASTM) International also have developed and published several standardized test methods, guidance, and reports dedicated to the physicochemical and biological characterization of engineered nanomaterials. It compiles general informative documents and guidelines offering an overview of existing methods to determine basic physicochemical and toxicological characteristics of nanomaterials. These documents highlight the relevance and the limitations of different techniques and include special considerations for testing of nanomaterials. The guidance covers all aspects of testing including nanomaterial characterization, sample preparation, toxicological evaluation, and risk assessment considerations.

5 Conclusion

The physicochemical characteristics at the nanoscale have the potential to influence physiological interactions from the molecular level to the physiological level. The rapid development and manufacture of nanoparticles for the use as drug carrier systems needs appropriate regulations. The measurement and characterization of nanomedicine poses several analytical challenges for scientists, developers, and regulatory agencies. Several practical guidelines for the characterization and quality control of nanoformulations are needed. Appropriate robust techniques for nanoparticles characterization are essential to ensure regulatory guidelines for efficacy and safety of nanomedicines. This chapter describes the important physicochemical properties of nanoparticles, followed by general overview to various methods, which are commonly used for characterizing nanoparticles. The short description of each technique together with their range of applications in nanomaterial characterization is described.

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Part II
Nanoparticle-Based Mucosal Delivery of Drugs and Biologics

Overview of the Advantages and Disadvantages of Different Mucosal Sites for the Delivery of Nanoparticles

Kan Kaneko, Nashwa Osman, Valeria Carini, Giulia Scagnetti, and Imran Saleem

Abstract Nanoparticles (NPs) often improve the efficacy of therapeutic actives, and their delivery to mucosal sites allows for unique and localized effects compared to parenteral delivery. Sites of mucosal surfaces includes the eyes, nasal cavity, lungs, and the entire gastrointestinal tract from mouth to anus, and offers extensive areas for the delivery of therapeutics. However, each mucosal site has unique physiological properties that affect aspects such as stability during the transit to the mucosal surface, release of the active molecules, and absorption of NPs into the body. The required NPs properties also differ based on if the goal is for absorption of intact NPs or release of the active molecules at the mucosal site. Therefore, the interaction of the NPs, with the medium that is in contact with the mucosal surface, the mucus layer, and the epithelial cells, must be considered during the formulation process. This chapter focusses on the advantages and disadvantages of delivering NPs through each major mucosal site and offers indications on NPs properties that may be ideal for each site.

Keywords Mucosal delivery · Nanoparticles · Ocular · Nasal · Lung · Oral Vaginal

Abbreviations

COPD	Chronic Obstructive Pulmonary Disease
GI	Gastrointestinal
GALT	Gut-Associated Lymphoid Tissue
IN	Intranasal
MALT	Mucosa-Associated Lymphoid Tissue

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NPs Nanoparticles
NALT Nasal-Associated Lymphoid Tissue
PP Peyer's Patch

1 Introduction

Nanoparticulate delivery systems have garnered much attention in the past few decades and constitutes a large area of interest in current research. Nanoparticles (NPs) exhibit unique properties through size, surface, solubility and other modifications, which can offer advantages relative to the conventional forms of drug molecules [1]. Through these unique characteristics and properties, NPs have been investigated for a range of therapeutic applications, including drug delivery, diagnostics and immunotherapy for various pathologies [2]. In addition, NPs can be delivered through different routes, which can result in unique responses and localization of the effect [1].

The mucosal surface of the body is considerably large and represents an extensive area for therapeutic delivery. Sites of mucosal surfaces includes the eyes, nasal cavity, lungs, and the entire gastrointestinal tract from mouth to anus. The delivery of NPs to these mucosal sites allows for unique localized effects and other advantages compared to parenteral delivery [3]. This chapter focusses on the advantages and disadvantages of delivering NPs through each major mucosal route.

1.1 Justification for Mucosal Delivery of Nanoparticles

From a logistical perspective, the mucosal sites are generally more convenient for administration, as it is minimally invasive and may enable greater access to therapeutics without requiring qualified personnel for administration [2]. It also translates to reduced risks for the patient, as, there is less opportunity for body fluid contamination and disease transmission without needles. The implications are also associated with economics, as there is reduced cost associated with administration due to the reduction in logistics which would otherwise be required for parenteral administration. Furthermore, the regulations and requirements for the manufacturing of parenteral formulations are more stringent compared to some of the mucosal routes.

An equally important aspect of the mucosal surface is the therapeutic effects associated with delivering to localized sites for localized ailments. The ability to restrict the exposure of the drug to the intended mucosal site for targeting means that it reduces the potential for adverse effects through reduced drug concentration outside of the target area, and equally increases drug efficacy through increased concentration at the target site. Due to the unique physiology at different mucosal

sites such as the proximity to important organs, type of epithelial layer or composition of immune cells, the delivery to different mucosal sites can also lead to unique effects compared to systemic administration [2].

Although NPs formulations such as polymeric NPs and liposomes can be tailored to accommodate for the specific requirements of different mucosal routes [1], each of these routes still present issues that must be overcome or makes it unfavorable for administration. It is important to acknowledge the balance of the advantages and disadvantages for each mucosal route, when considering NPs delivery.

1.2 General Physiology of Mucosal Sites

Although mucosal sites in the body differ considerably in many factors in accordance to their functions and location (Table 1), there are a number of general features which can be considered as broadly analogous for drug delivery (Fig. 1). Mucosal surfaces are generally composed of epithelial cells that act as a barrier between the body and the environment. Before any nanoparticulate material can reach this epithelium, it must generally pass through a viscoelastic layer of mucus that lines the epithelium and separates it from the environment [4]. The mucus is a hydrogel composed mainly of proteins known as mucins, and functions not only as a barrier to protect the epithelium from pathogens and pernicious material, but also prevents particle uptake by trapping and clearing them before they can interact with the epithelium [4]. When the NPs manage to reach the epithelium, the tightly connected cells that function to control the movement of material into and out of body generally limit NPs entry into the blood. Overall, the mucosal surfaces can act as barriers for NPs drug delivery through physical and chemical mechanisms.

2 Advantages and Disadvantages of Specific Mucosal Sites

2.1 Ocular

The unique anatomical and physiological structure of the eye comprises a myriad of structures that work together to provide the sense of sight, and aims to protect the eye against foreign substances. Despite numerous efforts, efficient ocular drug delivery remains challenging for researchers, and conventional invasive and non-invasive treatments, cannot guarantee high residence time of the drug in the tear film (2–5 min for topical applications of drug in the form of eye drops) [5, 6]. The low absorption represents the major issue yet to be overcome, and it is primarily due to clearance mechanisms that include efflux pumps, aqueous turnover, vitreous flow and ocular drug metabolism [7]. Recently, numerous NP-based formulations intended for both ophthalmologic and systemic diseases, have been developed aim-

Table 1 Summary of the physiology of mucosal sites

Site	Medium in contact with mucosal surface	Mucus	Epithelium	Unique features
Ocular	Air	• Secreted mucins, electrolytes, and water produced by the conjunctival goblet cells	• Single layer of basal cells and 4–5 cell layers of nonkeratinized, stratified squamous epithelial cells	• Very low residence time of drug (2–5 mins)
		• Ocular mucosa is slightly basic with pH ~7.8		
Nasal	Air	• High viscosity and elasticity, rich in mucin containing negatively charged acids,, salts, water, hydrolytic enzymes and antibodies	• Vestibule lining:	• Mucociliary clearance
			• Stratified squamous epithelium with hairs	• Thick mucus layer
			• Turbinate lining:	• Bypasses first pass metabolism
			• Pseudo-stratified columnar ciliated epithelium with mucous secreting cells	• Olfactory epithelium provides potential route for brain drug delivery
			• Olfactory epithelium:	
		pH of ~6.5	• Pseudo-stratified non-ciliated columnar epithelium.	
Lung	Air	• Mucus lined with lung surfactant that undergoes thinning toward the respiratory airways (60 µm to 2 µm)	• Pseudo-stratified columnar epithelium ciliated and mucous-secreting in the conducting airways.	• Mucociliary and alveolar macrophage clearance
				• Bypasses first pass metabolism
		• Limited enzymatic activity and rich in immunoglobulins	• Alveolar epithelium is composed of almost flat single cells.	• Large surface area and densely vascularized
		• Same pH as extravasated blood		
Oral cavity	• Air but covered with saliva	• The saliva has pH of 6–7 and contains electrolytes, proteins, enzymes, mucin and immunoglobulins	• Stratified squamous epithelium	• High clearance by saliva
				• Bypasses first pass metabolism

(continued)

Table 1 (continued)

Site	Medium in contact with mucosal surface	Mucus	Epithelium	Unique features
GI tract	• Gastric fluid: • 1–3 pH • Lipases and proteases Intestinal fluid: • 5.7–7.4 pH • Proteolytic enzymes more abundant • Bile salts	• Adherent and non-adherent layers of mucus with thickness of 50–500 μm • pH of 5.2–6.2	• Simple columnar epithelium • Intestinal cells express microvilli	• Highly degradative conditions • Transit time of 0.5–4 hours in stomach, 1–2 hours in small intestine and 12–24 h in colon • M cells can translocate NPs across intestinal wall at the PP
Vaginal	Air	• Menstrual cycle, menopause and pregnancy are responsible for the diverse composition of the mucus • pH 3.5–4.5	• Nonkeratinized, stratified squamous epithelium. • Highly folded epithelium.	• Large surface area and rich blood supply

ing to overcome ocular barriers, target specific ocular tissue and avoiding non-specific drug tissue accumulation. Several nanocarrier systems including polymeric NPs, liposomes, niosomes and dendrimers, have been widely studied as potential ocular drug delivery systems. The development of nanotechnology-based formulations also contributes to the creation of novel devices including nanoparticles-loaded contact lenses, and innovation in the field of imaging and screening.

The ideal NPs delivery system should enhance the retention, permeation and control the release of the drug, enabling high drug loading efficiency to reduce the instilled volume, and hopefully increase the patient compliance through avoidance of more than two administrations per day [8]. Furthermore, the NPs must protect the drug from metabolic degradation. In particular, liposomes have shown to provide protection of entrapped genetic material and enhance its adsorption [9]. These lipid bilayer vehicles can be considered as a possible strategy to formulate several potent actives, although liposomes still have limitations including limited drug loading efficiency, harsh and aggressive conditions for preparation, and difficulties related to the sterilization of the formulation. The susceptibility of phospholipids to oxidative degradation in air [10] can be easily overcome by using a non-ionic surfactant-based vesicular system called niosomes, which are more chemically stable and can encapsulate both hydrophilic and hydrophobic drugs [11].

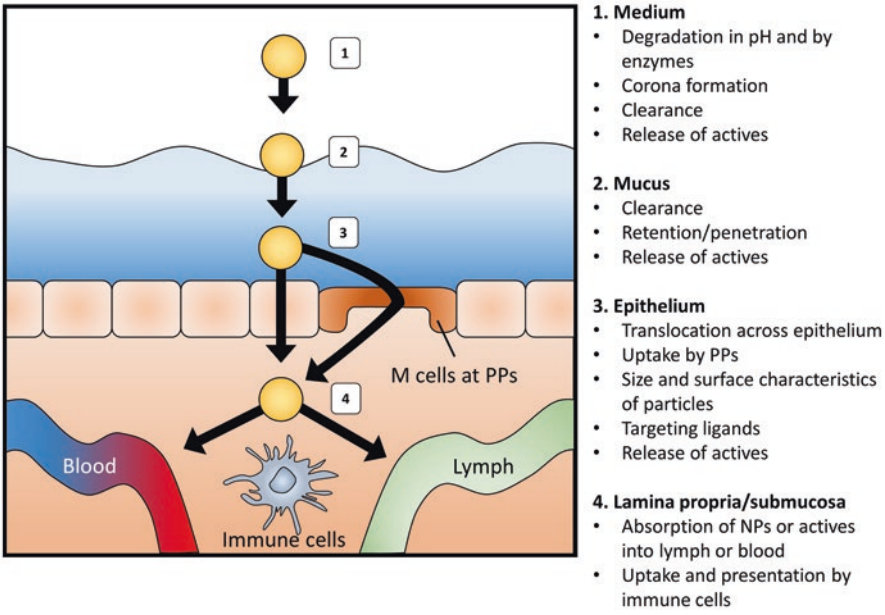

Fig. 1 Considerations for the delivery of NPs at mucosal sites. The properties of the medium in contact with the mucosal surface, the mucus, epithelium and lamina propria/submucosa, all contribute to the absorption of NPs and/or the incorporated active molecules. The presence of PP at the epithelial boundary is also a significant avenue for NP translocation across the epitheliums

Much of the published data regarding ocular drug delivery via NPs systems suggest that the particle composition, size and surface properties, play significant roles in the in-situ retention time and cellular uptake of the active. In order to avoid ocular irritation and blurred vision, the NP should have an appropriate particle size and a narrow particle size distribution. The drug time of action is particle size-dependent; smaller particles lead to higher absorption into ocular tissues from the precorneal pocket, larger particles lead to slower drug dissolution [12]. Moreover, surface properties including the particle surface charge, are key factors affecting the particle distribution between the vitreous humor and retinal layers [13]. Positively charged carriers show higher cellular uptake and retention time, and due to the negatively charged surface of the corneal epithelium, it is possible that the initial interaction is electrostatic in nature [14]. The literature also shows that formulations of positively charged liposomes containing a poor water-soluble drug, such as acyclovir, exhibit sustained penetration of the drug across the cornea, increasing the extent of absorption [15], which could potentially be useful for the treatment of herpes keratitis.

Another method for increasing the precorneal residence time of the active, is encapsulating within NPs with mucoadhesive properties. Polyethylen glycol (PEG), chitosan and hyaluronic acid are the most common polymers used to improve the mucoadhesion, because of their ability to contact intimately with corneal and conjunctival surfaces. Chitosan-coated systems compared to non-coated ones, exhibit

unique behaviors, which can potentially be utilized to target different regions of the eye. A comparative *in vivo* study for chitosan coated vs non-coated, indomethacin NPs, were conducted in rabbits and showed that the surface coating helped to increase the half-life of indomethacin relative to non-coated formulation [16]. Such NP formulations that can increase the residence time at the ocular surface could be one avenue for ocular NP formulations with improved efficacy.

To summarize, the ocular mucosa presents with a number of disadvantages; the main ones being the high degree of clearance, and limited systemic applications. Despite these limitations, there is potential for the development of suitable NPs capable of encapsulating a wide range of drugs that can increase the absorption of the active for local pathologies. The retention of NPs at the ocular surface is one of the potential strategies for increasing the absorbance of the active by enabling sustained release.

2.2 Nasal

Intranasal (IN) delivery of therapeutics is widely practiced for treating local nasal conditions such as sinusitis, rhinitis, coryza, nasal bleeding, and nasal polyps, using anti-inflammatory steroids, antihistamines, vasoconstrictors, and numerous other drugs. The IN route has also recently garnered attention as a potential alternative route for systemic drug delivery; most importantly, for drug delivery to the brain and for vaccination [17]. The formulation of active therapeutics into NPs for the nasal route is an avenue for improving the efficacy, as it has been shown to enhance the potential effects of active therapeutic molecules, compared to their conventional formulations [18].

The nose is a portal of entry for the respiratory system, and responsible for filtration and humidification of the inspired air. The nasal cavity extends from the nasal vestibule to the pharynx (around 160 cm^2 surface area) and is halved by the nasal septum. The mucus (approximately 5 μm thick) forms a viscous elastic layer, and contains salts and mucin that confers a slightly acidic pH (6.5) and a negative charge. In addition, hydrolytic enzymes such as aminopeptidases that degrade proteins, antibodies plus other molecules are especially abundant. The nasal cavity has three regions that differ in their epithelial and functional characters; the vestibule, turbinate and olfactory regions. The vestibule has the epithelial change from skin to stratified squamous epithelium, with abundant hair representing the first filtering mechanism for inhaled particles (aerodynamic diameter > 10 μm). The turbinate forms the main nasal cavity and highly perfused warming chambers. It is lined by pseudo-stratified columnar mucous-secreting epithelium that aids in trapping inspired particles. It has ciliated and non-ciliated cells, with both immotile and motile microvilli, that play a double-edged role by increasing the surface area of absorption of NPs, as well as limiting the drug absorption through mucociliary clearance. The olfactory region, formed from pseudo-stratified non-ciliated columnar epithelium, is a recognized target for brain drug delivery through olfactory

nerves and/or para- or trans-cellular transport. The nasal mucosa is part of nasal-associated lymphoid tissue (NALT) that is rich in M cells and dendritic cells (DCs) and has been investigated as a delivery route for NP vaccine formulations [17, 19–21].

One main advantage of delivery through the nasal route is the highly vascularized absorption area (150 cm^2), which can lead to fast circulatory drug levels [22]. The nasal environment is also relatively less harsh compared other sites such as the GI tract, and allows the bypass of first-pass hepatic metabolism. It is also a site in contact with the lymphatic system, opening the opportunity for the delivery of vaccine formulations [22]. Another avenue for the utilization of the unique nasal site is the delivery of active therapeutics to the brain, through the olfactory epithelium, thus avoiding the brain barrier. For the patient, the nasal route is easily accessible, allows for self-administration and is well-tolerated. However, there are numerous limitations and challenges for NP delivery at the nasal site. The limited drug absorption and rapid mucociliary clearance, means that designing nano-based formulations which provide drug stability and desired release properties suitable for local nasal delivery is still challenging [19]. To address these hurdles, various mechanisms have been employed to enhance the nasal drug solubility, retention and uptake. The use of the solubility and/or permeation enhancement agents have shown promising results. Solubility enhancers modify the formulation characteristics after delivery, to increase the availability of the drug [19]. Permeation enhancers alter the permeability of the nasal mucosa, temporarily reducing the mucociliary and enzymatic clearance, and improving drug bioavailability [22]. Examples include bile salts, peptidase inhibitors and cyclodextrins among others, which have been widely investigated [23]. Mucoadhesive materials have also been investigated to enhance the mucosal retention and reduce its clearance. Examples include naturally occurring polysaccharides such as chitosan, which exhibits biocompatible, mucoadhesive properties, and is commonly used as part of NP carrier formulations. Chitosan has also been used in a variety of dosage forms from a solution to dry powder [23–25]. In addition, PEGylated NP carriers have exhibited promising absorption profiles compared to non-PEGylated counterparts.

2.3 Lung

Administration through the pulmonary route has been successful for delivering therapeutics intended to treat local respiratory problems, such as asthma, chronic obstructive pulmonary disease (COPD), lung malignancies and lung infections, as well as systemic diseases through delivery of therapeutic molecules, such as proteins/peptides, genetic material, hormones or vaccines [26, 27]. NPs can be a successful platform for enhancing the efficiency of the pulmonary drug delivery, not only for the local conditions but also for systemic administration [28, 29]. The pulmonary route has very complex structure that is divided into two parts; conducting and respiratory areas. Each area exhibits different physiological and functional properties that present unique challenges for NP delivery.

The conducting area of the lungs extends from the nose, trachea, main bronchi, and branching until the respiratory bronchioles, resulting in a surface area of 2–3 m^2. One major immediate limitation for NP delivery into the lungs is the significant influence that the aerodynamic diameter has on the deposition within the different regions of the lungs. Particles in the size range of 1–5 μm are generally considered appropriate for lung deposition [30, 31], which means that NPs alone are not suitable for direct inhalation. There are however, solutions such as formulation of NP in microcarriers or inhalation via nebulizers, which can temporarily increase the aerodynamic diameter for appropriate lung deposition [32, 33]. The lining of the conducting airways is also a barrier for NP delivery as it is composed of pseudo stratified columnar epithelium, which secrete mucous, express motile cilia, and is lined with a surfactant layer. Epithelial tight junctions limit the translocation of molecules and NPs across the epithelium, and the strong mucociliary clearance mechanisms that filter the inspired air from any particles or bacteria, present major challenges for NPs delivery [34]. The humid environment represents another challenge for the hygroscopic NPs, which undergo increases in their particle size, and subsequently is favored for mucociliary clearance. The state of the conducting area of the lungs can also be affected by different diseases like asthma, cystic fibrosis and COPD [35], which may consequently increase the resistance for the air flow and limit the delivery of aerosolized NPs formulations.

The respiratory area of the lungs extends from respiratory bronchioles to the terminal bronchioles and alveolar sacs, with a wide surface area of approximately 120–140 m^2. The epithelium lining is very thin compared to the conducting epithelium (0.2–2 μm and 60 μm thickness. respectively) and is an attractive target for NP delivery. It includes alveolar cells type I (main cells, flat) and type II (irregular shape, secreting lung surfactant) with tight and gap junctions, with a thin layer of lung surfactant [36]. The alveolar epithelium has a plethora of wandering cells, for example, DCs, macrophages, mast cells and lymphocytes. This however represents a double-edged sword, as these cells contribute to the clearance of NPs, but at the same time, could be used as the initiator of immune responses in case of vaccination therapy [37, 38]. In terms of NPs uptake by the epithelium, the alveolar epithelium exhibits high permeability and dense vasculatures for its gas exchange functions, and subsequently makes it an attractive site for NPs delivery. NPs are known to be translocated past the epithelium through transcytosis or paracytosis, and is influenced by particle size and surface charge. Like the conducting area, the respiratory area of the lungs is also affected by different diseases, such as emphysema, pneumonia, lung cancer and tuberculosis [28]. Therefore, it is important to consider the interaction of NPs with the mucosal site in different disease states. Another question that should be addressed for pulmonary delivery is the risk of toxicity through oxidase stress, inflammation, fibrosis and genotoxicity, which could result from NP presence in the lungs. The health impact from particulates found in air pollution is becoming increasingly established, and chronic inflammation induced by presence of NPs in the lungs have been linked to lung diseases such as asthma, COPD and even lung cancer [39].

NPs of various forms have been proposed and investigated for pulmonary administration. Solid lipid nanoparticles and liposomes, which are made from phospholipids naturally present in the lungs, have been popular formulations for lung delivery, in addition to polymeric NPs. One application for NPs in pulmonary administration is for potential induction of immune responses in the lungs, as the pulmonary route is the entry site for pathogens [33, 40, 41]. Polymeric NPs incorporating a protein antigen from S.pneumoniae exhibited induced the production of secretory IgA and plasma IgG antibodies specific for the pathogen [38]. Virus-like particles are also of interest for pulmonary vaccine applications, as the mucosal administration mimics the natural pathogenesis of respiratory infections [42]. In addition to the prevention of infections, NP formulations incorporating antimicrobials have been investigated for potential use in established infections, with liposomal formulations incorporating ciprofloxacin and amikacin currently in phase III trials [43–45]. Treatment of inflammatory lung conditions has also been explored, with the administration of PLGA NPs encapsulating hydroxybenzyl alcohol incorporated polyoxalate showing attenuation of inflammatory processes in the lungs [46].

To summarize, the advantages of the pulmonary delivery are the large surface area for absorption, good vascularization, and relatively high permeability of the epithelium compared to other mucosal administration sites. There are also limited proteolytic enzymes that could degrade NPs and the encapsulated active. The pulmonary route can be used to treat both local and systemic diseases and subsequently absorbed actives do not encounter first pass metabolism. Pulmonary delivery method is non-invasive, uses smaller doses for local lung conditions which can result in less potential side effects to the patient [47]. In terms of the challenges for NPs delivery at the lungs, numerous physical and biological barriers can make NPs delivery difficult. The delivery of NPs, even to the epithelium is a challenge on its own, as airway narrowing and branching play a role in particle impaction away from the alveolar and respiratory regions. The high humidity within the lungs also affects hygroscopic particles, favoring their clearance. The intrinsic clearance mechanisms of the lungs can contribute to the loss of NPs in the forms of mucociliary clearance in the conducting airways, and NPs phagocytosis by alveolar macrophages in the respiratory airways. Lastly, pulmonary diseases can affect the state of the airways and subsequently delivery of the NPs into the lungs and the interaction of NPs with the mucus and surrounding cells. The NPs formulation should have sufficient biocompatibility and biodegradability, as to minimize any potential toxicity and inflammatory response that may elicit adverse effects, and careful exclusion of such materials should be ensured [47, 48].

2.4 Oral

The potential sites for oral delivery starts directly in the mouth cavity, and extends all the way to the rectum, forming the largest continuous mucosal surface in the body and functioning as the interface between the body and the environment [49].

Despite the large surface area, the unique environmental conditions in these areas is a challenge for NPs delivery.

However, there are a number of unique effects that can be induced from the oral route of using NPs [50]. The localized delivery, sustained release and potential for targeting are some of the NPs properties that could be utilized to improve efficacy. There are many specialized sites of immune cells found throughout the oral route, which can interact with NPs differently compared to the non-particulate form of the active molecule. The immune system of the oral route makes up a large part of the mucosa-associated lymphoid tissue (MALT), and has the capacity to dictate how the immune system responds to encountered antigen [2]. All of these unique effects, including drug delivery and immunology, can however vary depending on the region of the oral route; oral cavity, GI tract and rectum.

2.4.1 Oral Cavity

The oral cavity is the first region the NPs encounter through oral administration. The area is composed of stratified squamous epithelial lining that covers the highly vascular tissue, and features low proteolytic enzyme activity and a pH of 5.8–7.4. There are numerous delivery forms, such as sublingual, buccal, disintegrating, effervescent, and chewable systems. The oral cavity is subsequently considered as an appropriate area for the treatment of local pathologies, and also a potential portal for systemic delivery, due to the rich blood supply and permeability in the areas of non-keratinised lining [51].

The delivery of NPs to the oral cavity presents with a number of advantages over the regions further down the GI tract. Firstly, the method of administration is relatively convenient for the patient. There is no need to swallow tablets or capsules, which could be advantageous for the elderly or the very young. The conditions in the oral cavity are also less degradative compared to the stomach and intestine that exhibit low pH and degradative enzymes. The oral cavity permits the delivery of sensitive molecules, and also NPs, that could otherwise potentially be degraded [51]. In addition, the pharmacokinetics of the formulations in the oral cavity is less likely to be unaffected in the fed or fasted state, compared to formulations in the GI tract. The absorption at the buccal site also avoids first-pass metabolism, which allows for a favorable pharmacokinetic profile for affected drugs. An example of such drug is the sustained release of imidazopyridines, which has a rapid onset of action but can be limited by short half-life [52]. Another possible benefit is NP internalization by epithelial cells, allowing for the delivery of active molecules to the local cells [53]. Thepotential for uptake and low degradation offered by NPs at the oral mucosa allow for the treatment of numerous conditions, which are local to the oral cavity, enabling active drugs to exert effects without causing unwanted side effects at unaffected regions. Such local pathologies include inflammatory and ulcerative diseases [54], oral cancer [53], dental caries, and oral infections [55]. Owing to the dense population of dendritic cells in the sublingual region and the lymphatic link to regional lymph nodes, the oral mucosa has also been considered

as a site for vaccine delivery. A nanofibre-based mucoadhesive film consisting of a mucoadhesive layer, a backing layer, and a reservoir layer that incorporated PLGA-PEG NPs, exhibited penetration into the local tissue and subsequent delivery to local lymph nodes [56]. Such results gives credence towards the versatility of the oral cavity route for numerous applications.

One of the major disadvantages associated with delivery to the oral cavity is the continuous secretion and movement of saliva, which results in high clearance, and compromises the retention of the NPs within the oral cavity [57]. To address this limitation, there have been numerous research into mucoadhesive formulations, which can enhance the residence time of NPs and active molecules. For example, incorporating polymers such as chitosan [58], mucoadhesive films [59, 60], and buccal tablets containing NPs [54], could also allow for prolonged effects for drugs with short half-lives [51] and offers promising results for concepts which could eventually lead to products on the market.

The delivery of drugs through the epithelium presents another significant barrier, as the multiple layers of epithelial cells promotes low translocation through the epithelial layer [51]. However, the permeability across the epithelium depends on the area of the oral cavity and there have been reports of NP translocation across the epithelium at areas such as the sublingual area [56, 61]. Permeation enhancers which can overcome this limitation have also been suggested as a possible means of enhancing NPs or active molecule absorption, and offers a possible avenue for addressing absorption of non-lipophilic active molecules [51]. The further movement of permeation enhancers into the GI tract is not expected to be problematic as the GI membrane is thought to be robust enough to handle the temporary effects of permeation enhancers.

The lack of NPs formulations on the market, for the oral cavity, may be an indication of the difficulties of overcoming these limitations and suggests that there is a need for novel approaches which could enhance retention and permeability of the NPs within the oral cavity.

2.4.2 GI Tract

As with the oral cavity, the administration of NPs through the oral route is arguably the most convenient route for the adult patient due to possibility of self-administration and lack of pain, compared to parenteral routes. Not only do oral formulations promote compliance, but they also enable greater access, as they negate the requirement for qualified personnel for administration [49]. This also translates to reduced safety risks, as there is less opportunity for body fluid contamination and disease transmission without needles. From a regulatory and manufacturing perspective, oral formulations may also be favorable due to the production and preparation without the need for aseptic processes [62].

Physiologically, the intestinal tract is generally an attractive mucosal area for delivery due to the potential of high absorption for smaller molecules and abundant vasculature that exists under the large surface area of the intestinal tract. Despite

most of the absorbed material entering into the portal blood due to the relatively higher rate of flow compared to the lymph [63], the lymph is thought to be favorable for colloids or large molecules, as the capillaries of the lymphatic endothelium have greater permeability compared to the blood capillaries. The lymphatic pathway also avoids hepatic first pass metabolism, which can be a source of degradation for some molecules. From a drug delivery perspective, the GI tract is acknowledged as a difficult area for delivery that presents with challenging conditions, but NPs formulations can be useful for overcoming some of these limitations and achieving effective drug delivery [62].

One way that NPs can improve delivery of the active molecule through the GI tract, is through increasing solubility of the active drug. Many new drugs are hydrophobic, which can hinder delivery, absorption and subsequent bioavailability. By formulating the active molecule into a NPs form, saturation solubility and dissolution rate can be increased, enabling sustained release and potentially greater bioavailability [64]. Active molecules can also be encapsulated inside carrier NPs, not only improving the solubility, but also allowing for controlled release. It is also possible to formulate the NPs to initiate release upon changing pH conditions, such as when the formulation gets past the harsh acidic environment in the stomach into the small intestine.

One of the biggest advantages of NPs formulations is the ability to prevent or minimize degradation of the encapsulated actives in the degradative GI environment. The pH of the GI fluid varies along the GI tract, starting with highly acidic conditions in the stomach, to a neutral or slightly alkaline pH in the intestine and colon. The GI fluid also contains phospholipids, surfactants, enzymes and buffering agents, which serve to facilitate the degradation of ingested material. There are numerous approaches to formulating NPs that can maintain sufficient stability within these conditions, including NPs surface coating approaches such as with PEG [65] and chitosan [66], using particle ingredients resistant to disruption or degradation, and increasing the NP stability through covalent links [66].

Another way in which NPs can improve bioavailability is by targeting specified sites of the GI tract. Attachment of specific ligands on the surfaces of NPs can direct the NPs to certain cells and can improve the proximity of the NPs to the desired site and potentially increase the chances for absorption or interaction [2]. This targeting also applies to specific regions of the GI tract for targeting specific conditions such as for gastric ulcers in the stomach and ulcerative colitis in the small intestine. NP targeting can be achieved by pH, adhesion, or time dependent systems [49], which releases the active molecules in the affected area and reduces side effects elsewhere.

In addition to delivery of conventional therapeutic molecules, NPs vaccines through the oral route offers unique benefits in terms of the types of immune responses generated, as they not only induce mucosal immunity locally in the GI tract, but can stimulate other parts of the MALT through activated cells in the gut-associated lymphoid tissue (GALT) [67, 68]. The main form of lymphoid tissue in the GI tract are the Peyer's patches (PP), which are unique due to the presence of phagocytic M cells that demonstrate the unique ability to transcytose nanoparticulate matter, from the intestine to the underlying immune system through adsorptive

endocytosis, fluid phase endocytosis and phagocytosis [69]. NPs made from various materials, such as inorganic materials like gold and silica, and organic particles such as liposomes and polymeric NPs, have been investigated for oral use, and have exhibited immunostimulatory effects, that could be useful for immunotherapy and vaccine applications.

Despite the many benefits of NP administration through the GI route, it is one of the most complicated delivery routes. The absorption of active therapeutic molecules such as proteins have been challenging, with one of the main hurdles being the potential instability in the GI tract [70], which can degrade the active drug or the particle before sufficient absorption can occur. The gastric pH can range from 1.2 to 2.9, and the presence of degradative enzymes presents a challenge for delivery of active molecules encapsulated in NPs. The NPs must exhibit sufficient capacity to protect the encapsulated material in these conditions, as encapsulated materials can degrade through acid catalysis, and proteins can potentially lose activity through changes in the intra-molecular bonds that disrupt secondary and tertiary structures [71]. This is the reason that oral doses, especially for proteins, are required to be significantly higher compared to doses given by the subcutaneous route for comparable effect [72], as 94–98% of ingested proteins are digested by the GI proteases [71].

Polymeric NPs can be susceptible to surface and bulk erosion, resulting in loss of encapsulated material and loss of the initial particle characteristics [71]. Lipid NPs can be broken down by disruption of the membrane or surface by enzymes and surfactants contained in the GI fluid. Even without full degradation of the NPs, particle properties such as size and surface characteristics may change as a result of the different pH conditions, and presence of components in the GI fluid which may adsorb to the particle surface to change the surface characteristics or promote aggregation. This means that testing of potential GI tract formulations, including NPs, in bio-relevant fluids is required in order to evaluate the state of particle characteristics through the various conditions of the GI tract. An alternative solution is the formulation of NPs in conventional dosage forms such as tablets, which can release the NPs once it reaches the targeted site of the GI tract [73].

Assuming that the NPs and the encapsulated drug survives the degradative conditions, another major limitation of the oral route is the barrier presented by the mucus and epithelial layers. The mucosal surface of the GI tract is covered by a 50–500 μm viscoelastic layer of mucus [74].The outer loosely adherent mucus layer has a high turnover due to peristalsis, and the firmly adherent mucus layer is unyielding and cannot be removed mechanically without compromising the epithelium. Interaction of NPs with the mucus layer is influenced by certain particle characteristics, as hydrophobic particles with sizes smaller than 500 nm, have faster diffusion and increased penetration through the mucus layer [75]. There are however conflicting opinions on how surface charge might affect uptake. There have been suggestions that positively charged particles have a greater chance for uptake as the overall negative charge of the mucus may potentially result in a greater likelihood for electrostatic interaction and retention [4]. However results using different surface coating polymers have shown negative and uncharged particles to have greater affinity

for the underlying PP [75]. Recent literature suggests that particles which penetrate the outer loose mucus layer and adhere to the deeper, firmer layer are optimal for delivery to the underlying epithelium [4].

Despite the large surface area of the intestinal mucosa, there is very little particulate uptake through conventional intestinal epithelia due to the low rate of endocytosis occurring at the enterocytes [62]. There have been uptake of inert particles via transcellular and para-cellular pathways but this generally limits the uptake of NPs to sites such as the PPs, which only makes up 1% of the total intestinal surface and takes up less than 0.01% of the administered dose [76]. Furthermore, NPs aggregation upon exposure to GI fluid could have a large influence on the degree of uptake, as particle size has been correlated to transcytotic uptake by M cells [75]. Therefore, the failure of particles to maintain their size and surface properties could ultimately result in poor *in vivo* responses. Even after absorption, the active molecule travels directly to the liver where hepatic first-pass metabolism occurs, potentially reducing the active molecule concentration further. In addition to the various macroscopic barriers for absorption, the state of the GI tract is also influenced from ingested food [50]. The fed or fasted state can influence the motility of the GI tract and subsequently affect the retention and uptake of NPs.

2.5 Vaginal

The vaginal route has been widely investigated as an alternative way of drug administration, mainly for the advantages it presents in terms of avoiding the GI environment and the hepatic first pass effect. Recently, researchers have been focusing on the advantages of using NPs to improve vaginal delivery of drugs or the use of this route for immunization purposes [77].

The encapsulation of drugs in NPs such as liposomes, polymeric particles, inorganic NPs, niosomes and dendrimers offers many advantages compared to the traditional vaginal formulation [78]. The increase in solubility and bioavailability of the drug, together with the possibility of developing formulations that exhibit controlled [79] and prolonged [80] release of the drug, will lead to the decrease in the administered dose and of systemic side effects.

Firstly, although the use of NPs *in vivo* may be limited by their short residence times within the vagina, mucoadhesive polymers have been employed to overcome the poor retention issue that NPs may present, given the tight attraction between the mucus and the polymeric carrier [81]. Chitosan and alginate NPs showed prolonged contact with the mucus, thus being the first step for the delivery of drugs to the underlying tissues [82]. However, it is imperative to mention that mucoadhesive particles can damage the vaginal mucosa facilitating the penetration of pathogens and toxic materials into the mucus, leading to infections of the area [83, 84]. Lai et al. [85], demonstrated that the mucoadhesive properties of NPs can interfere with their capability of delivering drugs across the mucus to reach the epithelium; NPs often remain captured in the shed of the mucus without showing the desired effect [86].

NPs have exhibited promising activity for the delivery of macromolecules, such as proteins and nucleic acids, which are degraded, if administered alone via other routes. As commonly acknowledged, NPs have a protective effect against enzymatic attacks, given that they are too large to gain access to the drug entrapped within the nanocarrier [3]. Recent studies revealed that niosomes containing insulin have enhanced effects, compared to vaginal administration of the free insulin. Vaginal administration of insulin-loaded niosomes also exhibit similar bioavailability to subcutaneous administration [87]. Furthermore, the vaginal administration of molecules such as RNA entrapped in NPs, offers the advantage of avoiding nuclease enzymes that are present in the mucus, thus allowing RNA to reach the underlying epithelium without being degraded [88].

Several studies have been carried out to develop nanopharmaceuticals for the vaginal delivery of antimicrobial, antiviral and antifungal drugs, as useful strategies to prevent infections, or transmission of dangerous pathogens. Ensign et al., demonstrated that acyclovir encapsulated in mucus-penetrating NPs, when administered prior to the virus infection, would protect against the Herpes Simplex Virus (HSV) infection in 53% of the treated mice [89]. Malavia et al., developed liposome formulations that were capable of inhibiting HIV infections, having potential use in the prevention of HIV infection in women [90]. Moreover, it was demonstrated that the encapsulation of octylglycerol in liposomes enhanced its activity against HIV, HSV and *Neisseria Gonorreae* with a prolonged released of the drug compared to the traditional gel formulations [91].

Despite the several advantages associated with the delivery of NPs through the vaginal route, there is need for greater optimization of NPs formulations, as stability-related problems can occur due to short shelf-life [77]. One solution for improving stability is incorporating NPs in adequate micro-carrier systems for delivery. However, micro-carriers present with limitations of their own, such as when in need of achieving specific release profiles. In addition, the mucus layer that covers the vaginal epithelium represents another barrier required to be addressed for achieving a uniform distribution of the drug and its prolonged retention in the vaginal tract [92].

3 Considerations for Mucosal NP Delivery

For NPs to gain mainstream adoption as mucosal therapeutic delivery vehicles there are a number of hurdles to overcome. Each mucosal site has unique physiological properties that NPs formulations must cater towards (Table 1), but the ideal properties are mutual; NPs are required to exhibit sufficient stability during the transit to the mucosal surface, must be retained long enough for release, and must deliver or release the active molecule at the desired site and at the appropriate rate. The required NPs properties also differ based on if the goal is for absorption of intact NPs or release of the active molecules at the epithelium. Therefore, the interaction of the particle with the medium that is in contact with the mucosal surface, the mucus layer, and the epithelial cells, must be considered during the formulation process.

The GI tract is arguably the most studied route of administration for systemic delivery of the NPs or active therapeutic, due to the convenience and possibility of absorption, but other routes such as the lungs and nose are also commonly investigated for local pathologies. One of the main advantages for each mucosal site of administration is the localization of treatment, improving the drug concentration at the area, and subsequently reducing the potential for side effects associated with systemic distribution. Another advantage is the lack of needles required for administration, which offers ease of logistics, as well as pain-free administration. There are of course limitations associated with mucosal delivery sites, including the variable bioavailability. The distinctive features of the mucosal sites, and the advantages and disadvantages, have been summarized in Tables 1 and 2, respectively, and gives an indication of how varied the local conditions are.

Table 2 Summary of the advantages and disadvantages of NP delivery at different mucosal sites

Site	Advantages	Disadvantages
Ocular	• Treatment of local ocular pathologies without unwanted absorbance elsewhere	• Poor bioavailability due to clearance mechanisms • Low patient compliance • Low scope for systemic applications
Nasal	• Ease of administration • Potential for brain drug delivery • Highly vascularized	• Mucociliary clearance • Difficult penetration of mucous layer • Enzymatic degradation
Lung	• Rapid absorption • Highly vascularized • Large surface area • Limited enzymatic degradation	• Narrowing and branching of airways may favor particle impaction away from target site • Mucus and surfactants may cause NP aggregation • Mucociliary and alveolar macrophage clearance • Challenges in delivery and low patient compliance
Oral cavity	• Ease of administration • Avoids first pass metabolism	• High clearance due to secretion and flow of saliva • Limited absorption through epithelium
GI tract	• Ease of administration • High surface area • Unique immune make up in the GALT	• Hostile environment can degrade NPs and active molecules • Limited absorption of NPs through the epithelium
Vaginal	• Unique immune make up in the MALT • High residence time of drugs in the site of administration • Potential for prevention of local infections.	• Gender-specific • Hostile environment can degrade NPs and active molecules • Limited absorption of NPs through the epithelium • Mucoadhesive polymer can damage the mucus • Diverse composition of mucus according to age and menstrual cycle.

The immune response is another unique feature at these mucosal delivery sites. There are local differences in the composition of immune cells and tissues, and the resulting immune response can differ, based on the site. This is a point of consideration for the induction of the desired immune response by immunotherapy/vaccine NP formulations at the desired locations. The presence of immune cells also means that the awareness of the immunological consequences, such as inflammation, are also required for formulations even which are not primarily designed to induce immune response.

Future mucosal NPs formulations would therefore ideally address points of interest such as the stability of the particles before they reach the mucus layer, whether retention or penetration at the mucus layer is desired, interaction of the particles with the epithelial or immune cells for uptake, and subsequent release of the active molecules.

To conclude, the delivery of NPs to mucosal sites offer unique advantages and challenges. Each mucosal site differs in physiology and subsequently requires adaptation of the formulation to optimize the NP interaction with the barriers associated with absorption or delivery.

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Current Status and Perspectives in Mucosal Drug Delivery of Nanotherapeutic Systems

Vineela Parvathaneni, Nishant S. Kulkarni, and Vivek Gupta

Abstract Currently, nanoparticulate therapeutic systems are gaining importance and are capable of being delivered through various routes of administration while facilitating both systemic and local drug delivery. Delivering therapeutics to mucosal surfaces of various regions in the body is of special interest because it provides the ability to treat a wide range of disorders. However, due to the mucosal barriers encountered at respective organs/body cavities, it is challenging to deliver therapeutics to mucosal area. Hence, drugs demand certain carrier systems to overcome the barrier and facilitate drug delivery to the site of action. There are several strategies which enable efficient mucosal delivery of nanoparticles (NPs) and enhance the residence time of those systems at the mucosal site. Even though numerous approaches have been used with nanoparticle delivery systems, currently available strategies require further improvements to accomplish mucosal drug delivery. There is still a large gap in understanding the correlation between mucus clearance rates and NPs' performance. This chapter summarizes various approaches utilized for enhancing mucosal delivery of nanoparticulate systems and strategies to evade mucus clearance.

Keywords Nanoparticles · Mucoadhesion · Mucus-penetrating particles · Surface modification · Specific-interactions

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1 Introduction

Nanoparticulate delivery systems offer various advantages in delivering drugs through various routes of administration. These systems can be exploited to facilitate local drug delivery and target specific tissues to ensure efficacy and safety [1, 2]. Depending on the application, various modifications have been introduced in organic nanoparticle fabrication [3] as well as inorganic nanoparticle fabrication [4] through application of chemistry, engineering and manufacturing principles [5].

As majority of the bodily organs are either lined or covered by a mucus membrane, delivering therapeutics to mucosal surfaces of the lung airways, gastrointestinal tract, female reproductive tract, nasal cavity and ophthalmic areas, is of prevalent attention. However, the viscoelastic and sticky nature of mucus layer, which lines mucosal tissues, acts as a barrier for efficient delivery of therapeutics [6]. Moreover, limited permeability of delivery systems through the mucus barrier and hydrophobic nature of drugs result in relatively fast clearance from the site of action. This necessitates innovation of carrier systems capable of overcoming this rapid clearance and simultaneously providing prolonged retention time at the site of action. To achieve this, delivery systems like nanoparticles (NPs) should be capable of not only achieving prolonged retention at the site of action but also be able to penetrate through at least the outer mucus layers. While many advances have been made, conventional NPs are unable to overcome this barrier, which necessitates the need for engineering specially designed nanoparticulate systems or utilization of mucoadhesive polymers.

The current nanoparticle-based formulations require drastic improvements to achieve their intended goals of developing a unique delivery system for fulfilling the gap in accomplishing mucosal drug delivery [7]. This chapter describes the various strategies for mucosal delivery of NPs while overcoming its barrier properties; advantages and disadvantages of these different approaches. Readers are directed to earlier chapters of the book for information about physiology and functioning of mucosal barriers. Few of the recent studies implementing these strategies have also been discussed in further sections.

2 Available Strategies for Mucosal Delivery of NPs

Exploring nanoparticulate delivery systems for an existing drug molecule is gaining attention in the pharmaceutical research field. However, conventional particles (CPs) without any surface modifications get trapped in the mucus and are cleared rapidly resulting in shorter residence times. Hence, researchers have been exploring ways to deliver NPs for mucosal delivery through novel research efforts [8]. Through the application of wide array of engineering approaches and utilization of polymers with mucoadhesive properties, NPs can be made to bypass/penetrate the mucosal barrier. These NPs with efficient mucus penetrating properties are termed as mucus

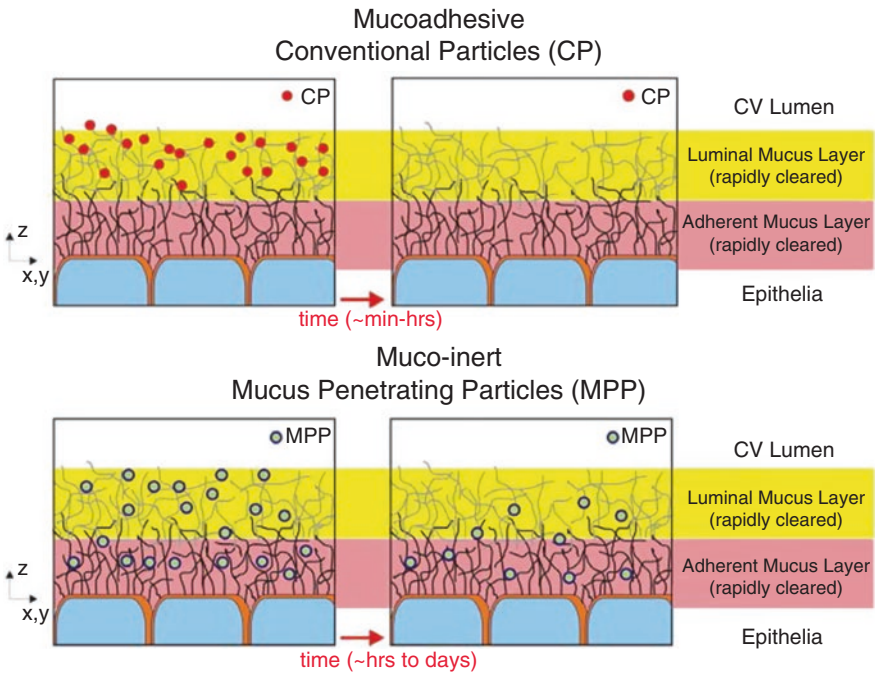

Fig. 1 Schematic illustration of the fate of mucus-penetrating particles (MPP) and conventional mucoadhesive particles (CP) administered to a mucosal surface of gastrointestinal and cervicovaginal tracts. MPP entering the underlying adherent mucus layer (AML) through travelling readily across the luminal mucus layer (LML) compared to CP. Rapid clearance of LML, only MPP can deliver the dose to AML and underlying epithelia where in CP are cleared along with LML providing longer residence time for MPP at the mucosal surface. Similarly, in the respiratory airways, CP are unable to traverse through luminal stirred mucus gel layer, while MPP penetrate the mucus layer and enter the underlying layer, Adopted from [9]

penetrating particles (MPPs) [9]. Figure 1 summarizes the fate of mucus penetrating particles versus conventional mucoadhesive particles [9]. Other available approaches include the use of a modulating agent or mucolytic enzyme-decorated carrier systems. Summary of available approaches for mucosal delivery of NPs is presented in Table 1. Figure 2 indicates the significance of drug delivery systems such as NPs in oral mucosal delivery [10].

2.1 Conventional Strategies for Enhancing Mucosal Residence Time

Conventional approach of mucosal drug delivery refers to the use of mucoadhesive systems. The principle behind this strategy would be enhancing mucosal residence time of dosage forms such as buccal or oral mucoadhesive tablets. Mucoadhesion is

Table 1 Summary of strategies available for Mucosal Delivery of NPs

Strategy	Evidence	Pros	Cons	References
Mucoadhesion	Adhere to the mucus layer	Increased residence time	Trapping of particles inside the mucus	[11, 12]
PEG Modification	Reduced NP electrostatic and hydrophobic interaction potential	Increased NP mobility	NP surface modification needed	[13]
			Increased complexity	
Pluronic Modified NP	Amphiphilic polymer masking hydrophobic interaction sites	No NP surface modification	Potential solubility issues related to amphiphilic polymer micelle formation	[13]
		Works with hydrophobic NP's	Treatment 'window' (dynamic barrier)	
	Increased polystyrene NP mobility		Effect on surface charged NP's unclear	
NP + mucolytic agent	Breaks S_S bonds in multimeric mucin	Apparent increased NP mobility (moving with mucus rather than through)	Increases sol phase viscosity	[13]
			Increased viscous drag	
			Increased interaction potential	
	Liquefies mucus gels		Corona build-up and increased NP size	
	Increased mucus mobility		Reduced first line of defense due to mucus structure breakdown	
NP + osmotic agent	Increased cellular fluid secretion	Generally safe	Initial mucus de-swelling	[13]
	Increased pore size	Reduced steric barrier		
	Reduced steric barrier		Mucus swelling increasing distance for NP diffusion	
		Decreased sol viscosity	Network interactive barrier unchanged	
NP + hydrating solution	Epithelium fluid uptake	Generally safe	Mucus swelling increasing distance for NP diffusion	[13]
	Tidal flow	Flow directed towards the epithelium	Network interactive barrier largely unchanged	
	Mucus gel swelling			
	Increased pore size	Reduced steric barrier		
		Decreased sol viscosity		

(continued)

Table 1 (continued)

Strategy	Evidence	Pros	Cons	References
MECS	Crosses the mucus barrier in a comparatively efficient manner by cleaving mucus substructures and form tiny holes or passages through the mucus	Exhibits broad specificity, cleavage of peptide bonds of basic amino acids, leucine, or glycine.	Breaking down the mucin structure bears the risk that these hostile intruders (pathogens) could come into contact with the epithelium	[14]

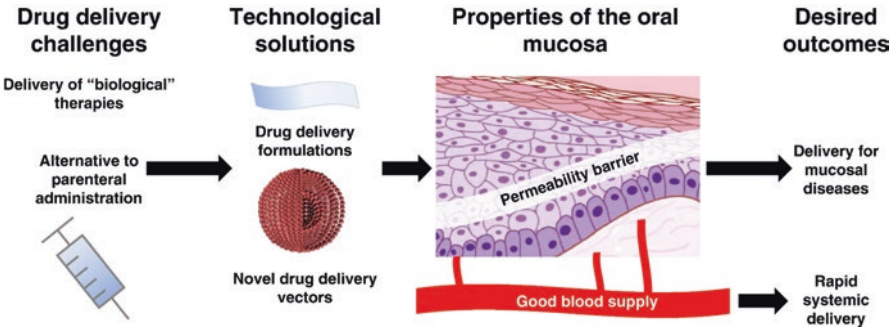

Fig. 2 Graphical representation illustrating the significance of novel drug delivery systems in oral mucosal drug delivery. Newer drug delivery formulations or nano carrier vectors such as NPs capable of overcoming the challenges encountered in delivering therapeutics. In addition, enabling both systemic delivery and local delivery at mucosal surfaces through non-invasive route of administration, Adopted from [10]

the process of adhesion of molecule to mucus layer and is defined as attractive interaction at the boundary between a pharmaceutical dosage form and mucosal membrane [15]. This interaction can occur through the involvement of various mechanisms including physical and mechanical interactions such as intercalation of polymer chains with mucin chains, involvement of hydrogen bonds, electrostatic and hydrophobic interactions etc.; and a clear understanding of those mechanisms is crucial in developing a mucoadhesive drug delivery system [16]. The idea of mucoadhesion was pioneered in ophthalmic drug delivery systems in the early 1980s and this was succeeded by numerous studies, which exhibited the potential of mucoadhesion for effective drug delivery in the fields of buccal, nasal, ocular and vaginal delivery systems, to name a few [17].

Mucoadhesive drug delivery systems offer numerous advantages through prolonging the dwelling period of delivery system at the site of action thus further enhancing the drug absorption. In addition, application of dosage forms on various mucosal surfaces (buccal, nasal or rectal) avoids first pass metabolism as drug delivery occurs across mucosa while protecting drug from harsh environments like gastrointestinal enzymes, resulting in a faster onset of action [18]. Encapsulation of

Table 2 Types of mucoadhesive interactions

Type	Examples	Pros	Cons	References
Non-specific interactions	Anionic polymers, cationic polymers and non-ionic polymers	Form stronger hydrogen bonds with mucus	Shorter retention times and lack of specificity	[12, 17, 20]
Specific interactions	Lectins, thiolated polymers and Polyox water soluble Resins (WSR)	Not affected due to high mucus turn-over rates	Lack of capability to penetrate across the mucus layer due to strong interaction Immunogenic potential (lectins)	[12, 17, 20]

drugs inside the polymeric matrix enables their protection from degradation [19]. These delivery systems are fabricated by mucoadhesive polymers which impart the delivery system its mucoadhesive properties. Ideal properties required for mucoadhesion as described in the Sect. 2.1.1. include the ability to interact with mucus layer through adhesion, possessing high molecular weight and swellable properties [18]. These properties facilitate NPs for their site-specific uptake compared to other systems such as microspheres as in case of transport of NPs across intestinal barrier [19].

Mucoadhesion can occur through either specific or non-specific interactions. Based on the type of interaction they can be categorized as first generation, second generation novel polymers, which will be discussed in further Sects. (2.1.2 and 2.1.3). Different types of mucoadhesive interactions possible are listed along with their advantages and disadvantages in Table 2.

2.1.1 Factors Affecting Mucoadhesion

a) **Molecular weight:** The higher the molecular weight of mucoadhesive polymer (>100,000), higher the mucoadhesive strength of the polymer [21].
b) **Flexibility:** Diffusion of the polymer chains in interfacial area is vital so that the polymer chains contain an extensive degree of flexibility and are capable of entangling with the mucus [22]. Higher the flexibility of a polymer, better will be its diffusion into the mucus network [23].
c) **Cross-linking density:** Increased density of cross-linking lowers water diffusion rate into the polymer network, which in turn causes inadequate swelling of the polymer and diminished rate of interpenetration of the polymer into mucus layer [23].
d) **Hydrogen bonding capacity:** Polymers must have functional moieties that are able to form hydrogen bonds and should also have flexibility potential enough to improve hydrogen bonding in addition [23].

e) **Hydration:** When a mucoadhesive polymer is hydrated, it swells and causes induced polymer chains mobility, exposing bioadhesive sites for hydrogen bonding and/or to form electrostatic interactions between the polymer and the mucus network [23]. A critical degree of polymer hydration is required for optimal swelling and mucoadhesion to occur [24].
f) **Charge:** Presence of anionic charge is reported to be a stronger characteristic for efficient mucoadhesion as compared to cationic charge due to their strong hydrogen bonding ability with the mucin in mucosal layer [11, 24]. However, few cationic polymers have shown greater mucoadhesion in presence of neutral to slightly basic media [25]. For example, high–molecular-weight cationic polymers such as chitosan have exhibited better adhesion in neutral or alkaline environment [26]. pH at the interface of bioadhesive and mucoadhesive membranes influences the adhesion properties of polymers as ionization of the functional groups of polymers depends on it [11, 27].
g) **Concentration:** Concentration of the polymer refers to the available polymer chain length to interact with mucin layers to exhibit stronger mucoadhesion. Hence, higher concentration of the polymer results in better penetration and adhesion. If the polymer concentration is too low, interactions with mucin network are unstable. However, critical concentration should be considered for each polymer as above that the polymer produces a coiled structure resulting in poor penetration ability [27].

2.1.2 Non-Specific Interactions of Mucoadhesive Particles With Mucus

Non-specific interaction involves adhesion of mucoadhesive systems to mucus through non-specific bonding. This includes Van der Waals interactions and hydrogen bond formation. First-generation mucoadhesive polymers which include anionic, cationic and non-ionic polymers, can form these kind of interactions with mucus. Stronger the hydrogen bonding, stronger will be the mucoadhesion. Hence, functional groups play an important role in deciding whether or not to impart strong adhesive properties to the polymer. Moieties such as carboxyl, hydroxyl and amino groups are capable of facilitating such interactions [11, 28]. In addition, polymers possessing functional groups within their structure are referred as polyelectrolytes, where anionic polyelectrolytes form stronger adhesion compared to neutral polymers. Typical examples of polyelectrolytes include poly (acrylic acid) (PAA) and sodium carboxymethylcellulose (NaCMC). These polymers exhibit outstanding mucoadhesive features by forming strong hydrogen bonding interactions with mucin network in mucosal layer. Chitosan is the most widely used cationic polymer, well known for its intriguing properties and mucosal binding through ionic bonds between the amino group and sialic acid residues [12, 20]. However, all of the first generation polymers adhere to the mucus non-specifically lacking specificity as the interactions involved in mucoadhesion are of non-covalent nature .

2.1.3 Specific and Targeted Mucus Interactions of Mucoadhesive Particles

Newer second-generation polymers are employed to impart specific mucoadhesive properties to drug delivery systems by overcoming the disadvantages of non-specific mucoadhesive polymers while being site specific and unaffected by high mucus turn-over rate [11, 29]. Second-generation polymers such as lectins, thiolated polymers, Polyox Water Soluble Resins (WSR), and tomato lectin specifically interact with the mucosal cells and are thus more targeted [30]. Lectins are natural, structurally varying proteins which can bind to specific carbohydrate residues on mucosal cells reversibly. Following binding to mucosal cells, lectins can either stay on surface or get internalized through endocytosis via receptor mediated adhesion. Thus, lectins can provide site specific controlled drug release. However, presence of potential immunogenic reaction is a disadvantage of lectins [12, 20].

Thiolated polymers are derived from water soluble polymers such as polyacrylates or chitosan [12]. They imitate the mucus glycoproteins covalently bound in the mucus layer. Free thiol groups of the polymers form disulfide bonds with cysteine present in mucus [20]. This specific adhesive property assists in better mucoadhesion and alteration of the drug release pattern due to enhanced cross linking [12]. Commonly used thiolated polymers include chitosan–iminothiolane, poly(acrylic acid)–cysteine, poly (acrylic acid)–homocysteine, chitosan–thioglycolicacid, chitosan–thioethylamidine, alginate–cysteine, poly (methacrylic acid)–cysteine and sodium carboxymethylcellulose–cysteine [20].

Polyox WSR is a novel, high molecular weight polyethylene oxide homopolymer which is readily water soluble and comprises of functional groups available for hydrogen bonding [12]. Tomato lectin is another novel polymer, which is different from lectins, and can bind to the small intestinal epithelium selectively [12, 20].

2.1.4 Shortcomings of Conventional Mucoadhesive Strategies

Mucoadhesion requires a prolonged contact time for effective binding at the site of action which may lead to local ulcerous effects due to ulcerogenic characteristics of the drug itself based on the site of mucoadhesion [31]. For example, buccal site suffers from inconvenience because of taste and irritability potential of mucoadhesive oral dosage forms, presenting a major limitation to this approach [32]. Rectal and vaginal sites suffer from inconvenience of administration and patient compliance. In addition, transit time of mucoadhesive systems is predicted by the mucus turnover rates as these systems interact with mucin network of mucus layer [9]. Due to the adherence of mucoadhesive polymers to mucus, they lack the capability to penetrate across the mucus layer and enter the epithelia underneath, thus making them challenging for intracellular delivery of therapeutics [9]. Also, manufacturing complexities prove bioadhesives as a complicated drug delivery approach [16].

NPs coated with either non-specific or specific bioadhesives have shown potential for mucosal delivery but these approaches demand further research and progress

before being approved by the regulatory agencies and become available to the patients [33]. Multiple engineering approaches have been attempted for fabrication of mucoadhesive nanoparticles which can adhere and cross mucus barriers and accomplish prolonged residence time of particles at mucosal surfaces and reach the intended target across the epithelium, respectively [9, 34].

2.2 Engineering Particles to Cross Mucus Barriers

NPs of mucus-penetrating potential are suitable for treatment of disorders at mucosal surfaces [35]. Modulating surface properties of nanoparticles made from polylactic acid, chitosan, poly (lactic-co-glycolic) acid (PLGA) aids in attaining desired hydrophilic/hydrophobic properties through including molecules on their surface. The surface moieties influence particles' penetrating behavior through the mucus.

Several approaches have focused on modifying the NPs" surface to escape entanglement in the mucin networks of mucus [13]. These systems can penetrate the mucus with minimal interactions and exhibit [14] slippery surface [36].

2.2.1 Modulating Hydrophilic/Hydrophobic Surface Properties

Mucus as a biological barrier hinders the mobility and penetration of NPs through involvement of mucin interactions with the particle surface. Hence, strategies to modulate surface of NPs came into existence such as PEGylation to alter the hydrophilicity of nanocarriers. Length, structure and grafting degrees of surface ligands govern the surface properties of NPs. Further, charge and hydrophobicity of NPs have a significant impact on their mucus penetrating behavior. Hence, modulation of NPs' surface properties for attaining hydrophilic and uncharged surfaces to successfully diminish the adhesive interactions between mucin and particles through reduced hydrophobic or electrostatic interactions present a promising strategy for efficient mucosal delivery [37]. A few polymers that have been used to modulate the surface properties of NPs are outlined below.

PEG-Modified NPs

Modification of nanoparticle surface with molecules of smaller sizes than the mucus network and coating with poly (ethylene glycol) (PEG) cause rapid diffusion of particles through mucosal secretions [38]. These PEG-modified NPs have shown improved distribution through mucosal surfaces along with greater therapeutic efficacy in treating diseases such as cervical cancer and also lung cancer using gene therapy [39, 40]. Creating copolymers of PEG with polymers through adsorption onto carrier particles alters their characteristics to achieve an effective particle size

and surface charge [41]. Surface modification using PEG imitates virus-like diffusion thus allowing for an improved transport through mucosal surfaces.

In a study by Cu and Saltzman, PEG conjugation to COOH-functionalized polystyrene particles was found to increase their diffusion in cervical mucus [42]. Moreover, PEGylation of NPs also augments their stability with regards to both physical (aggregation) and chemical properties in mucus along with improved transport. Stability is vital while particles transverse through a thick mucus layer before reaching underlying layers [9]. Few other research works have revealed that coating NPs with a high density of low molecular weight PEG is able to reduce the interaction of particles with mucin network. Adhesion is incomplete due to diminished polymer chains interpenetration into the mucus due to the low MW of PEG. Moreover, PEG density is adequate to protect the hydrophobic core [38, 43]. PEG can also be conjugated with other polymeric materials such as poly sebacic acid (PSA) [44], polyethylenimine (PEI) [45] and poly-l-lysine (PLL) [46]. Additionally, PEG can also be adsorbed on the particle surface through hydrophobic or electrostatic interactions [37].

Pluronic F-127 Modified NPs

Pluronics are triblock copolymers consisting of a hydrophobic poly (propylene oxide) (PPO) core with two hydrophilic PEG arms. PPO core of pluronics can adsorb onto hydrophobic nanoparticle surface, while the PEG chains safeguard the particle surface from possible electrostatic and hydrophobic interactions with mucin [47].

Few studies in literature reveal that pluronics containing PPO segments with MW of more than 3 kDa can produce MPPs. Pluronic F-127 falls under that category [48]. Yu et al established mucus-penetrating nanoparticle system composed of two poorly water soluble drugs and coated with select pluronic F127 and reported a muco-inert surface of the coated particles [34]. However, not all hydrophilic and neutral modifications can enable mucus penetration. For example, hydrophilic and uncharged polyvinyl alcohol (PVA) coated polystyrene NPs were found to be mucoadhesive independent of MW and PVA concentration resulting in poor mucus penetration [37].

2.2.2 Limitations of Nanoparticles With Surface Modifications

PEGylated NPs can mobilize rapidly in mucus coating and enter other sites in the body due to their size and surface properties. Rate of diffusion of NPs depends on hydration of mucus layer, leaving modified NPs with no significant benefit in cases of less hydrated mucus conditions such as cystic fibrosis (CF) and chronic obstructive pulmonary destruction (COPD) [49, 50]. Even though larger size NPs are preferable in terms of drug loading efficiency and desired release kinetics, optimal size is required for mucosal delivery applications. Due to amplified resistance forces,

NPs of large size, even well-coated ones are unable to diffuse and overcome mucociliary clearance when delivered by the pulmonary route (REF). Depending on the mucosal tissue properties, different nanoparticle diffusion rates are essential to cross different mucus barriers. For instance, a 700 μm and a 10 μm thick mucus is found in colon and eye respectively where even the viscosity of the mucus and mucin types differ thus necessitating NPs of different diffusion rates. Moreover, for efficient uptake into underlying epithelial cells, larger nanoparticle size is anticipated to lessen the endocytosis rate [9, 51, 52].

2.3 Co-Association of NPs and Mucus Modifying Agents

Although there are several strategies available to modify particles so they can penetrate mucus while preventing them from being entangled in mucin networks, there are a few unresolved questions. These include induced changes in mucus properties in different pathological conditions and non-feasibility to manufacture surface engineered NPs at an industrial scale. The introduction of mucus modulating agents can improve mucosal nanoparticle drug delivery and overcome the above challenges [13]. Mucus modulating agents alter mucus barrier properties considering modification of its steric and interactive components. These agents induce changes in mucus matrix architecture, pore size and reduce mucus-NPs interactions [13]. In case of mucosal diseases such as CF and other lung diseases the mucus barrier properties alter significantly in terms of mucus volume, composition or physiological function like mucociliary clearance [53, 54]. Hence, these conditions demand use of mucus modulating agents capable of both improving nanoparticle transport and mucociliary clearance in lung diseases. For example, guluronate oligomers have the potential to alter mucus barrier function and are under investigation for their role as active pharmaceutical ingredients to improve mucociliary clearance in CF [55, 56].

Major motivations for the use of mucus modifying agents in association with NPs include the ability of these agents to improve mobility of NPs in mucus which can be produced easily [57] while keeping costs low [58] compared to complex systems [57]. NPs. Complex drug delivery systems involve numerous components and need for surface modifications make the large scale production more complicated [57, 58]. Considering all commercial aspects such as producing NPs with required specifications while keeping the unit cost low and scaling up from small batch production, it is of significant interest to identify strategies that are able to manufacture NPs which to function effectively. Utilization of a mucus modulating agent compromises the active mucus barrier encountered by a nanoparticle in mucosal delivery and enhanced drug uptake can be achieved [13].

In a strategy involving co-administration of the mucus modulating agent and nanoparticle, they were administered as separate dosage entities at the mucosal surface either simultaneously or sequentially [13]. In another co-formulation strategy, both NPs and mucus modifying agents were present in the same delivery system [13]. In many other studies, a non-covalent association between mucus modifying

agents with the nanoparticle surface were established like in the case of polyplexes, where surface alteration with mucus modifying agent was performed and thus was part of the nanoparticle itself [59].

For a successful and efficacious co-association of nanoparticle and mucus-modifying agent, they are essential to reach the mucosal surface in a functional state without being altered by the physiological processes encountered. Mucus modulating agents, such as mucolytic agent or mucus hydrating agent, that are already in clinical use can be utilized for co-administration with NPs [13]. In the section below we briefly mention these mucus modulating agents that can be co-administered with NPs.

2.3.1 Use of Mucolytic Agents

Mucolytic agents work by depolymerizing (lyse) mucins or other polymeric components of the mucus or sputum, while DNA and actin are the non-mucin targets. Mucolytics can be used as a therapy alone or in combination with other therapeutic agents to improve its delivery through mucosal secretions [13]. In addition to exploring mucolytic agents for their capability to enhance therapeutic delivery across mucosal surfaces [60, 61], they have also gained importance in the framework of pulmonary delivery for treating lung diseases, especially CF and COPD, where abnormally thick and viscous mucus is encountered in the airways [62]. These agents are currently used to lessen the bulk viscoelasticity of CF sputum through cleaving its constituents and improve sputum removal and lung function [63].

It is important to understand the basis of macro-rheology of mucus due to its significance toward the progress and assortment of possible beneficial approaches. For example, CF patients are required to administer mucolytic agents by inhalation for an enzymatic cleavage of mucus components for facilitating proper mucus clearance from lungs through coughing. For instance, in CF sputum, reduced water content and augmented cellular debris is observed which leads to an upsurge in physical entanglements, diminished usual mesh pore spacing, and increase in the viscoelastic property of the sputum [64].

N-acetylcysteine (NAC) and recombinant human DNase are the commonly used mucolytic agents. NAC acts by decreasing viscoelasticity through replacing the disulfide bonds of mucin networks with free sulfhydryl moieties, which further helps in disrupting the structure of the mucosal gel [65]. NAC is known to reduce the viscosity of mucus/sputum both *in-vitro* [66] and *in-vivo* [67]. Inhalation of a lysine salt of NAC, nacystelyn, has also been found to reduce sputum viscoelasticity and solid content in a dose-dependent manner [68]. Mucolytic agents also provide enhanced penetration rates of drug and gene carrier particles into the mucus [65]. Thus, the use of mucolytics as an adjuvant to particle transport is gaining importance. In 2011, Suk et al evaluated the enhancement of particle penetration through CF sputum in the presence of a combination of NAC with dense PEG coatings on particles. They reported enhanced sputum penetration by larger particles by combination strategy which are otherwise trapped in CF sputum [63].

2.3.2 Increased Mucus Hydration

Increasing mucus hydration is mostly applicable in treating lung diseases such as chronic bronchitis and CF which involve mucus dehydration compared to typical state [54, 69, 70] with limited applicability in other conditions. Increased hydration of mucus reduces barrier properties by either imparting the mucus a gel swelling property or by decreasing the viscosity of the sol phase inside the mucosal gel layer [71]. In case of gel swelling, increase in the average pore size of gel matrix occurs. Blackmon and co-workers recently demonstrated real time increase in pore size, and reduction in steric barrier properties due to mucus swelling in model mucus secreting cell cultures such as Calu-3 upon exposure to osmotic agents like hypertonic saline. Steric barrier imposed by the pore size in mucus will affect interaction with NPs. Hypertonic saline initiates cellular fluid secretions thus enhancing mucus hydration and reducing viscosity of the sol phase in the mucus layer. They have used coherence tomography to image and directly monitor mucus hydration [72]. Ibrahim et al also examined hydration effects on the mucus barrier properties, using mannitol as an osmotic agent to augment mobility of nanoparticle gene carriers through the sputum [71]. Further, mannitol as an osmotically active agent can be administered as a dry powder for inhalation [73].

As an alternative strategy, hypotonic aqueous formulations are used as vehicles for nanoparticle administration at the absorptive mucosal surfaces of the vagina and colorectum [13]. Hypotonic solutions are known to dilute the sol phase, and can cause induced uptake of fluids by the epithelial cells, thus creating a tidal flow to direct the nanoparticle formulation through the mucus [13]. In one such study, Ensign et al demonstrated that muco-mobility of mucus penetrating NPs was enhanced in presence of hypotonic aqueous formulations, which was hypothesized to be the result of mucus gel swelling and corresponding increase in pore size in mucus gel [74].

2.4 Design of Mucolytic Enzyme Decorated Carrier Systems

Design of mucolytic enzyme decorated carrier systems (MECS) provides another promising approach to formulate carrier systems capable with enhanced bioavailability across the mucus barrier. These systems comprise of micro and nanoparticulate systems as well as self-emulsifying drug delivery systems (SEDDS) decorated with enzymes like papain (PAP) or bromelain (BRO) which can cleave peptide bonds of mucus glycoproteins in mucin [75]. MECS are capable of crossing the mucus barrier efficiently by cleaving substructures of mucus which are obstructing their path toward the epithelium. These enzymes exert their effects by hydrolyzing peptide bonds of mucus glycoproteins and forming tiny holes or passageways across the mucus. In various research, *in-vitro* and *in-vivo* studies have demonstrated that MECS are efficient with greater mucus penetrating capability over nanocarriers deprived of enzyme decoration [14]. Due to MECS' ability to cleave the mucus

network locally, the protective barrier properties of mucus are undisturbed, which makes MECS a feasible strategy for long-term treatments [14]. Passive systems and carrier systems of surface charge modifications are only capable to effectively permeate the mucus up to 200 nm particle size. But, MECS can penetrate the mucus even if the particles are larger in size [14] by enabling MECS to transport between the larger subunits of mucus. Due to the expansion of these mucus meshes, MECS are capable of penetrating the mucus [76]. Enzyme decorated SEDDS can be prepared through incorporation of enzyme into the lipophilic core [77, 78]. Leichner et al developed a papain loaded SEDDS system, reported increased mucus penetration and mucosal residence. Papain aids in enhancing mucus permeation through its mucolytic activity [77].

Polymeric MECS should possess mucus penetrating properties along with a slippery surface and characteristics of enzymatic decoration. Using negatively charged particles exhibit superior transport in mucus compared to positively charged particles due to electrostatic repulsion [79]. By combining a negatively charged polymer, such as PAA with a polyvalent cation like calcium chloride as a cross-linker, MECS with passive permeation characteristics can be formulated. It was also reported that particles with polymers of opposite charges producing a neutral surface net charge had shown better permeation properties relative to either negatively or positively charged NPs enabling it as another capable carrier structure [80].

For the preparation of MECS, the approach of polymer-enzyme complex formation between PAA and PAP was utilized by Dautzenberg et al and covalent attachment of PAP to PAA by Müller et al [75, 81]. Development of novel MECS by combining different enzymes further enhances their mucus penetrating effect in case of additive or synergetic effects. Moreover, MECS could provide an useful drug carrier system in treating mucus related diseases such as CF and COPD [14]. Figure 3 summarizes the available strategies utilized to improve nanoparticulate delivery for drug delivery across the mucus.

3 Research Strategies for Mucus Penetration

3.1 Diffusion Experiments

Diffusion of polymeric particles through mucosal layer can be quantified by *in-vitro* as well as *in-vivo* methods. A traditional method for the same is the use of diffusion chambers. In this method, rates of permeation of particles through mucus layer placed in the diffusion chamber can be measured. Sinko and co-workers used reconstituted porcine gastric mucin gel to measure the diffusion of polystyrene particles of different sizes across a layer of mucus-mimetic gel layer. They used a Transwell-Snapwell diffusion chamber where mucin gel was placed between the two chambers [84]. Even though diffusion experiment is simple, it is sensitive against parameters such as thickness of mucus sample and unstirred layer outside filters, changes in mucus characteristics and blockage of filter pores by mucus [49].

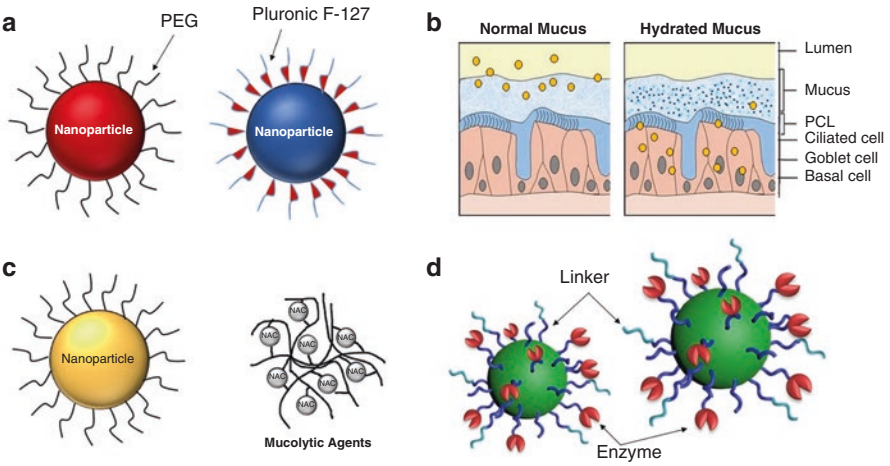

Fig. 3 Schematic of available strategies for mucosal delivery of nanoparticles. (**A**). Surface modified nanoparticles either with PEG or pluronic F-127. (**B**). Mucus hydrating agents as mucus modulators to enable penetration of nanoparticles through mucus layer. (**C**). Co-administration of nanoparticle and mucolytic agent. (**D**). Nanoparticles decorated with mucin cleaving enzymes and can cross the mucus barrier through the mucus, Adopted from [82] (B), [83] (D)

There are other studies which have overcome the problems encountered with use of diffusion chambers. These include fluorescence recovery after photobleaching (FRAP) and multiple particle tracking (MPT). These methods are capable of recording active transit of NPs in the mucus layer using fluorescence microscopy.

In case of FRAP, fluorescently labeled NPs are exposed to a laser beam to produce a moving white spot. By recovering the fluorescence intensity from diffused fluorescently labeled molecules due to the flow of NPs, diffusion coefficient is attained [85]. Shen et al. examined the diffusion of plasmid DNAs in mucus by means of this FRAP method [86]. Nordgård et al also explored the use of FRAP to inspect the impact of guluronate oligomers on NPs mobility in mucus layers, and determined that guluronate oligomers were capable of advancing NPs movement in gastric mucus of native pigs [55]. Even though mobility of labeled molecules can be determined in mucus and biogels by using FRAP method, the drawback with this method is its inability in providing quantified diffusion rates for individual particles and determination of average diffusion rates only. This limits FRAP method's applicability for complex mucus experiments [87].

Recently, Hanes et al established multiple particle tracking (MPT) technique to help with measurement of NPs' mobility in mucus [88]. Diffusion behavior of NPs in the mucus can be calculated by rotating diffusion tubes [89] and mucus slices [90]. Moreover, MPT techniques are capable of recording the trajectory of each individual particle using inverted fluorescence microscope. Also, one can analyze NPs in some complex biological secretions [91]. Other *in-vitro* techniques for

mucus penetration measurements include capillary penetration using magnetic beads; and a magnetic field and nuclear magnetic resonance (NMR) with either pulsed-field or pulsed-gradient spin-echo [92].

3.2 Cell Models

Cell models constitute a monolayer of epithelium or a multilayer of cells with mono or co-cultured cells on a semipermeable membrane. These models can be used to evaluate the epithelial uptake and/or absorption of therapeutics. Based on the required level of epithelial cell integrity and cellular differentiation of the model, selective culturing conditions have to be chosen. Use of co-cultured cell models potentially provides more physiologically relevant and biologically responsive models for performing the studies [92].

3.2.1 HT29-MTX Cell Model

HT29 (human colonic cell line) differentiates into mature goblet cells in presence of methotrexate (MTX), capable of secreting mucus. Hence, HT29-MTX cells help in studying the impact of mucus layer on NPs' mobility [93]. However, there are limitations such as the exposure of NPs to only single type of intestinal epithelial cells. Therefore, performing *in-vivo* experiments is the best alternative approach for performing such transport studies [37].

3.2.2 Caco-2/HT29-MTX Co-culture Cell Model

The co-culture model consists of simultaneous culturing Caco-2 intestinal epithelial cells, and mucus-producing HT29-MTX (goblet cells) which can provide a better drug absorption model including the mucus barrier [94]. Improved in-*vivo/in-vitro* correlation application can be preserved by co-culturing Caco-2/HT29-MTX at a proportion of 90%/10% or 75%/25% preferably [95].

3.2.3 Caco-2/HT29-MTX/Raji B Triple Culture Model

In this model, human Burkitt's lymphoma Raji B cells which represent M cells are co-cultured with Caco-2, and mucus-producing HT29-MTX. This triple co-culture is capable of establishing a model to closely mimic human intestinal epithelium. As M cells play a key role in transporting antigens from the intestinal lumen to immune cells [96] and are located in the epithelium overlapping Peyers' patches, this co-culture forms a significant model to study intestinal translocation. It has been reported that

NPs can enter intestinal epithelia through M cells [37]. This model was established through co-culturing of Caco-2 and HT29 cells into transwell filters followed by adding Raji B cells to the basolateral chamber [97].

3.3 Animal Models

Animal models are necessitated to better comprehend the fate of these NPs and also to understand a way to translate these results in humans. Hence, most of the recent studies have implemented animal models to inspect the pharmacokinetic and pharmacodynamic behavior of NPs. These models include mainly isolated intestinal experiments, *in-situ* experiments and *in-vivo* experiments.

3.3.1 Isolated Perfused Intestinal Model

In experiments where cell monolayer models were used, there are few experimental limitations, including lack of three-dimensional structure and cells with variable differentiation. Hence, isolated intestinal experiments (including everted intestinal sac and permeability study by Ussing chamber [98]) are adopted to determine mucoadhesive properties of NPs. Nevertheless, this model requires the intestine to be removed, opened, washed and segmented, which may alter intestinal absorption properties, thus failing to forecast or correlated *in-vivo* performance of NPs [99].

3.3.2 In-situ Models

Intestinal loop models help to study systemic absorption of drugs. In this model, a slice of the small intestine is removed from the abdominal cavity, followed by ligating at both ends of that portion to make an isolated "loop" and the test NPs are injected into the loop directly. After certain period, the animals are sacrificed; and additional morphology or quantitative analysis can be conducted by removing the intestinal loop from the body cavity [100]. In 2013, Li et al used this model to investigate the ability of core/shell corona nano-lipoparticles in facilitating the insulin permeation across the ileum epithelia [101]. Also, in 2013 Chen et al have studied the influence of mucus on NPs absorption and the amounts of particles trapped in mucus using this model [102].

3.3.3 In-vivo Models

Even though there are many recent advancements available to utilize *in-vitro* models, *in-vivo* assessment is essential for validating the actual performance of drug delivery systems. For instance, as in case of oral delivery of NPs, it is complex

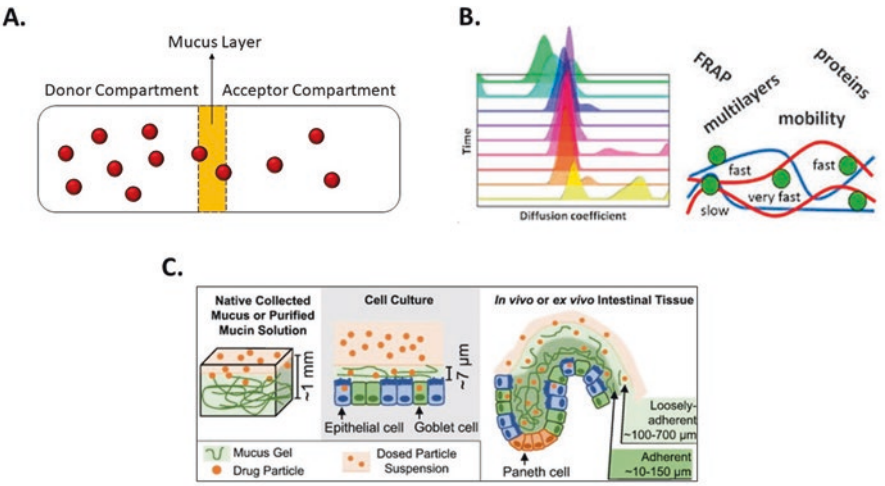

Fig. 4 Biophysical approaches used to evaluate the transport of nanoparticles through mucus barrier. (**A**) Schematic diffusion chamber experiment showing nanoparticle (NP) diffusion from the donor compartment, across a mucus layer, and into the acceptor compartment. (**B**) Schematic of fluorescence recovery after photobleaching (FRAP) experiments to determine the diffusion of fluorescently labelled NPs. (**C**) In vitro, in vivo, and ex vivo mucus models to study drug carrier diffusion using either isolated mucus gels or mucus present on cell cultures, tissues and animal models, Adopted from [106] (B), [107] (C)

to maintain the composition and thickness of the actual mucus and simulate in *in-vitro* models [37]. In 2001, Lamprecht et al studied particles for targeting inflamed colonic mucosa in rats and observed large number of particles trapped in mucus [103]. In another study by Arbos et al, it was found that poly(methylvinylether-co-maleic anhydride) NPs were unable to move toward the enterocytes of small intestinal and colon lining in rats and also observed highest localization of NPs in the mucus layer [104]. However, an inadequacy presented in all *in-vivo* models so far presents an extrapolation to human studies uncertain [105]. Figure 4 summarizes the biophysical approaches that are utilized to evaluate the efficiency of the fabricated nanocarriers in penetrating the mucus.

4 Conclusion

Mucosa provides a potential site for delivery of numerous therapeutics in treating a wide range of disorders. The significant role of nanoparticulate systems in current therapies make them promising carriers for mucosal delivery. However, the barrier property of mucus has to be addressed through proper strategies to result in an efficient mucosal drug delivery system to deliver NPs. Even though there are numerous approaches which have been used, there still exists a large gap in understanding

mucus clearance rates. Future studies require well-designed *in-vivo* studies in order to validate the mucus penetrating capability of various nanoparticle strategies rather than relying on only *in-vitro* systems.

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Buccal Delivery of Nanoparticles

Andrea C. Ortiz and Javier O. Morales

Abstract The buccal route offers an alternative for drug administration due to its advantages, including the avoidance of the gastrointestinal tract, the hepatic first-pass, enzymatic degradation and chemical instability of certain molecules that would pose a challenge to formulate orally. Moreover, the oral cavity has a lower enzyme content than the rest of the gastrointestinal tract, predictable transit times, easy administration, and provides the opportunity to readily halt drug administration. Additionally, the oral cavity is an organized system with stratified epithelium that allows manufacturing of pharmaceutical forms for drug delivery.

Due to the possibilities offered by this route, recent research efforts have been conducted towards the use of nanotechnology to enable buccal drug delivery. In this chapter, we discuss the anatomy of the oral cavity, relevant characteristics of the epithelium to drug delivery and delivery system permeation, types of nanocarriers that have been reported to-date and toxicity studies addressing nanotechnology.

Keywords Buccal absorption · Nanocarriers · Nanoparticle safey · Ex vivo permeation · Mucoadhesion · Buccal drug delivery

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1 Introduction

Different routes of administration have emerged to overcome difficulties of the oral or the intravenous route. However, there are many molecules (mainly class II and IV of the Biopharmaceutical Classification Systems and biologics alike) that pose challenges to formulate and thus to administer orally [1–3]. These molecules frequently exhibit very low oral bioavailability or, conversely, they have to be administered intravenously. Injections, however, are invasive and poorly accepted by patients [4]. For such reasons, alternative routes of administration are largely studied, and one of them is the buccal route. Buccal drug administration has been extensively studied due to patient comfort, bypassing the gastrointestinal tract and thus the hepatic first-pass effect [5, 6].

The mouth is a well-organized system, with a variety of functions, which closely work together to prevent absorption of foreign substances, maintain the oral micro ambience and support the digestion process [7]. The buccal epithelium is located in the inner mucosal side of cheeks, and together with the sublingual epithelium, it is non-keratinized as opposed to other regions of the oral cavity [8]. The buccal route has advantages such as the avoidance of the gastrointestinal (GI) tract and thus avoidance of low pH exposure, a relatively low enzyme content compared with the intestine, predictable transit (contact) times, an excellent vascular and lymphatic supply, ease of administration, and long cellular turn over (5–6 days) which may facilitate long term delivery in retentive dosage forms [9].

The use of the buccal route is geared by the avoidance of the hepatic first-pass effect, enzymatic degradation and chemical instability of certain molecules that would pose a challenge to formulate orally [10]. In addition, this route of administration is comfortable and accepted by patients, especially those who have difficulty swallowing [11]. For this route of administration, a number of pharmaceutical forms are available, including tablets for sublingual administration, chewable tablets or mouth swabs. These dosage forms, however, have limitations due to the environmental factors of the oral cavity, enzymes or the effect of saliva, and thus limited buccal bioavailability can be obtained from these forms [12]. Therefore, challenging molecules (BCS class II, IV, or biologics) may need advanced drug delivery systems that favor residence time and permeation through the buccal mucosa.

For this reason, nanoparticles have been a strategy for the administration of drugs, since they can be designed for controlled release, protection of the active components from enzymatic agents, and localized retention [13]. In addition to these advantages, manufacturing methods can be scalable [14–16] and applicable to different medications and disease conditions [17]. There are different types of nanoparticles including organic [18–20] and inorganic [21–23], which have been studied for different routes of administration. Specifically, polymeric nanocarriers have been the most studied for evaluating the buccal route of administration [8, 24–27].

In this chapter, we will discuss the anatomy of the oral cavity, important properties of the buccal mucosa, methods by which drug delivery has been enabled by means of nanotechnology, and the potential toxicity of these systems.

2 Buccal Epithelium Anatomy and Physiology

The oral cavity is easily accessible and is not invasive, making it an excellent candidate for alternate route of administration. Moreover, different tissues in the oral cavity can promote high systemic absorption. In addition, the administration of drugs by this route avoids the hepatic first-pass effect and degradation promoted by the different pH and enzymatic environment in the rest of the GI tract [28]. Moreover, blood irrigation, epithelium thickness, and a non-keratinized epithelium are factors that favor the buccal route of administration [4]. All these factors can affect drug permeation through the oral mucosa [29].

The various tissues in the oral cavity include the lips, cheeks, hard and soft palate, tongue and the floor of the mouth [29]. Moreover, three types of oral mucosa have been identified, with a similar distribution in adults and children [4]: (i) lining mucosa (60%), (ii) masticatory mucosa (25%), and (iii) specialized mucosa (15%). The lining mucosa comprises the non-keratinized sublingual and buccal epithelium. The masticatory mucosa comprises the hard palate and gums (both keratinized). Finally, the specialized mucosa, exhibiting both keratinized and non-keratinized regions, is found in the dorsal surface of the tongue [30].

The buccal mucosa is a stratified squamous epithelium followed by a basal membrane, lamina propria, and a submucosa as its innermost layer (Fig. 1) [4, 31]. It is generally understood that the permeation barrier resides on the top quarter or third of the stratified epithelium [32]. The oral mucosa protects the underlying tissues from mechanical damage and from entry of toxic materials and microorganisms [33].

From a drug delivery standpoint, the buccal mucosa offers advantages over other epithelia in the oral cavity including a larger surface area ($50.2 \pm 2.9\ cm^2$) [34] and an intermediate permeability compared with the low permeability of gingival and palatal epithelia [35]. Furthermore, the buccal epithelium is highly vascularized and any drug diffusing across the buccal mucosa can directly access systemic circulation via capillaries and venous drainage, bypassing the hepatic first-pass metabolism [36].

A continuous desquamation process occurs in this epithelium, resulting in a long cellular turnover, that is similar to the rest of squamous epithelia of the body [37]. In addition, due to homeostasis, the buccal epithelium can quickly heal after damage [38]. It is due to this constant differentiation and stratification, that the epithelium is highly permeable but less than the intestinal epithelium [38]. On the other hand, the sublingual mucosa has a more limited surface area, a more continuous liquid flow, and it is thinner in comparison to the buccal mucosa, which would favor

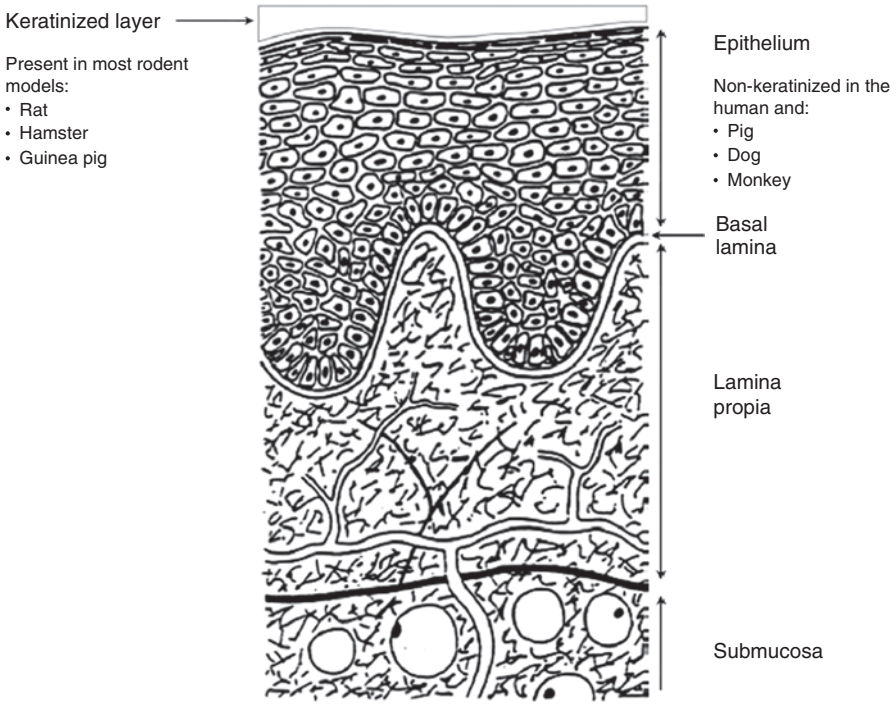

Fig. 1 Diagram of a cross-section of the buccal mucosa. The keratinized layer is only present in most rodent models while humans have a non-keratinized buccal mucosa. Reprinted from Morales and McConville [5]

rapid and better action [39]. Keratinized areas of the oral cavity (gums and palate) have a larger content of cholesterol and ceramides (much like the skin), whereas non–keratinized domains such as the buccal and sublingual mucosa have a greater presence of phospholipids, esters of glycosylic ceramides and cholesterol. This accounts for the main differences in permeability between these tissues in the oral cavity [37].

In relation to the physiologic changes generated from childhood to adulthood, there is a decrease in the thickness of the buccal epithelium as we get older [40]. In a human study, it was observed that the epithelial cells become flatter with age, which suggests that the area and the perimeter of the epithelium becomes greater. However, despite the difference in oral development, these do not significantly affect the buccal epithelial tissue [41]. Furthermore, studies in animals have shown that during aging there is a decrease in cell density in the buccal cavity [42] and a decrease on the mitotic cell activity [27]. In this regard, it has been observed that freshly excised animal mucus membranes are widely used due to the similarity with human *in vivo* absorption (Fig. 1). Although rodents are the first line of animals used for buccal delivery studies, they are not good representatives due to their keratinized buccal epithelium. Within rodents, rabbits are better suited for buccal studies due to

their para–keratinized buccal membrane [43]. In general, larger animals exhibit a non-keratinized stratified buccal mucosa, which is similar to the human anatomy and differs mainly in the thickness and permeation properties of the tissue. However, monkeys and dogs, due to ethical and economic aspects, are not commonly used [44]. It has been widely demonstrated in literature that the oral mucosa of the pig seems to be the most suitable animal model, due to its availability, thickness, and permeation properties of the buccal mucosa [45–49].

For all these reasons, the oral mucosal route is a strategy that can be exploited for the administration of different drugs, and with a particular focus on challenging drugs (BCS class II, IV and biologics). A leading strategy in recent years has been the use of nanocarriers to enable buccal drug delivery systems.

3 Nanoparticles as Drug Delivery Systems for the Buccal Route

Nanotechnology has contributed with different delivery systems and drug release strategies to yield absorption through the buccal route. For the limitations of oral administration and the advantages of buccal delivery, the molecules of interest to incorporate in nanocarriers are those that have a marked GI metabolism and are strongly affected by the hepatic first-pass effect. Moreover, drugs that have low solubility and permeability could also be a good fit for buccal absorption. Furthermore, the buccal route of administration is better accepted and fulfilled by patients in comparison to an invasive route of administration, such as the intravenous [50].

Several research groups have studied the release of various molecules in different nanocarrier systems; however, most researched delivery systems are comprised of polymeric or lipidic nanoparticles. Within polymeric delivery systems, Mouftah *et al*. studied the release of heparin encapsulated in polymethacrylate nanoparticles for buccal administration. Heparin is a relevant molecule due to its macromolecular structure, and thus of sensitive chemical and physical nature. *In vitro* drug release studies revealed a very slow release reaching a 6% release plateau, which can be modified according to the composition of the polymeric matrix. The slow release was associated with strong electrostatic interactions between the negatively charged heparin and the polycationic polymers [51]. This was also demonstrated by Choi *et al*. where the release of human growth hormone from polymeric nanoparticles with a chitosan coating was evaluated. They determined that drug release decreased when chitosan was used in the formulation, due to the electrostatic interactions between the hormone and chitosan [24]. Another study evaluated the release of nystatin incorporated in alginate nanospheres coated with chitosan for buccal delivery. Drug release studies showed a burst release followed by a slow and sustained release. This effect was also attributed to an electrostatic interaction between the drug and the nanoparticle coating. In addition, the authors suggested

that the release processes involved solvent penetration into the matrix, gelation of the polymer, dissolution of the drug and diffusion of drug through the resultant polymer layer [52].

El-Nahas *et al*., evaluated in a segment of chicken pouch mucosal membrane as model mucosa, the behavior of polymethacrylate-derivative polymeric nanoparticles. In addition, the authors studied silymarin, a complex mixture of four flavonolignan obtained from *Silybum marianum* and a poorly water soluble drug. The oral mucosa presents a rich blood supply and allows direct systemic access through the internal jugular vein; moreover, absorption through this epithelium avoids the hepatic first pass metabolism of silymarin and improves its systemic bioavailability. The authors studied the release through the method of dialysis bags, from polymeric nanoparticle compared to a solution. It was observed that the permeability of silymarin increased 30 times with respect to a drug solution. Moreover, an increase in permeation flux and permeation coefficient was observed only for the nanoparticle formulation. It was also described that the smaller size of nanoparticles favored faster drug release due to their greater surface area and that all the nanoparticle systems had a sustained release in relation to silymarin solution [53]. Among lipid nanoparticles, Hazzah *et al*. studied the targeting of curcumin to oral mucosa from solid lipid nanoparticles. The investigation evaluated how the type of fat and stabilizer in lipid nanoparticles affected curcumin release. It was determined that curcumin dispersion in lipid nanoparticles significantly reduced its release in comparison to the curcumin suspension. This could be attributed to the lipid phase encapsulating the drug that lowered its release; furthermore, the type of stabilizer can generate more rigid nanoparticle structures and limit drug release [54]. This investigation is relevant as curcumin is a highly lipophilic molecule (a BCS class II drug model) and its oral administration has been highly challenging, with no commercial products to date [55]. Thus, curcumin can be representative of such molecules for potential buccal delivery while being a potential product on its own.

In the field of nanoparticle development as buccal drug delivery systems, a thorough characterization of the release mechanisms is required as it can determine the success of the system. There are different types of processes controlling drug release from buccal formulations. These mechanisms include drug diffusion, dissolution, swelling or erosion of the matrix, and osmotic effects [56, 57]. These processes can occur together, but the slower process is what governs release. Consideration should be given to the burst release of the drug from nanocarriers, because if the molecule associates to the instrument membrane to a large extent, it will diffuse and saturate the surface, and then when exposed in the medium, an abrupt release will be observed and a high initial concentration can be found [58]. This ultimately leads to the drug reaching the different areas of GI tract.

Once the release profile of the nanocarriers are known, absorption enhancers can contribute to improving drug permeation [59]. Bile salts, cyclodextrins and chitosan have been used in previous investigations as buccal permeation enhancers [60]. Kontogiannidou *et al*. evaluated different absorption enhancers, N-trimethyl chitosan (positively charged), sulfobutyl ether-b-cyclodextrin (negatively charged) and hydroxypropyl-b-cyclodextrin (neutral). Porcine buccal mucosa was used due to its

non-keratinized and highly irrigated epithelium by a dense network of capillary vessels, and as previously indicated, its morphology and permeability is comparable with that of the human buccal epithelium. The acceptor compartment was filled with phosphate buffered saline pH 7.4 and the donor compartment with 5 mg/mL of ropinirole hydrochloride plain solution in presence of different concentration of absorption enhancers. It was found N-trimethyl chitosan to be the best enhancer in promoting drug absorption. This was attributed to the positive charge of N-trimethyl chitosan, which would interact better with the negative charge of the mucin and thus allowing penetration. N-trimethyl chitosan also allows for interaction with lipids and increases drug uptake through the buccal epithelium [58].

All the studies listed above follow drug absorption by quantifying drug in the epithelium or permeated through in permeation experiments. However, it has been described that nanoparticles can remain intact through the buccal epithelium. For example, studies of inorganic nanoparticles, including silver nanoparticles (19 nm of diameter) [61] and titanium dioxide (30–150 nm) [62] indicate a relationship between permeability of nanoparticles through the buccal mucosa and their physical and chemical properties. In addition, polymeric nanoparticles have been found to show a permeation behavior governed by a combination of particle diameter and their agglomeration properties [63, 64]. It has also been described that anionic and cationic polymeric nanoparticles have different permeation mechanisms. Cationic nanoparticles are more efficient in permeating isolated porcine oral tissue in Franz cell diffusion experiments. Anionic nanoparticles of size 200 nm were found to agglomerate and were not effective in permeating; however, the smallest anionic nanoparticles (20 nm) were able to permeate by the transcellular route. On the other hand, although the 200 nm nanoparticles had a tendency to agglomerate, they had the ability to penetrate by endocytic mechanisms. Robbleg *et al*. describe that the morphology of buccal superficial cells determines the size dependent uptake of nanoparticles in the oral cavity [63]. Fig. 2 shows the furrows between microplicae with diameters in the range of 210–410 nm. Therefore, aggregates of nanoparticles larger than this size make it difficult to permeate the buccal mucosa.

The administration of a nanoparticle suspension is complex due to the clearance function of saliva and the presence of food in the oral cavity. Despite the stabilization provided by nanoparticles, their potential has been investigated in secondary nanocarrier vehicles to enhance their residence time in the buccal epithelium [26, 65, 66].

4 Mucoadhesion

Since the oral cavity is protected by the buccal mucosa, it is possible to design strategies for the interaction between the pharmaceutical dosage form and the mucus. The mucoadhesive principle is based on the composition of mucus, which is viscous in nature [67]. It is composed mainly of water with high concentrations of mucins, inorganic salts, proteins and lipids [68]. For the purpose of mucoadhesion, mucins

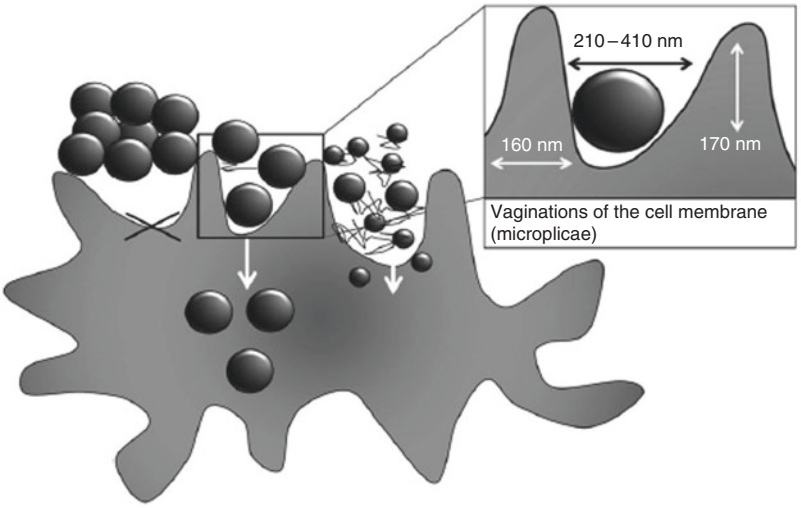

Fig. 2 The morphology of buccal superficial cells determines the size-dependent uptake of nanoparticles in the oral cavity. Reprinted from Roblegg *et al*. [63]

are responsible for the interpenetration with the other molecules. Mucins are glycoproteins with various structures that are specific to each region of the body [69]. These have a protein core and carbohydrate side chains, which are responsible for the specific non–covalent bond that can be produced when contacted with the mucoadhesive vehicle [69]. The role of mucins in the mucus is constituting a highly entangled system exhibiting physical junctions, disulphide bonding, and stabilized by inter hydrogen bonding or other non-covalent bond [68]. Weak interactions such as hydrogen bonds and Van der Waals forces are believed to play a key role in the formation of the mucoadhesive bond as well. As for covalent bonds, these can occur between the functional groups of mucin and mucoadhesive material. Polymers that form weak interactions are described as first generation mucoadhesives and second generation are those that rely on covalent bonds to establish a strong mucoadhesive bond [70]. The main effect of mucoadhesion is to increase the retention time of the dosage form, in order to favor the contact and subsequent permeability of the drug towards the mucus membranes. In addition, an intimate contact occurs, by which the active molecule is protected from the oral environment. Mucoadhesive polymers represent an important example of molecules that can significantly improve characteristics as buccal delivery systems. Some examples of first generation mucoadhesives are cellulose derivates (hydroxypropylmethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, among others), sodium alginate, polyvinyl alcohol, xanthan gum, chitosan, polycaprolactone and acrylic acid copolymers. Chitosan, and other polycations, due to its positive surface charge has been described as an excellent carrier for drug delivery. This is because it acts as a vehicle, permeation enhancer and mucoadhesive (Fig. 3) [71, 72].

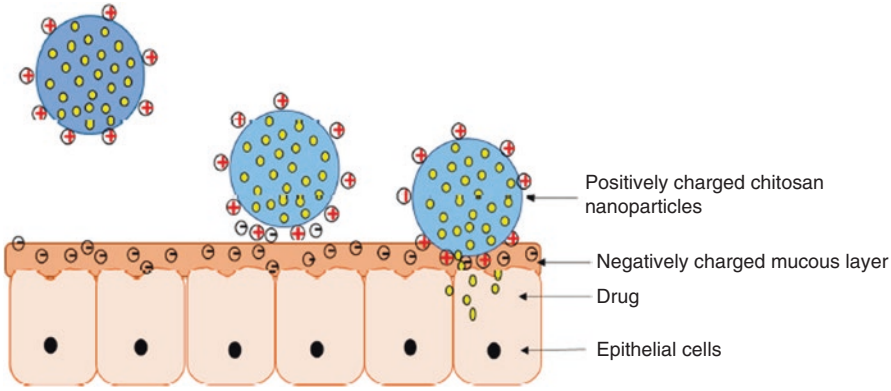

Fig. 3 Schematic representation of chitosan loaded nanoparticles structure and interaction with the mucus layer. Reprinted of Mohammed *et al*. [56]

Recently, mucoadhesives of second generation have been described in literature due to their mechanism of action [73, 74]. The mucoadhesive polymers in this category correspond to thiolated polymers, lectins and by harnessing the phenomenon of bacterial adhesion (the capacity of bacteria to adhere is due to the presence of fimbriae) adapted to buccal delivery systems.

The use of nanoparticles or secondary vehicles that are composed of mucoadhesives will then favor drug delivery. As mentioned as will be indicated later, using nanoparticles composed of chitosan can increase retention in the mucosa. A study carried out by Tejada *et al*., showed that the presence of functional groups loaded in the polymer chains increases the interaction with mucin. Therefore, the formation of strong hydrogen bonds and ionic interactions between the functional groups of the polymer and the mucosa has a clear effect on the strength of the mucoadhesive layer compared to the polymer with the lowest charge [75]. Reda *et al*. evaluated polymeric nanofibers loaded with ketoprofen and attributed that the mucoadhesive property of the nanofibers tested could be due to the polyanionic nature of both polymers used. Although there are anionic groups in the structure which could be repelled by the negative charges of mucin, these can form hydrogen bonds and increase residence time. In addition, it was argued that the large surface area of nanofibers can interact with the biosurface, resulting in interaction with the mucosa after the absorption of fluids due to the presence of numerous nano–size interfibrillary pores that cause mucoadhesion [76].

In the case of lipid nanoparticles, a study showed that Gelucire® 50/13 nanoparticles have a lower mucoadhesion compared to the same formulation with poloxamer 407 (synthetic block copolymer of ethylene oxide and propylene oxide) in their composition. This result is attributed to the fact that poloxamer is more hydrophilic and, therefore, has greater capacity to generate hydrogen bonds with mucosal components [54].

These results are consistent with the mucoadhesive theory where the material is key in increasing the interaction of the delivery system with the mucosal components, and thus the retention time [77].

5 Secondary Vehicles for Nanocarrier Buccal Administration

The secondary vehicle is defined by the nature of the nanoparticle and by the effect that it is desired to obtain. Thus, various types of secondary vehicles can be found in the literature, including buccal films [9], gels [78], and buccal tablets [79]. For example, buccal films are specifically preferred in the case of local delivery for oral diseases as they offer the advantage of formation of a thin layer on the mouth lesion, thus protecting the wound surface and reducing the pain [80, 81]. In 2017, a review was conducted on different methods for film manufacture for buccal administration. This work emphasized the need for biologicals to be delivered by the buccal route, which is an approach to the requirements of the area [10]. The methodology of ink injection loaded with drug has been described to print nanocarriers, to be carried on films. This research studied the stability of the protein used after the injection process. The authors conclude that it is possible to achieve high printing efficiencies and manage the structural viability of the printed protein [82]. Moreover, recent work has highlighted the potential of inkjet printing of nanocarriers as a means of incorporating nanocarriers on films for their buccal administration [83].

Recently, Kraisit *et al*. evaluated the swelling and mucoadhesion of a polymeric film with chitosan nanoparticles loaded with propranolol hydrochloride [84]. Firstly, the swelling property were relevant because it determines polymer chain relaxation and promotes penetration of the nanoparticles into the buccal mucosa. The swelling index increased in the presence of nanoparticles in the film at early time, but then reached an equilibrium at later times. The authors attributed this effect to different types of interactions that occur with the materials used [85]. Porcine mucosa was used for mucoadhesion studies and it was found that films containing nanoparticles presented greater mucoadhesive properties in terms of work of adhesion and maximum adhesive force [84]. Moreover, Nair *et al*. evaluated the permeability of acyclovir-loaded nanoparticles in a polymeric film for buccal delivery in a rabbit buccal mucosa *ex vivo* model. The permeation studies were conducted in Franz diffusion cells and it was observed that drug permeation was sustained in films with respect to drug alone. Furthermore, it was observed that the greater amount of drug in the nanoparticles, the permeated amount also increased. Additionally, it was also suggested that the incorporation of either free drug or nanoparticles had little or no effect in the mucoadhesive strength of the film. The release of nanoparticles from formulations is governed by the separation of nanoparticles from the polymer matrix and their diffusion from the matrix. Furthermore, kinetic studies suggested that the acyclovir release from nanoparticles followed first order kinetics in low concentration (0.5 mg/cm^2) and a Higuchi model at high drug concentration (1 mg/cm^2) [86]. In another investigation, Barzoki *et al*. studied the pattern of insulin release from

nanoparticles after incorporating them into a film. They made chitosan/gelatin films and concluded that the physical and chemical properties of insulin are influenced by the film polymer, the nanoparticle and the drug [87]. In another study, Morales *et al*. evaluated the incorporation of insulin-coated nanoparticles in polymeric films and studied insulin permeation in a human buccal epithelium model. Additionally, similar to other studies, the kinetics of insulin permeation from films was determined by the film (secondary vehicle) composition and the enhanced permeation was attributed to a great concentration gradient built by the delivery system [9].

Another type of secondary vehicle for nanoparticles are gels, which are believed to function by polymer interaction with mucin, resulting in the adhesion of the formulation. However, this pharmaceutical form has the disadvantage that it can be affected by the movement in the oral cavity and saliva, which could risk a more unspecific contact with the appropriate area in the buccal region and a more heterogeneous dose release control [68, 88]. Marques *et al*. developed and characterized lipid nanoparticles in a carbopol hydrogel and determined that the incorporation of nanoparticles to the mucoadhesive hydrogels has desirable rheological properties (texture and mucoadhesion) that can benefit therapeutic efficacy, since it increases the retention time and ease of application. Carbopol can promote longer residence times in the mucosa, favoring sustained release from the nanoparticles [17]. Raafat et al. evaluated the mucoadhesive properties considering the effect of saliva on nanocomposites and observed that the presence of Ag nanoparticles slows the release of propranolol from the nanocomposite formulation. Therefore, the nanoparticles in the secondary vehicle can act as a physical barrier for drug diffusion and, consequently, can regulate its release profile [25]. Elkomy *et al*. incorporated lipid nanoparticles to a carbopol/poloxamer gel and evaluated their pharmacokinetics in human volunteers, in relation to the administration of a tablet. They observed that the bioavailability of the oral tablet was similar to that of the gel [78]. Giovino *et al*. described nanoparticles loaded with insulin and included them in a chitosan film for buccal drug delivery. From the results, the authors concluded that the system demonstrated excellent swelling and mucoadhesive properties due to the presence of hydrophilic compounds in the formulation, which allowed the interaction with the mucosa. In addition, the release of insulin was controlled by chitosan erosion. Finally, the permeation was studied, and it was shown that there is a potential use for buccal administration based on the extent of permeation describing a flux of 0.1 μg/cm^2/h and apparent permeability of 4×10^{-2} cm^2/h [89].

El-Nahas *et al*. proposed the use of chitosan/gelatin microparticles with sylimarin Eudragit – loaded nanoparticles and used this material as substrate for compacting into tablets. Pharmacopeial specifications were evaluated (weight variation, content uniformity, friability, hardness test) and a functional tablet formulation was obtained. Finally, the tablet with nanoparticles showed interesting mucoadhesive properties and allowed a greater drug penetration than sylimarin-loaded nanoparticle and sylimarin suspension when studied on chicken pouch membrane using Franz diffusion cells [53].

Another advanced buccal drug delivery system is the IntelliDrug Device (IDD), an innovation developed to treat chronic diseases and addictions [90]. The device

consists of a microprocessor, a drug reservoir and a valve for drug release. The drug reservoir can be a drug solution or nanoparticles loaded with drug. IDD is a device inserted in the dental arch, much like a tooth, and does not interfere with the patient's comfort. The mechanism by which this device functions allows to manage wirelessly the speed and amount of drug released. This also allows knowing the need for drug refill, which is done remotely and is also capable of informing the patient that a refill is required [91].

Although the buccal route presents advantages including avoidance of the hepatic first-pass effect, enzymatic degradation and chemical instability, comfortable, easily self-administrable and accepted by patients. The administration of nanoparticles and complementary systems can be enhanced by taking advantage of the characteristics of the oral cavity. As discussed before, characteristics such as the low presence of enzymes, structure of the epithelium and components of the mucosa can be used to increase drug residence time and drug bioavailability. Therefore, granting these characteristics can lead to advanced buccal drug delivery systems.

6 Toxicity and Safety Aspects

Considering that buccal delivery systems are oral administration systems, it is very important to conduct toxicity studies *in vitro* and *in vivo*. The *in vitro* assays provide a first approximation to the possible behavior of these systems in the buccal mucosa. However, *in vivo* studies must be conducted to have a better understanding of the materials used in complex organisms. For this, *ex vivo* assays have been used in porcine buccal epithelium, as it is structurally and enzymatically similar to that of the human oral cavity.

Roblegg *et al*., evaluated the effect of saliva on the availability of nanoparticles to the buccal mucosa. They determined that the effect of saliva is varied and depends on the composition of the nanoparticle, but its main toxic effect was related to agglomeration [64]. As mentioned in the previous section, nanoparticles can enter the epithelium and the size of the nanoparticles should be considered because it can affect permeability. Given the size and agglomeration, nanoparticles could permeate or be included in the invaginations of the cell membrane and be retained from deeper penetration (Fig. 2) [62]. Teubl *et al*., in a study in 2015 demonstrated that particles of TiO_2 showed a minor impact on the viability and the membrane integrity of human buccal epithelial cells. However, it was observed that there was a significant increase in the metabolic activity of mitochondria (by MTS assay) after an incubation time of 4 hours. This phenomenon can be explained by the first reaction of the cell against xenobiotics. It was proposed that nanoparticles cause ROS generation and cellular defense mechanisms were activated. However, the metabolic activity decreased to normal levels after 24 hours, showing that the cells remain viable after exposure [62].

Andreani *et al*., studied silica nanoparticle for oral insulin delivery and evaluated their toxicity *in vitro* in Caco-2 and HepG2 cell lines. In comparison with the con-

trol group no significant differences were observed, which was attributed to the surface charge of nanoparticles. In this investigation, silica nanoparticles exhibit a negative zeta potential at pH 7. Several studies have reported that negatively charged nanoparticles exert very little or no toxicity on biological membranes, in comparison to positively charged particles [92]. In 2017, Iglesias *et al*. evaluated cytotoxicity and genotoxicity of nanoparticles for oral drug delivery. They studied the effect of NPs (negative zeta potential) on cell viability using the proliferation assay in Caco-2 cells over 3 h, without any significant evidence of hindering proliferation of cells in any of the conditions tested. Additionally, it was shown that the nanoparticles did not induce relevant genotoxic lesions during the time of the study. The authors reported that the oxidized DNA bases induced by NP after 24 h of treatment may not have any biological relevance due to the low level of damage and the high concentration required [93]. Klemetsrud *et al*. studied the behavior of different types of polymer coated liposomes for use in the oral cavity and they evaluated cell viability in TR146 cells. Interestingly, chitosan was the only polymer exhibiting a marked negative effect on the cell viability. In this research, chitosan was the only positively charged polymer studied, and as presented above, positively charged nanocarriers can cause cell necrosis and this could lead to the observed reduced cell viability [94, 95]. Chitosan is generally classified as a nontoxic, biocompatible and biodegradable polymer; nevertheless, it has been described that its modifications can ultimately determine its toxicity. Chitosan malate for example, has shown toxicity in cells at longer contact times in slightly higher concentrations than the hydrochloric salt utilized in a study [96]. In other research, a high number of ionized groups, *i.e.* deacetylation degree, has been found to decrease the cell viability [97, 98].

To date, many mucoadhesive and permeability studies have been carried out, which have delivered encouraging results to continue investigating buccal delivery system. In relation to the above, mucus is relevant in the delivery of nanosystems, as mucus has glycoproteins with which nanoparticles can interact. Therefore, it seems that a negative zeta potential can be less toxic. However, oral mucosal toxicity studies are few and such studies are necessary to expand the knowledge and translational use of this drug delivery system.

7 Conclusion

The oral cavity can be used as an effective route of administration due to its ease of access and physiological features that can be harnessed as tools for drug delivery. Mucoadhesion is one of the main strategies to enable buccal drug delivery, where polymers can be selected to increase the delivery system retention time in the buccal epithelium. In addition, this route may allow the delivery of biologics, as described by authors with recent examples of high buccal bioavailability. This brings advantages over the conventional oral route, since the administration of biologics is complex.

While promising, the buccal route has limitations that have been addressed with the use of nanoparticles, films, gels or other secondary vehicles enriched with nanoparticles that favor buccal drug delivery. Although buccal toxicity studies have been conducted on nanoparticles, the information still is early and specific to certain types of nanoparticles, and as such, future studies should be envisioned to tackle other types of nanoparticles and toxicity aspects not yet fully elucidated.

Finally, future investigations are expected to explore buccal absorption enhancement through nanoparticle and secondary vehicle design in order to fully harness the potential of this route, particularly for the recent successes observed in biologics buccal drug delivery.

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Respiratory Drug/Vaccine Delivery Using Nanoparticles

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Abstract Respiratory diseases account for a very significant portion of worldwide morbidity and mortality but to-date only a limited number of therapeutics are available for direct delivery via inhalation. Nanotechnology offers a range of potential

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benefits to facilitate the targeted delivery/co-delivery of existing therapeutic agents and to support the delivery of more advanced biotherapeutics e.g. proteins, gene medicines. The clinical and commercial translation of inhalable nanomedicines is not trivial and presents significant formulation, manufacturing, assessment and regulatory challenges. Herein, we explore the range of respiratory diseases being targeted using nanoparticle-based delivery systems for therapeutics and vaccines, the composition and manufacture of these nanoparticles, their integration into relevant inhaler devices, the methods being used to characterize these nanoparticles *in vitro* and *in vivo* and the regulatory requirements governing inhaled nanomedicines.

Keywords Respiratory drug delivery · Nanoparticles · Nanomedicine Therapeutics · Vaccines · Inhalation · Aerosol

1 Clinical Applications of Nanomedicines for Respiratory Disease

Respiratory diseases account for a very significant portion of worldwide morbidity and mortality but to-date only a limited number of therapeutics are available for direct delivery via inhalation. There are multiple factors potentially underpinning an unhealthy human lung. Lung cancers and single gene disorders (e.g. cystic fibrosis (CF), alpha-1 antitrypsin deficiency (AATD), primary ciliary dyskinesia (PCD/Kartagener's disease) and pulmonary alveolar proteinosis/surfactant deficiency are non-infectious pulmonary diseases that could each benefit from inhaled therapies. These diseases all have disparate aetiologies and thus require bespoke nanomedicines. Similarly for allergic and inflammatory diseases of the lungs such as asthma and chronic obstructive pulmonary disease (COPD), both of which occur due to environmental exposure of susceptible individuals to allergenic or noxious stimuli. Infective exacerbations often complicate these pre-existing lung pathologies and thus anti-infective measures are often required in addition to disease-specific treatments.

A wide range of therapeutic modalities have been explored to-date as inhaled nanomedicines including small molecule drug actives, peptides, proteins, antibodies and nucleic acid-based therapeutics. Effective inhalation of particles is generally seen for particles with aerodynamic particle sizes between 1–5 μm. Particles in the nanoparticle size range (<1 μm) generally need to be delivered either within nebulized droplets of inhalable size or incorporated into microparticle formats for delivery via dry powder inhaler (DPI) [1–5]. Inhalable microparticulate/nanoparticulate dry powders are often prepared using spray-drying. Examples of therapeutics that have been formulated into inhalable formats for targeted lung delivery include various antibiotics (e.g. vancomycin, clarithromycin, tobramycin, azithromycin, rifam-

picin [6–8]), antimicrobials such as ofloxacin and moxifloxacin [9], as well as the anti-inflammatory fluticasone propionate, the steroid budesonide and β2-agonists for the treatment of COPD and asthma [10–13]. Various lung cancer and anti-metastatic therapeutics, including doxorubicin, paclitaxel, cisplatin, gemcitabine, camptothecin, fluorouracil and azacytidine have been similarly formulated (reviewed in [14]). Notwithstanding the clinical benefits of these therapeutics, the development of newer and more effective medicines for various lung diseases is desirable, and nanoparticle technologies could enable their development. A selection of examples is presented below.

Each nanomedicine needs to be tested empirically for its physicochemical and morphological characteristics as the behaviour of seemingly quite similar cargoes e.g. double-stranded DNA oligos and miRNA mimics, can vary greatly even when using the same nanoparticle formulation. Although these various cargoes are vastly different in their modes of action, the goal of good formulations is to ensure that all of them are soluble, stable (i.e. nuclease- or protease-resistant), non-toxic and maintain their activity when they are delivered to their site of action. Whether the therapeutic cargo of a nanomedicine is designed to function extracellularly, such as for a nanomedicine carrying an antiprotease to be delivered to the lumen of the lungs, or intracellularly within specialised lung cells in the bronchial epithelium or alveolar compartment, will also affect decisions regarding the most appropriate size, charge and method of delivery for a specific nanomedicine.

Age and gender can occasionally be important factors associated with the development of certain respiratory diseases. In aged adults of either sex, idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible chronic lung disease for which there is no cure, whereas lymphangioleiomyomatosis (LAM), for example, occurs almost exclusively in middle-age women. Regardless of the different causes and manifestations of these various lung disorders each could have the potential to benefit from custom-designed nanomedicines to reverse, inhibit or repair aberrant processes. Novel nanomedicine therapies for IPF are in development. Using a murine bleomycin-induced model of IPF, Garbuzenko *et al.* [15] have demonstrated that nanostructured lipid carriers loaded with prostaglandin E and siRNAs targeting matrix metalloproteinase 3 (MMP3), chemokine (C-C motif)ligand 12 (CCL12) and hypoxia-inducible factor 1 (HIF1)alpha, delivered locally to the lungs by inhalation reduced mouse body mass, limited hydroxyproline content in the lungs, restricted lung tissue damage and prevented animal mortality. Sirolimus (also called rapamycin), is a macrolide compound that acts as an inhibitor of the mTOR pathway and is the FDA-approved drug treatment for LAM. mTOR inhibitors also have therapeutic application for bronchiolitis obliterans syndrome, a chronic form of lung allograft rejection that occurs due to mesenchymal cell-mediated airway fibro-obliteration. Gold nanoparticles loaded with everolimus (a mTOR inhibitor) were shown to inhibit mesenchymal cell proliferation *in vitro* [16] and were non-toxic when administered by inhalation to mice [17].

Other pulmonary diseases suitable for treatment via inhaled nanomedicines, could include acute or traumatic lung diseases such as pneumonia or acute respiratory distress syndrome (ARDS), respectively wherein the therapy must be immedi-

ately applied and be fast-acting. Like pneumonia, tuberculosis (TB) is an infectious lung disease; however, TB is more commonly described as a chronic rather than an acute lung disease caused by bacterial infection. Cross-linked poly-β-cyclodextrin (pβCD) nanoparticles, as one example, have been explored for pulmonary delivery of anti-TB drugs. Interestingly the pβCD nanoparticles themselves have intrinsic antibacterial properties against *Mycobacterium tuberculosis* (Mtb); they can interfere with Mtb infection of macrophages by provoking macrophage apoptosis [18].

1.1 Aerosol Vaccination Using Nanoparticles

Aerosol-mediated delivery of vaccines to the mucosal surface of the airways has already demonstrated significant potential as a successful vaccination strategy [19–22]. The potential for nanoparticle-based vaccines themselves have broadly demonstrated efficacy [23], however their specific potential for prophylactic or therapeutic application in vaccination via the respiratory mucosa is yet to be fully realized. That notwithstanding, it is reported that there is a significant amount of nanoparticle focused research underway in the development of new and revisited vaccine strategies targeting the respiratory mucosa [24–26]. That research builds on the learnings from the development of the many existing worldwide regulatory-approved nanomedicines [27, 28] and acts to fill the pipeline of potential new nanovaccines.

In vaccinations, nanoparticles have the potential to perform several functions, ranging from being simple vectors for controlled release of encapsulated antigenic material, to presentation of antigens or cell targeting moieties on their surfaces, to imaging and additionally, the provision of adjuvant effects. Depending on the therapeutic needs, nanoparticle design and material choice, nanoparticles can serve to prolong antigen release or promote lung retention over time [29, 30]. Presentation of antigens or cell targeting moieties on the surfaces of these nanoparticles can enhance effectiveness through means of cell receptor targeting. This surface modification of nanoparticles may serve multiple purposes such as increased compatibility or interaction with the target cell type [31] or alternatively, as a means of presenting antigens intended to illicit an immune response [32].

As discussed above (see Clinical applications of nanomedicines for respiratory disease), targeted deposition within the airways is achievable through generation of appropriately sized aerosols/particles with which to deliver the nanoparticles. Identifying the target cell and their location within the airways may aid in further optimising the immune response by delivering the nanovaccine to that location. For example, the distribution of sialic acid influenza receptors varies greatly with location within the airways, thus delivery of an otherwise promising influenza nanovaccine to the wrong location may result in attenuated or nil effect [33]. Other means of preferential targeting of aerosols within the airways have been described in the literature [34, 35]. It has been shown that targeting of aerosolised super paramagnetic iron oxide nanoparticles (SPIONs) within the airways is feasible, albeit using

complex and potentially expensive procedures [36, 37]. In practice, this targeting ability is likely to facilitate crude targeting of conducting versus peripheral airways and not cell-specific targeting. Nevertheless, improvements in the state of the art would represent an important advance in the success of aerosol-mediated vaccines in that targeting specific cell types may have a significant bearing on the ultimate immune response, and conferred level of protection.

2 Types of Nanoparticles Being Used for Pulmonary Drug Delivery

The challenges faced in pulmonary drug delivery of novel therapies might be overcome using advanced formulations including encapsulation of drug actives within biodegradable polymeric nanoparticles [38] as well as/or "pure" (non-polymeric) drug nanoparticles [39]. As noted above these nanotechnologies must be delivered in either a droplet or powder with appropriate aerodynamic properties i.e. aerodynamic diameter 1-5 μm.

It is worth noting that very few materials and excipients are approved for inhaled drug delivery [40]. However, a wide range of materials/carriers are being explored for drug delivery via the inhaled route. These carriers include nanoparticles – polymeric and lipidic, liposomes, dendrimers, micelles, nanoemulsions and nanosuspensions. In particular, materials already approved for other routes of administration e.g. biodegradable and biocompatible polymers such as poly(lactic-*co*-glycolic acid) (PLGA) and liposomes, have been extensively studied for the delivery of a range of cargoes to the lungs. These materials offer a number of advantages over other materials, such as metallic or carbon nanomaterials, due to their biodegradation and in some instances thier established biocompatibility profile. Advantages associated with these different formulation technologies are outlined in Table 1, with exemplar references to their application for inhaled nanoparticles.

2.1 Liposomes and Lipid Based Formulations

Inhalable solid lipid nanoparticles (SLNs) and liposomes have been extensively explored as nanomedicines for lung delivery and some have reached clinical trial stages. The first liposomal product to be marketed was Alveofact® used to treat acute respiratory distress syndrome (ARDS) in infants by pulmonary instillation [60]. Typically, they are considered less toxic than their polymeric counterparts as physiological lipids are often used. Examples of studies targeting the respiratory system include that of Wang and colleagues [41] who used curcumin-loaded SLNs and observed reduced inflammation and cytokine expression in murine asthma models thus highlighting the favorable therapeutic and toxicity profiles of SLNs.

Table 1 Nanotechnology delivery systems explored for inhalation and their associated advantages

Excipient type	Advantageous characteristics	Ref.
Liposomes/solid lipid nanoparticles (SLN)	Can be designed for controlled release of drug cargo and are of particular interest due to their biocompatibility and non-irritant degradation products. Consideration has to be given, however, to the lipid miscibility of drugs when formulating SLN.	[41–44]
Polymeric materials	Currently being investigated for pulmonary drug delivery due to their versatility such as modified surface properties, high encapsulation of the drug, protection of the cargo from degradation, prolonged drug delivery, an expanded shelf life and functional groups available for the attachment of cargo, targeting and imaging agents. The most common examples are poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), alginate, polyethylenimine (PEI), dendrimers, chitosan and gelatin. A number of polymeric materials such as PEI are employed principally as gene delivery vectors and are less suitable for small molecule delivery	[34, 35, 45–50]
Micelles	Nanostructures made of amphiphilic molecules, like polymers or lipids. Polymeric micelles are emerging as a promising platform for drug delivery.	[51–53]
Nanoemulsions	High solubilizing and drug protection features. Have the potential to deliver proteins as well as other new or classic active drug compounds to the lungs.	[54, 55]
Nanosuspension	Can be prepared without using large volumes of organic co-solvents, and concentration of nanosuspension is not limited by solubility in the carrier, thus, a wider dose range can be achieved. Good content uniformity and can facilitate penetration of deep lung and smaller airways, leading to a more even drug distribution and resulting in a more accurate modeling of the drug distribution and efficacy. Several excipients such as sugars, lipids, amino acids, surfactants, polymers and absorption enhancers have been tested for their efficacy in improving drug pulmonary administration.	[56, 57]
Magnetic/Metallic	Metal based nanoparticles are used in theranostics in lung cancer. Use for local delivery to the lung is somewhat limited by potential immune and cytotoxic effects.	[58, 59]

Liu and colleagues [42] also assessed the *in vivo* potential of ciprofloxacin containing liposomes in rat models where reduced pulmonary irritation was observed with drug encapsulated into liposomes compared to drug alone controls. A liposomal ciprofloxacin formulation advanced to phase three clinical trials for non-cystic fibrosis bronchiectasis where it reduced pulmonary exacerbations and had a similar adverse effect profile to placebo [61]. In our own work we have demonstrated that liposomal encapsulation of recombinant secretory leukocyte protease inhibitor (SLPI) offered improved stability, reduced clearance and increased residency time in the lungs after local delivery [62, 63].

2.2 Polymeric Based Formulations

Properties of polymeric nanoparticles appear to have differing levels of influence on toxicity and therapeutic outcomes when investigated *in vitro* and *in vivo*. Grabowski and colleagues [45] for example showed that surface charge influences *in vitro* cellular uptake and transport across mucus layers. This property however was not as significant a factor when the same group [64] compared these findings to an *in vivo* accumulation and elimination study, thus highlighting the necessity for both thorough *in vitro* and *in vivo* investigations. Toxicity of a range of biodegradable nanoparticles (PLGA coated with; chitosan, poloxamer 188 or polyvinyl alcohol (PVA)) to that of non-biodegradable inorganic (TiO_2) and polymeric nanoparticles (polystyrene) was determined in Balb/cJ mice (4–6 weeks old) and biodistribution in NMRI nude mice (4 weeks old). Nebulization of non-degradable nanoparticles resulted in increased numbers of macrophages, protein quantification and levels of inflammatory markers such as IL-6, MCP-1 and TNFα. Histological examination showed signs of tissue damage in the non-biodegradable group with thickening of interstitial walls and erythrocyte accumulation in comparison to minimal changes in the group treated with biodegradable nanoparticles.

Dendrimers are highly branched macromolecules with varying architectures to suit their cargo needs. Their absorption, distribution, metabolism and elimination (ADME) profile is dependent upon these various structural features. Kaminskas *et al* demonstrated controlled release and longer lung residence time by conjugating PEGylated Poly-L-Lysine (PLL) dendrimer to the chemotherapeutic agent doxorubicin. In addition, they found local lung delivery led to a greater reduction in tumour burden of >95% compared to only 30–50% reduction by intravenous delivery [48].

Chitosan and its derivatives demonstrate potential for chemical modification and functionalization [46]. Its mucoadhesive and membrane permeability properties, in addition to its antimicrobial nature, mean that chitosan has been extensively explored for respiratory diseases and associated infections. Work by Garg and colleagues [65] in murine TB models shows how chitosan nanoparticles loaded with the antitubercular agents isoniazid and rifampicin are both therapeutically effective and minimally toxic *in vivo* in comparison to free drug. Nebulized nanoparticles were selectively phagocytosed thus preferentially targeting macrophage rich organs such as the lungs. This highlights also the close links between clearance and therapeutic pathways for nanomedicines.

3 Nanoparticle Targeting

Functionalization of nanomedicines can be used to confer targeting specificity into the nanomedicine and/or enable particles to overcome biological barriers to achieve efficient delivery. Non-specific distribution and unwanted elimination of nanoparticles pose a significant challenge for those developing nanomedicines, including

those being developed for respiratory delivery. When designing a functionalized nanoparticle for respiratory delivery it is critical to understand the nature of the target site/cell and the anatomical/physiological environment presented by the lungs, including the pathological presentation.

One of the key biological barriers in the respiratory tract is the presence of mucus or surfactant on the surface of the airway cells. The upper airway epithelial cells are covered with a viscous layer of mucus containing glycoproteins and lipids, which trap unwanted particles (dust, pathogens etc.) and is then cleared via mucociliary clearance. In the alveolar region of the respiratory tract the alveolar cells are covered with a layer of surfactant composed of lipids and proteins which serves to prevent alveolar collapse [66]. Crossing of these barriers is crucial for many nanomedicines in reaching their target site either at the cell surface or intracellularly in order to exert their effect. Mucoadhesive surface modifications to nanomedicines increases retention time in the lungs and allows a localized, controlled release of the nanomedicine by circumventing clearance mechanisms. Mucus-penetrating modifications function by improving the ability of the nanomedicine to permeate through the mucus and surfactant layers which line the upper and lower lung environment.

Chitosan, which as noted above has known mucoadhesive properties, has been extensively explored for respiratory and nasal delivery. While it has been used alone, particularly as a gene delivery vector, it is becoming increasingly popular to functionalize nanoparticles with chitosan in order to improve their bioavailability [67]. Chitosan modification of PLGA is a common approach which successfully combines the key features of both delivery vectors. Chitosan functionalized-PLGA particles are cationic in nature and confers PLGA particles with greater mucoadhesive properties, thereby increasing site retention and drug delivery efficiency [67–69].

Polyethylene glycol (PEG) modifications have been shown to reduce nanoparticle associations with mucin glycoproteins by shifting the particle to a more neutral charge, thereby enhancing their movement through the negatively charged mucus layer and can alter their cellular uptake by decreasing their interaction with cytoskeletal actin filaments. By minimizing adhesion to the viscoelastic mucus, fluorescent polystyrene sphere PEGylated particles could rapidly penetrate up to 35-fold faster than uncoated particles through to the airway cell epithelium. However, this is particle size dependent with particles >500 nm becoming immobilized in the mucus [70–72]. Suk *et al* showed PEGylation of PLL and PEI particles improved their ability to traverse mucus from cystic fibrosis (CF) patients, led to enhanced gene transfer *in vivo* and could express functional CFTR expression as a potential CF therapy [71]. Due to the potential for cytotoxic responses to arise relating to the molecular weight and density of particle PEGylation, alternative functionalization methods using PEG are being developed [72].

Functionalizing nanoparticles with ligands for specific receptors such as transferrin, folic acid or intercellular adhesion molecule-1 can potentially confer a high level of cell-specific targeting [73–75]. For example, the mannose receptor (MR) is highly expressed on alveolar macrophages which could be used to target nanomedicines to these cells. Targeting the phagocytic system of the alveolar macrophages

(AM) can prove therapeutically advantageous as this is the driving cell population for inflammation related diseases including respiratory infections. Liposomes encapsulating ciprofloxacin, coated with mannose showed a significantly greater level of alveolar cell uptake compared to uncoated liposomal particles and efficient antibacterial activity at doses lower than those used clinically [76]. Costa *et al* showed attachment of the mannose to the surface of SLNs resulted in increased macrophage uptake compared to unmodified lipid nanoparticles as a potential therapeutic for TB [77]. We have studied the effect of coating liposomes with mannose on cell association with differentiated THP-1 macrophages using a high content analysis (HCA) approach (Fig. 1) [43]. This work demonstrated that mannose-coating of liposome carrier particles could enhance cell association with the THP-1 cells in a time-, concentration and linker-length dependent manner. In addition, the mannosylated liposomes showed immunosuppressive characteristics including hampering of NFκB activation compared to non-mannosylated liposomes [43].

4 Nanoparticle Characterisation

Drug/drug-loaded nanoparticles are produced using a variety of methods. These can involve the generation of small particles from a bulk material (top down) or the production of individual nanoscale particles (bottom up). The bottom-up approaches generally produce nanoparticles by crystallization and solvent removal, while the top-down approaches include milling and homogenization [78–80]. Ultimately the method chosen depends on whether a pure active nanoparticle or a drug-carrier nanoparticle is being formulated and what the nature of the actives and carriers are along with accessibility of equipment and instrumentation. Once manufactured a key requirement is the effective characterization of the nanoparticles.

A comprehensive list of characterization approaches for nanomedicines is now emerging and includes characterisation of the shape, size of primary particles, aspect ratio, degree of aggregation and agglomeration, size distribution, specific surface properties, surface chemistry (e.g. surface charge, functional groups, catalytic activity), crystal structure, and surface modification (chemical composition, type of modification). Key to robust nanoparticle characterization is selection of the appropriate analytical technique. Some methods are of course common to all nanomedicines and others are more specific for nanoparticles being developed for inhalation. More general methods for sizing of nanoparticles include dynamic light scattering (DLS) [81], Nanoparticle Tracking Analysis (NTA), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) as well as Atomic Force Microscopy (AFM). Crystal structure of drug nanoparticles and encapsulation efficiency of drug-loaded nanoparticle formulations must also be determined. Encapsulation efficiency can be determined by quantifying the amount of drug present, which is expressed as a fraction of the initial drug loading. Of course, all nanoparticle formulations for clinical use will have a requirement for appropriate *in vitro* and *in vivo* testing to determine efficacy and safety. More specifically for nanoparticles designed for respiratory delivery there is their inte-

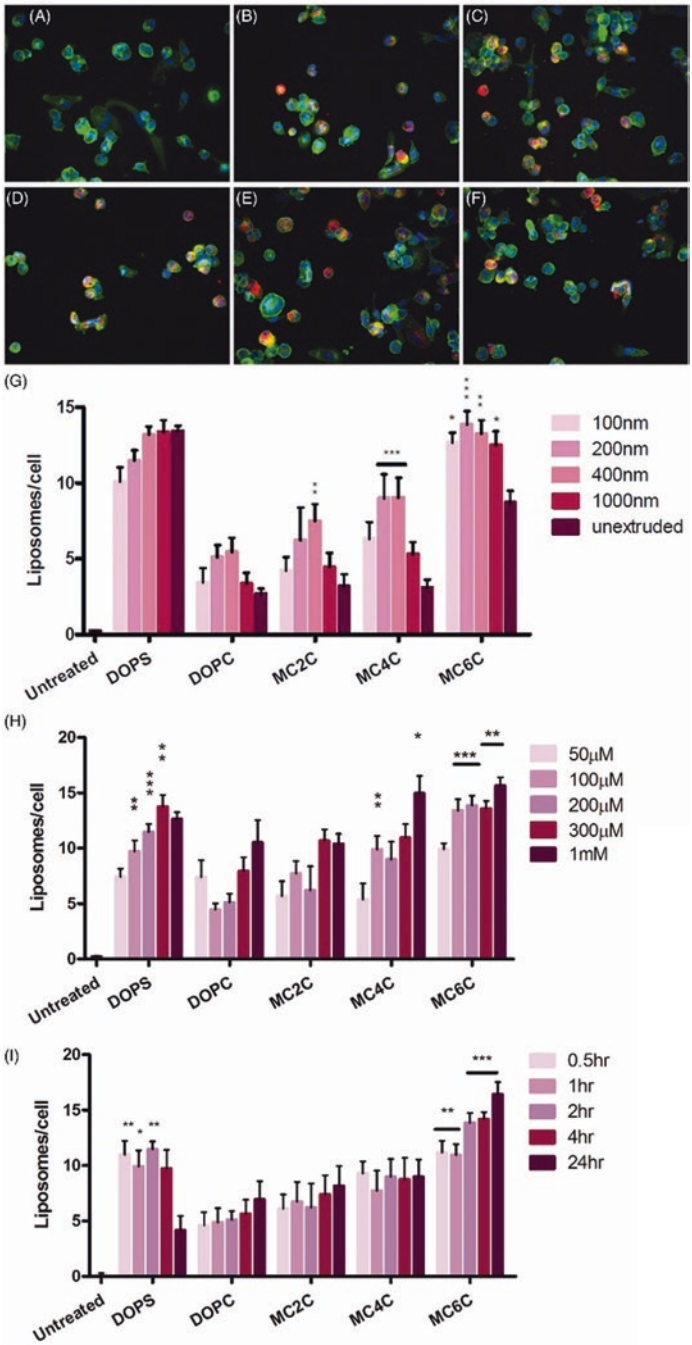

Fig. 1 Cell association analysis by INCELL HCA of rhodamine-labeled liposomes with differentiated THP-1 cells. Cells were incubated without liposomes (untreated) or with fluorescently tagged (rhodamine; red) anionic (1,2 -dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)),

gration with appropriate inhaler devices and the subsequent aerosol testing as outlined below. A number of organizations are now focusing on the specific requirements around nanomedicine characterisation and development including The National Cancer Institute (NCI) established the Nanotechnology Characterization Laboratory (https://ncl.cancer.gov/) in conjunction with the Food and Drug Administration (FDA) and the National Institute of Standards and Technology (NIST). In the European Union the European Nanomedicine Characterisation Laboratory (EUNCL) (http://www.euncl.eu/) was established. These organizations provide extensive guidelines to academic researchers and industry detailing the type of characterization which might be expected when developing a new nanomaterial or nanomedicine. For inhaled nanomedicines there are additional characterization requirements around drug-device integration and aerodynamic properties as outlined below.

5 Integration of Nanomedicines With Devices for Inhalation

Appropriate selection of an aerosol generating device is of vital importance in the development of lung targeted nanomedicines. Beyond the standard regulatory requirements for drug device combination products, where reliability and reproducibility of performance is key [82], here, the compatibility of the aerosol generator and the nanomedicine must be considered.

Across the selection of aerosol generator technologies available (dry powder inhaler (DPI), pressurized metered dose inhaler (pMDI), soft mist inhaler, venturi nebulizer, ultrasonic nebulizer, passive mesh nebulizer and active vibrating mesh nebulizer) the nanomedicine may be exposed to many forms of heat, shear forces or evaporative stresses. The consequences of exposing the nanomedicine to these stresses may include changes in morphology, loss of conjugates, formation of particle aggregates, or concentrating effects [83]. Further, the formulation requirements for DPI, for example, may complicate or add expense and time in development [84].

The ultimate choice of aerosol generator will vary depending on its compatibility with a specific nanomedicine formulation and should be the first assessment to be completed in the development of any combination nanomedicine product. Characterisation of morphology, aggregation and therapeutic effect pre and post

Fig. 1 (continued) non-mannosylated neutral (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)) and mannosylated liposomes (composed of 7.5% mannosylated lipid with different carbon linker lengths MC2C, MC4C and MC6C), fixed and stained for nuclei (Hoechst; blue) and F-actin (phalloidin-FITC; green). Images were acquired by an INCELL 1000 cell analyser with a 20× objective. Representative images show (A) untreated cells and cells treated with 200 nm DOPS liposomes at concentrations of (B) 50, (C) 100, (D) 200, (E) 300 and (F) 1000 μM for 2 h. Liposomes were counted per cell using INCELL 1000 analysis software following treatment with liposomes ranging (G) in size at 200 μM for 2 h, (H) in concentration at 200 nm for 2 h and (I) in incubation time at 200 nm and 200 μM. Data represented as mean ± SD (n = 6). Statistical differences were determined by two-way ANOVA with Bonferroni's post hoc test (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001) versus unextruded liposome (for size comparisons, G) or DOPC-treated counterparts (for concentration and time comparisons, H and I). (Reprinted with permission of the publisher Taylor & Francis http://www.tandfonline.com from [43])

aerosolisation must be considered at a minimum and will act to de-risk further development activities [30, 31, 85]. Additionally, it may occur that the nanomedicine formulation is grossly unsuitable for use with a particular aerosol generator technology, for example, venturi-type nebulizers are known to be incompatible with suspensions. In this scenario, the evaporative effects within the medication cup of the nebulizer results in a concentrating effect, where the buffer is preferentially aerosolized and the suspended therapeutic is contained in an ever reducing buffer volume [83]. This changing concentration effect may lead to an increase in aggregation, formulation instability or even changes in aerosol droplet size over time [86].

As a next step, the effect of the nanomedicine formulation on device performance and aerosol characteristics should be assessed. It is known that the physicochemical characteristics of a pharmaceutical formulation may have a profound effect on the output rate (mass or volume aerosolized per unit time) and droplet size produced by some aerosol generators [87, 88]. These characteristics may be influenced by the surface tension or viscosity of formulation excipients, and also the concentration of actives in a formulation. The output rate may be seen to increase or decrease over that expected. Time to delivery of a dose is a critical consideration in the day to day treatment of patients and may have an influence on patient adherence with prescribed therapy [89]. Extended delivery times may also be a factor in device selection depending on the therapeutics' half-life in the body.

These effects may also manifest as altered droplet size distributions compared with those expected from the same device aerosolizing small molecules, for example albuterol sulphate. This is an important consideration, especially if the nanomedicine aerosol is intended to deposit in specific regions within the respiratory tract for greatest effect [19, 20]. At a minimum, droplet size should be assessed using standardised, regulatory approved means such as laser diffraction or cascade impaction [90]. This is done in order to confirm that the aerosolised droplets containing the nanomedicine are within the respirable range of interest [2, 91].

Once a compatible aerosol generator has been identified, the next consideration is the delivered dose to the lungs or target area. The literature is replete with studies describing the differences in delivered dose between device types, patient interventions and patient interfaces [92, 93]. Patient type is a key factor also, with the choice of the most appropriate aerosol generator sometimes being limited by the environment in which the therapy is being administered. For example, anaesthetized patients receiving invasive respiratory support through mechanical ventilation are likely to require a different device than a spontaneously breathing, conscious patient. These respiratory support interventions require the use of a variety of complex equipment, each of which have been shown to have a significant effect on the delivered dose [93, 94]. These differences in dose delivery are magnified further between infant, paediatric, adolescent and adult patients. With effects conferred by differences in breathing patterns, airway geometries and device/patient interfaces, there is a huge range of potential delivered doses reported in the literature [95–98]. Understanding the delivered dose is critical in formulation design. It inputs into dose concentration and dose volume calculations. It may also lead to prescriptions of device type, interface or intervention in ensuring that the appropriate dose is delivered.

6 In vitro and In vivo Assessment of Inhaled Nanoparticles

6.1 In Vitro Testing

6.1.1 Cell Culture Models

The lungs are complex not only in structure but in the assortment of cell types that are present, of which there are about 40 varying types [4]. Various cell lines have been developed which act as models for different cell phenotypes in the lungs and a key feature of cell culture models for assessment of inhaled nanoparticles is that cells are often cultured at an air-liquid interface to recapitulate the lung environment. Calu-3 and 16HBE14o- cell lines are capable of forming polarized monolayers and tight junctions providing a suitable model of the bronchial epithelium. Calu-3 cells are also capable of forming an airway surface layer, an impacting barrier to nanoparticle delivery [99]. Alveolar epithelial cells can be replicated using human primary cells, the adenocarcinoma derived A549 cell line or the NCI-H441 cell line. Primary cells and NCI-H441s form a competent barrier [100] whereas the A549s do not [101]. Primary airway epithelial cells have been isolated from human and animal sources. These primary cultures consist of several cell types and provide a better approximation of the *in vivo* environment. Normal human bronchial epithelial cells (NHBEs) can be fully differentiated with mucin and cilia production when grown on an extracellular matrix at an air-liquid interface. However, this process takes a relatively long time with cells being cultured for 30 days.

There are several immune cells present in the lung including mast cells, dendritic cells, and alveolar macrophages. The THP-1 monocytic leukemia cell line is a commonly used alveolar macrophage *in vitro* model and can be differentiated into macrophages using phorbol-12-myristate-13-acetate (PMA) [102]. Primary human alveolar macrophages have also been isolated from patients with disease states such as COPD. These cells can be difficult to culture and may show an inability to phagocytose [103]. Primary monocyte-derived macrophages obtained from human blood and differentiated with granulocyte-macrophage colony stimulating factor (GM-CSF) have also been used as lung models but are not alveolar macrophages [40].

Cell-cell interactions can mean results regarding inflammation and responses to particles vary. Over the years a number of co-culture models have been developed to enable the study of the interaction of particles with lung cells. These co-culture models facilitate cell-cell interactions and could thereby better recapitulate the *in vivo* environment. Some examples include the system developed by Rothen-Rutishauser et al. in 2005 [104] consisting of A549s, macrophages and dendritic cells. The cells are grown on and underneath filter inserts with pores of 3 μm in size. The model has been used to investigate particle interactions with the lung and the immune system. More elaborate 3D models are also being developed and used which seek to recapitulate the extracellular matrix of the lungs using materials such as Matrigel® [105], poly(ethylene glycol) [106], and collagen [107] to support cell culture *in vitro*. While these systems offer significant advantages over traditional 2D cell culture, they do not take into account dynamic processes of respiration.

Microfluidic-based devices are seeking to address this including the lung-on-a-chip developed by Huh et al. [108–112] which in the author's own words is "a microfluidic device that replicates the microarchitecture and dynamic microenvironment of the alveolar–capillary unit of the living human lung" [112].

6.1.2 In Vitro Toxicity & Immunogenicity of Inhaled Nanoparticles

There is still significant research required to properly elucidate the physiological processing of nanomaterials that serve as drug carriers [113] and drug alone nanoparticles [114]. The ideal properties required for nanoparticle drug-delivery systems: size on the nanoscale, large surface area facilitating drug solubility and/or surface modifications to aid targeting, good cell interaction are the same characteristics that can contribute to a nanoparticle's deposition and pulmonary toxicity profile [115, 116]. Additionally, the properties of the nanomedicines themselves and the region in the lungs where they are deposited will influence the degradation and clearance properties of the nanoparticles as demonstrated in Fig. 2 [117].

Biodegradable polymers are often considered as safer alternatives compared to non-degrading carriers but to-date none have been regulatory approved for inhaled use. There are reports of inhaled PEI and PEGylated drug-loaded nanoparticles

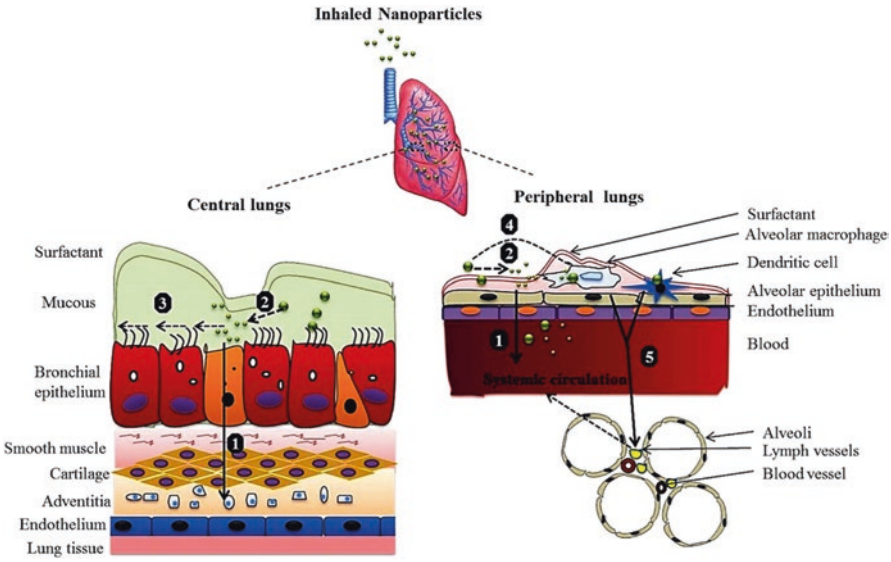

Fig. 2 Several extracellular and cellular barriers determine nanoparticle deposition, degradation and clearance: In the central lungs nanoparticles firstly encounter (1) epithelial lining and can traverse bronchial epithelial cells either in-tact or (2) larger particles may be broken down into smaller ones. Traversing the bronchial epithelium means that the nanoparticles avoid (3) mucociliary clearance in the central lungs and (4) phagocytic uptake by alveolar macrophages in the peripheral lungs or (5) dendritic uptake, translocation to the lymphatic system and systemic circulation. Reprinted with permission of the publisher Elsevier from Haque *et al.*, 2016 [117]

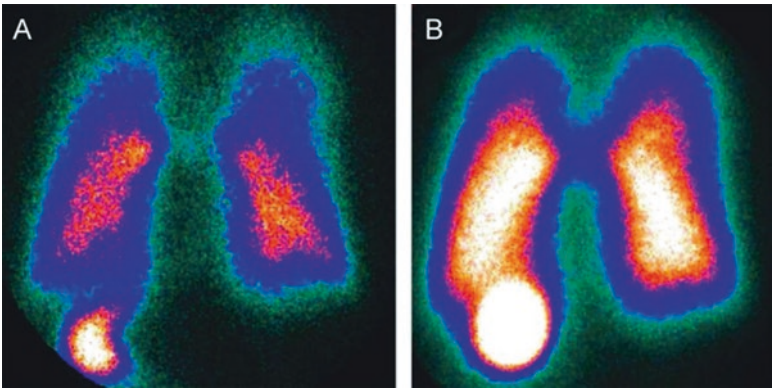

Fig. 3 Gamma scintigraphy images comparing nebulization efficiencies with (**A**) jet nebulizer and (**B**) vibrating mesh nebulizer during non-invasive ventilation using a radiolabelled diethylenetriaminepentaacetic acid in saline solution. Reproduced with permission of Daedalus Enterprises Inc from [147]

inducing inflammation in the lungs and disease-associated inflammation contributing to altered clearance mechanisms and pulmonary toxicity [118]. *In vitro* toxicity has also been observed for example for PLGA, chitosan and PLGA Pluronic®F68 (PF68) in epithelial A549 cells [45].

Briefly, *in vitro* testing facilitates simple, cost and time efficient determination of toxicity endpoints at a generic cytotoxicity or genotoxicity level [119]. A number of techniques are employed from traditional cell-culture studies to more advanced *in vitro* kits that serve as more realistic representatives of the lung and consider both lung physiology and surrounding environment [120]. As outlined above traditional cell-culture methods (monolayers, co-cultures and more recently 3D cell-culture models) may be used to mimic the lung environment and determine potential pulmonary toxicity. Cellular toxicity or indeed antigenicity [121] may be determined via simple metabolic assays [122] and more complex multi-parameter toxicity kits that also consider the influence of nanomaterials on factors such as cell morphology, size, membrane integrity, cell density and lysosomal mass-pH [47]. Metabolic activity is often determined via mitochondrial activity using an MTT assay. Cellular necrosis, another indicator of cytotoxicity is also detected colorimetrically using the lactate dehydrogenase (LDH) assay [123]. Stress response tests are based on the presence of reactive oxygen species (ROS) [124]. Detection of inflammatory markers (IL-6, IL-8 and TNF-α) present in cell supernatants, via a series of antibody and enzymatic detection reactions involving an enzyme-linked immunosorbent assay (ELISA) test, may determine immune responses to materials [123]. Despite the apparent usefulness of the aforementioned *in vitro* tests, nanoparticle interference remains a problem with the overall test success and validity determined by the nature of the nanomaterial and the test itself. Properties such as nanoparticle hydrophobicity, surface charge, catalytic activity and adsorption capacity can interfere with test findings thus providing false positive or negatives with regards to *in vitro*

Table 2 A summary of nanoparticle interference in commonly used *in vitro* toxicity assays (Adopted from [123])

Assay	Detection principle	Interference	Result	Ref.
MTT	Mitochondrial activity determined via colorimetry	Substrate adsorption	Altered cell viability	[125, 127]
LDH	LDH release determined via colorimetry	LDH inhibition	Altered prediction of necrosis	[128]
ELISA	Cytokine secretion determined via colorimetry	Cytokine adsorption	Altered cytokine concentration	[129]
H_2DCF-DA	ROS production determined via fluorescence	Fluorescence quenching	Unreliable indication of oxidative stress	[130]

MTT:(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); LDH: lactate dehydrogenase; ELISA: enzyme-linked immunosorbant assay; H_2DCF-DA: 2′,7′-dichlorodihydrofluorescein diacetate; ROS: reactive oxygen species.

activity particularly for metallic nanoparticles as outlined in Table 2 [125]. Although some researchers have conducted sterilization, microbial and endotoxin analysis of their nanoparticles most published nanoparticle studies are still manufacturing under non-sterile conditions. Whether this is a cause for concern and influences *in vitro* and *in vivo* toxicity findings remains debatable as even the lung itself is not truly a sterile environment thus nanomaterials may be simply disrupting the natural microbiome [126].

The scientific community are keen to pursue developing, standardising and validating more advanced *in vitro* detection methods in particular with continuous public concern regarding animal welfare [131]. Centers such as the European Centre for the Validation of Alternative Methods (ECVAM) are driving this process of international standardisation and validation [123]. Current acceptance for translation includes nanomedicines that meet the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Standard 8 and those of the ASTM Committee E56 (https://www.safenano.org/knowledgebase/standards/astm-international/). Within Europe projects such as *Future Nanomedicines* and *NanoReg2* aim to define the European strategy for regulatory testing of clinically translatable nanomaterials [132]. There are advances and improvements in marketed *in vitro* tests, for example commercially available differentiated epithelium MucilAir™ can mimic healthy or diseased tissue including expected respiratory system characteristics such as ciliary beating, ion transport and metabolic activity [120]. How a material behaves *in vivo* may differ considerably to *in vitro* due to the presence of biological fluids (plasma, mucus, lung surfactants) and pulmonary enzymes [118]. Co-cultures as referred to earlier, comprising two or more cell types facilitate closer replication of physiological conditions *in vivo* but require complete validation of cells used in both mono and co-cultures [116].

6.2 *In Vivo Testing*

In terms of a target site for *in vivo* testing generally the respiratory system is relatively accessible with many veterinary devices used in studies involving mice [64, 65], other rodents, dogs [133] and sheep [134] in particular. Rodent models are not ideal in terms of representing human physiology and anatomy, which can lead to poor recapitulation of the human lung nanoparticle clearance and overall pharmacokinetics. Sheep are more useful models with respiratory system dimensions and physiology more representative of that in humans [134].

6.2.1 In Vivo Toxicity & Immunogenicity of Inhaled Nanoparticles

When determining *in vivo* toxicity, consideration must be given to a number of factors including: route of administration; dose delivered and exposure levels; overall health of the respiratory system; particle size and the material itself among others. Nanoparticles administered via inhalation have been associated with systemic, inflammatory and neurotoxic effects following penetration of the central nervous system [135]. The dose delivered and number of exposures also may induce accumulation of polymer which in turn precipitates pulmonary edema, and release of pro-inflammatory chemokines and cytokines. Similarly the health of the respiratory system to which the nanoparticles are being delivered can influence their retention time and thus therapeutic effect [136]. Toxicity of nanoparticles *in vivo* is generally determined by the detection of relevant biomarkers including cytokines, chemokines and the presence of inflammatory cells including macrophages and neutrophils in the lung tissue and broncheoalveolar lavage fluid [117]. Studies to-date on inhaled nanoparticles have primarily focused on the pharmacokinetics and drug release profiles of the therapeutic cargoes rather than the fate and products of degradation of the nanomaterial [45, 64, 117, 137]. If these nanoparticles are to be transferred to the clinic, complete characterization of their toxicity profiles, an understanding of the mechanisms involved in nanomaterial clearance and correlation of *in vitro* - *in vivo* assessment is essential [117]. A combination of *in vitro* and *in vivo* methods may be employed with endpoint pulmonary toxicity determined by viability, apoptosis, oxidative stress, inflammatory response and mucus interaction [116]. The reader is referred to an extensive list of *in vitro* and *in vivo* toxicity endpoint studies for both polymer and lipid nanoparticle systems as presented in the review by Fattal and colleagues [116].

6.2.2 Biodistribution of Inhaled Nanoparticles

Inhalation allows for targeted delivery of nanoparticles to the lungs, however, the respiratory system is not an isolated one. Determining the fate of drug-loaded nanoparticles including their site of deposition, residence time in the lungs, the

nanoparticle drug (nanomedicine) half-life, off-target effects and rate of clearance are important in terms of effective delivery as well as protection from toxic accumulation. Biodistribution also provides vital information for optimal patient care when selecting the appropriate inhalation device and dosing regimen in terms of drug compatibility and inspiratory effort of the patient particularly for respiratory patients with compromised lung function.

Tracking of nanoparticle fate following inhalation can be done by either pharmacokinetic methods or imaging techniques. The method employed depends upon the type of information that is required i.e. the level of cell/tissue targeting, the effect of nanoparticle/dose manipulation or the effect of lung disease state [138–140]. Selection of the appropriate animal model is also of critical importance when assessing total and regional deposition to inform human studies. Rodent models are a popular choice for biodistribution studies, being relatively inexpensive with easy availability of reagents and kits with which to analyse endpoints. However, as noted above the anatomy of rodents differs significantly with human anatomy. Rodents have monopodial branching compared to the dichotomous branching in human lungs, rodents lack respiratory bronchioles and have a reduced number of alveoli affecting air flow. These structural differences mean particle entrainment and change in velocity does not occur in rodents until they reach the central to lower airways with deposition occurring due to sedimentation unlike deposition due to impaction often seen in humans, therefore care must be taken when extrapolating the regional deposition of particles from rodent studies to humans [141, 142].

After respiratory delivery of nanoparticles, histological analysis permits determination of drug concentration and assessment of focal lung regions deposition which has occurred. In addition collection of the bronchoalveolar lavage fluid (BALF) provides useful information on nanoparticle-drug distribution. This involves instilling and then aspirating sterile 0.9% saline solution from the whole lung followed by centrifugation and cell staining. Microscopy, flow cytometry and immunohistochemistry of the BALF can provide evidence of any inflammatory responses to the nanoparticles and/or the distribution of nanoparticles within the BALF cells and any preferential cell uptake that might be exhibited [26, 71, 143]. Application of these techniques proved useful in the comparison of polymer, i.e. PEI and PLL particles, which were uncoated or PEGylated. The nanoparticles were able to efficiently cross the human CF mucus barrier *ex vivo* which, when taken further into mouse *in vivo* studies, showed the ability to compact and deliver plasmid DNA to airway epithelial cells. Furthermore, histological biodistribution comparisons between the nanoparticles showed PEGylated particles were able to achieve small airway deposition compared to uncoated particles which had significantly reduced small airway distribution; coated particles were retained in the lungs for longer duration demonstrating the utility of these techniques in delivery method selection [71].

Conjugation of a tag or incorporation of photostable fluorescent dyes into nanoparticles can be a powerful tool in studying their biodistribution and fate of the nanoparticle and/or therapeutic cargo in the lungs. Conjugates such as Alexa fluor can be covalently linked to permit biofluorescent visualisation of nanoparticle bio-

distribution. By selecting varying fluorophores across the spectra multiplex, analysis can be carried out where varying formulations of nanomedicines can be examined for site specific targeting. Schneider *et al* labelled PLGA and polystyrene polymers with differing Alex fluor conjugates prior to particle manufacturing and intranasally delivered either mucoadhesive or mucus-penetrating nanoparticle variations of the polymers to assess which provided better biodistribution and crossing of the tracheal mucus layer in mouse models. The mucus penetrating formulations (PEGylated) in both cases demonstrated enhanced ability over mucoadhesive formulations in diffusing across the tracheal mucus layer [144].

Visualisation of luciferase with instruments like the In Vivo Imaging System (IVIS) generates biodistribution data based on a bioluminescent signal by the marker at the subdivided anatomic zones of the lungs, showing where drug nanoparticles are located [145]. Luciferase plasmid encapsulated in dimerized HIV-1 TAT peptide delivery vectors was administered intratracheally into tumour-bearing lung cancer mouse models which showed a dose dependent uptake at the tumour site over a 14 day monitoring period and limited off-target distribution [146]. Application of both bioluminescence and biofluoresence distribution techniques elegantly demonstrated the lungs as a viable administration route over systemic delivery of vaccine formulations. Li *et al* showed intratracheal delivery of Alexa647-OVA+ cells and luciferase expressing OT-I CD8+ cells in mouse models were able to traffic from the lungs to the mediastinal lymph nodes. Subsequent intratracheal delivery of peptide encapsulating lipid nanocapsules (interbilayer-crosslinked multilamellar vesicles) primed 13-fold more T-cells compared to controls in both the lungs and distal sites, as well as eliciting memory immunity and showing greater biodistribution compared to parenteral delivery [26].

Gamma-scintigraphy is a two-dimensional technique with the radionuclide either adsorbed onto the surface of the nanoparticle or combined in the drug solution for nebulisers providing detailed information on total nanoparticle delivery and subsequent distribution. Galindo-Filho and colleagues demonstrated the utility of this technique showing vibrating mesh nebulisers achieved >two-fold pulmonary aerosol deposition compared to standard jet nebulisers using a radiolabelled diethylenetriaminepentaacetic acid in saline solution (Fig. 3) [147]. Use of gamma scintigraphy in healthy individuals also demonstrated a 2.3 fold increase in pulmonary deposition of nanoscale salbutamol drug compared to standard micronized dry powder delivery [11]. However, limitations with this technique include the overestimation of deposition due to poor separation between the peripheral and small airways which can affect calculation of Permeation Index [148, 149]. Three-dimensional imaging can overcome this issue with Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) by conjugation of radionuclides or incorporation into the formulations respectively; by taking multiple planar images computer algorithms are able to generate the 3D images. Vij *et al* delivered PEGylated PLGA nanoparticles encapsulating ibuprofen to COPD mouse models and were able to determine by X SPECT-CT particle localisation to sites of pulmonary inflammation in real-time and subsequent decrease in detection marker indicating drug efficacy showing the additional capacity of this technique in terms

of theranostics [150]. However, radionuclides used in these methods have relatively short half-lives limiting their use in longitudinal studies and sophisticated instrumentation is required meaning they are mostly used in basic scientific human trials [143, 150, 151].

6.2.3 Computational Modelling

Due to the ethical issues, expense, resource requirement and the lack of good iterative and standardised *in vivo* models there is a significant focus now on computational modelling (*in silico* testing) of deposition [152]. The models are based on either mechanical or mathematical modelling of the airways to predict whole lung and/or regional levels of deposition. Formulations such as Sar-Gel, when coated to the interior of 3D printed airway models, allow qualitative assessment of biodistribution based on colour change upon interaction with moisture present in an inhaled solution [153]. While these types of models are useful they do not generate quantitative data meaning slight or subtle differences may be missed and they are not suitable for all types of inhalers. Computational *in vitro* studies are now looking at a combined mechanical/mathematical approach generating Functional Respiratory Imaging (FRI). Manipulation of experimental conditions such as varying formulations or inhaler device and disease progression can all be modelled to assess the effect on nano medicine biodistribution.

7 Industrial and Regulatory Aspects of Nanotechnology in Inhaled Product Development

The ability to manufacture and commercialize nanoparticles on a large-scale is essential to facilitate clinical translation. The successful scale-up of nanoparticle-based delivery systems relies heavily on collaborative efforts between academic researchers, industry, contract research organisations (CROs), government agencies and regulatory authorities in addition to financial investment, to facilitate transfer of scientific know-how to a therapeutic product [154]. Translation of standard evaluation assays for nanoparticle characterization is not straightforward due to the distinctive nature of nanoparticle physicochemical properties. This contributes to interference in assay findings as discussed earlier [155]. Finally personnel exposure to the nanoparticles during manufacture, healthcare and patient exposure during administration, and environmental and public exposure following disposal and excretion via waste water must also be carefully considered [156].

As mentioned above it is essential that complete *in vitro* and *in vivo* characterisation of the final product is conducted and this is costly not only in terms of time but also the requirement for personnel with appropriate skills and expertise in the area [154]. As outlined previously in the characterisation section the NCI-NCL and the

EU-NCL are cooperative centers that focus on harmonisation of nanomedicine development and pave the pathway for regulatory approval of nanomedicines [154]. The aim is to facilitate a "safety by design approach" for novel nanoparticles and enhance their potential for efficient and effective development and scale-up of new nanomedicines [157].

To address concerns regarding the safety and toxicity profile of nanopharmaceuticals requires the active participation of both the regulatory and scientific bodies and the academic/industrial researchers involved to develop and reinforce safety measures and regulatory frameworks to insure the public health [158, 159]. Research is now showing that harmless bulk materials tend to become more toxic when they are made into ultra-fine particles. The broad opinion is that the smaller the particles, the more reactive and toxic are their effects. The European Medicines Agency (EMA), in conjunction with experts of other Regulatory Agencies from US, Japan, Canada and Australia, joined the international reflection hosted by the FDA on how to define the characteristics of medicines based on nanotechnology. The group of regulators discussed and shared information on relevant on-going guidelines [160] and scientific and legislative initiatives in the various regions in order to facilitate worldwide harmonization regarding nanotechnology.

According to scientific guidelines from the EMA, the quality and performance of nanomedicines depends on the particle size, sample composition, chemical and physiochemical status, surface characteristics and interactions with biological environment. In harmony with this classification, devices are tested in accordance with the European Committee for Standardisation (CEN) Standard for Respiratory Therapy Equipment EN 13544–1 [161]. In 2006, new regulatory guidance was issued by the EMA and Health Canada [162]. This guidance reflected the dependency of safe inhaler device use on the selected formulation/device combination.

Orally inhaled products (OIPs) act in combination as device and formulation, however, from a regulatory perspective, are treated as drugs by the ruling pharmacopoeia's given that the drug is the main causative agent in the product [82, 90, 163, 164]. Given the multi-dose dispensing nature of many OIPs, the uniformity of delivered dose is of importance in all inhalation products, to ensure the patient receives a consistent drug dosage. As discussed previously, particle size has an important influence on localization of the drug therefore particle size and aerodynamic particle size and distribution characterisation must be carried out on all OIPs. The pharmacopoeia monographs and their expected results should be used at all stages of the product life cycle from development to batch-to-batch analysis once in production.

Aerodynamic particle size distribution provides important information on deposition of the drug due to the plume generated by the inhaler. Cascade impactors can provide this information as well as aerodynamic particle size by use of an airstream to entrain the particles and assess where they reach due to inertia. This method also permits assessment of drug mass that reaches the target site in the lungs providing the 'respirable fraction' of the drug [161, 162, 165].

8 Conclusion

To conclude, the potential for nanoparticles in successful respiratory therapeutic and vaccination strategies is significant. Unlike systemic delivery approaches, using nanoparticles via inhalation facilitates local, targeted delivery of the nanomedicine to the disease site overcoming many of the issues associated with parenteral delivery. The ability to combine several functions into a single nanomedicine could play a significant role in bringing nanomedicines to the fore as a go-to inhalable technology platform. The optimal integration of nanomedicines with devices for inhalation is a multifaceted and complex process, but reliable and reproducible dosing can be achieved through optimised combination product development, ensuring that nanomedicines have the greatest opportunity to demonstrate their potential as a next generation approach to therapy.

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Oral Vaccine Delivery: The Coming Age of Particulate Vaccines to Elicit Mucosal Immunity

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Abstract With the evolution of different challenging diseases, there is an urgent need of vaccine development against them to save millions of lives around the world. Particlulate delivery system plays an important role by acting as self-adjuvant in form of particles and thus assisting the immunogenicity of vaccines. Particulate vaccines have shown to have improved uptake by antigen presenting cells as compared to the soluble antigen. Traditional injectable vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infections at the mucosal site. Mucosal vaccines have been reported to provide additional secretory antibody mediated protection at the mucosal site of entry of the pathogen. In this chapter, we discuss the benefits of particulate drug delivery systems for oral delivery, the role of immune system in the gut, and a case study ofa novel particulate

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vaccine formulated into oral dissolving film for immunization via the buccal route. Key formulation components, process parameters and their biophysical characterizations have been discussed as well.

Keywords Microparticles · Spray dry · Oral dissolving films · Mucosal immunity Buccal immunization

Abbreviations

APCs	Antigen-Presenting Cells
BBB	Blood-Brain Barrier
BSA	Bovine Serum Albumin
DCs	Dendritic Cells
EPR	Enhanced Permeability and Retention
GALT	Gut Associated Lymphoid Tissue
IFNϒ	Interferon Gamma
IL	Interleukin
MHC	Major Histocompatibility Complex
MLNs	Mesenteric Lymph Nodes
MALT	Mucosal Associate Lymphoid Tissues
MIS	Mucosal Immune System
NALT	Nasopharynx-Associated Lymphoid Tissue
ODF	Oral Dissolving Film
PPs	Peyer's Patches
PLGA	Poly(lLactic-cCo-gGlycolic aAcid)
RVG	Rabies Virus Glycoprotein
TLRs	Toll-Like Receptors
Th1	Type 1 Helper T Cells
Th2	Type 2 Helper T Cells
VLPs	Virus-Like Particles

1 Introduction

Vaccines play a pivotal role in the management of various infectious diseases and are considered to be as successful tours de force in medicine [1]. Concerted efforts in the development of vaccines have benefitted both humans and animals in dealing with deadly infectious diseases. Vaccine development was historically based on Louis Pasteur's concept of isolation, attenuation and injection. Over the years, it has been observed that vaccines either loses its immunogenicity or cause adverse effects like fever and anaphylactic reaction [2–4]. There is always a risk in the use of live

attenuated viral vaccine i.e. it may undergo reversion to virulence. In an attempt to mitigate the adverse effects and in order to avoid the use of weakened viruses, recent research has led to the development of subunit vaccines. Subunit vaccine consists of single or a few highly purified antigens designed to induce a specific immune response. Advances in technology enabled the production of subunit vaccine in bulk quantity. Unlike traditional vaccines, it exhibits superior safety profile and reduced reactogenicity [5].

It has been observed over the years that subunit vaccines were less immunogenic than traditional vaccines due to the absence of pathogen-associated molecular patterns and poor antigen presentation to the immune sentinels i.e. antigen-presenting cells (APCs). These obstacles necessitated the use of adjuvants in vaccines and delivery of antigens in particulate form [5–7]. Adjuvants currently used in vaccines are more efficient in inducing an antibody-mediated immune response and produce the weak cell-mediated immune response. However, cell-mediated immune response is equally important in treating various infections caused by viruses and intracellular bacteria. Successful demonstration of increased vaccine efficacy promotes the delivery of vaccine antigen in the particulate form [8]. The objective of vaccination is to generate an innate and adaptive immune response to an infectious pathogen. The interface between the innate and adaptive immune response is APCs, particularly dendritic cells (DCs) [9]. DCs recognize the pathogen *via* pathogen recognition receptors namely toll-like receptors (TLRs) and undergo maturation, then redistributes the major histocompatibility complex (MHC) molecules from intracellular compartments to the surface of cells [10].

Immune cells e.g. DCs preferentially uptake virus sized particles (20–200 nm) and macrophages preferentially uptake larger sized particles (0.5–5 μm). Particles larger than 0.5 μm undergo cellular uptake *via* phagocytosis and are directed to phagosomes resulting in cross-presentation of antigen and production of cellular immune response. Nascent phagosomes undergo several transformation stages during maturation and interact with early endosomes, late endosomes and lysosomes and finally turn into phagolysosomes [11]. Phagolysosome formation results in extensive remodeling and turns the pH of its lumen acidic [12]. It triggers the proteolytic activity and generates small antigenic peptides thus avoiding the complete degradation of epitopes [13]. The antigens that enter into phagosome and released into the cytosol are degraded further by proteasomes into small fragments and shuttle to the cell surface using MHC I molecules resulting in cell mediated immune response. Alternatively, the uptake of smaller particles is primarily mediated *via* endocytosis and subsequently directs the antigen to early endosomes. The antigens that enter the cells *via* endosomal pathways are typically degraded in the vesicles and displayed on the MHC II molecules and thus activate the $CD4^+$ T cells. By tailoring the properties of particulate vaccine, antigenic peptides can escape the endosomes and be released into the cytosol. Antigens get degraded *via* proteasomes in the cytosol and displayed on the cell surface *via* MHC I molecules [10]. In contrast, the antigenic peptide that remain in the endosome, and not released into the cytosol, are presented on the cell surface *via* MHC II molecules.

DCs and macrophages express various surface receptors to recognize the antigen. Targeting the antigen using ligand specific for DCs or macrophages can further enhance the immunogenicity of vaccines and potentially reduce the dose. Interestingly, targeting of soluble antigens using antibody ligand has been found to elicit a strong cellular immune response compared to insoluble antigens [14]. Particulate nature of vaccines enabled the targeting of vaccine antigen by coupling a ligand specific for sentinel cells [14]. Ligands deliver the antigen to the target site such as phosphatidylserine incorporated in liposomes promoted interaction with surface receptors on monocytes. Ligands terminally linked to mannose, fucose or N-acetylglucosamine facilitates binding to mannose (DEC-205 and DC-SIGN) and lectin-like receptors on DCs. For example, grafting of antibody-ligand targeting DC-SIGN on poly(lactic-co-glycolic acid) PLGA based nanoparticles enhanced the antigen delivery and cellular uptake *in-vitro* [15, 16]. Different route of vaccine administration could target different secondary lymphoid tissues. For example, Langerhans cells in the skin, $CD103^+$ DCs in connective tissue and mucosal DCs in the gut. Delivery of antigens *via* different route may also affect the skewing of an immune response. Vaccine immunogenicity is not only affected by its composition of particulates, but its efficacy can be affected by the particle size, shape, and rigidity. Various publications have reported the effect of size, shape, and rigidity on the immunogenicity of vaccine [17]. These parameters provide the means to tune the vaccines immunogenicity, bio distribution, cellular uptake and antigen presentation.

Size: The size of particle affects its transport to the lymph nodes and the immune response. Smaller particles less than 50 nm converge to the lymph nodes *via* interstitial flow, however, larger particles shuttle to the lymph nodes using active transport by tissue resident DCs. Shima *et al*, also reported that smaller sized particles uptake was higher compared to larger sized particles. However, the delivery of antigen to APCs was higher for larger sized particles. For example, the uptake of 40 nm particles was twice compared to 200 nm particles but the delivery of antigen was three times higher for 200 nm particles [18]. The size of particulate vaccine is an important correlate of the extent of antigen presentation (MHC I & MHC II) and influences the type of immune response generated. Nanoparticles induce stronger activation of DCs and skewed the immune response towards Th1 evidenced from elevated expression of interferon gamma (IFN-γ), and IgG2a antibody titers relative to IgG1. It has been suggested that accumulation of small particles in the lysosomes may cause it to burst due to overload, resulting in the release of particles in the cytosol and explains the reduced MHC II loading of antigen. In contrast, the microparticulate vaccine skewed immune response towards type 2 helper T cells (Th2). Microparticles induced more MHC II molecules than MHC I and stimulate interleukin (IL-4) cytokine expression favoring Th2 antibody-mediated immune response [19].

Shape: Spherical nano and micron size particles induce stronger activation of DCs due to increased upregulation of co-stimulatory molecules (CD80 & CD86) compared to non-spherical particles [20]. This observation suggests that spherical particles are more efficient in antigen presentation. Further studies revealed that

spherical particles induce IFN-$_{\Upsilon}$ cytokine expression and IgG2a antibody titers and are more effective in skewing the immune response towards Th1 and $CD8^+$ T cells. In contrast, rod-shaped particles skewed the immune response more towards Th2 and are more effective in inducing IgG1 antibody titers.

Rigidity: The rigidity of particle affects its cellular uptake and immunogenicity. Studies showed that rigid particles activated DCs more strongly as measured by upregulation of CD40 and CD86 levels. On interaction with the cells, rigid particles wrapped up easily around the cell membrane, alternatively, soft particles tend to spread out across the membrane resulting in increased energy consumption. This led to the inference that rigid particles had significantly higher cellular uptake relative to soft particles due to less energy consumption [21]. In addition, rigid particles are efficiently presented by MHC I molecules on the surface of DCs and skew the immune response towards Th1. A study showed that reducing the rigidity of liposomes by adding cholesterol of low transition temperature imparts flexibility and lessen the Th1 mediated immune response [22]. For instance, generation of $CD8^+$ T cells and Th1 directed immune response, important in cancer vaccine, can be achieved by using spherical, nano-sized rigid particles. Alternatively, development of Th2 biased antibody-mediated immune response, useful for infectious diseases (e.g. hepatitis B vaccines), can be generated using rod-shaped, micron-sized flexible particles [23].

2 Particulate Vaccines

The quest to design an efficient delivery system for biologics is continuously evolving to be at par with the advances in drug discovery, gene mapping, proteomics and cancer targeting. Conventional therapy involves the administration of potentially high doses of the therapeutic moiety multiple times resulting in a multitude of adverse effects. Consequently, there is an increasing need for research in the field of controlled release systems. One of these methods involves formulation of a particulate delivery system where the drug/s of interest can be incorporated within various types of biodegradable polymer matrices [24].

Particulate delivery systems using biodegradable polymers have been investigated for sustained release and for targeted delivery to the site of action. Some of the currently used polymers in research can be broadly divided into being derived from either natural or synthetic materials.

Natural polymers include polypeptides and proteins (e.g. albumin, gelatin and collagen), polysaccharides (e.g. hyaluronic acid, starch, and chitosan), virus envelopes (e.g. Sendai viral envelopes) and living cells (e.g. erythrocytes, fibroblasts) [25]. Biodegradable synthetic polymers used for the formulation of polymeric nanoparticles for drug and vaccine delivery include aliphatic polyesters of hydroxyl acids, such as poly (lactic acid) (PLA), (PLGA), poly (caprolactone), poly (orthoesters), poly (alkylcarbonates), poly (amino acids), polyanhydrides, polyacrylamides and poly (alkyl-cyanoacrylates) [26, 27].

The particulate delivery systems offer significant advantages compared to the traditional delivery systems, such as:

- Capability of delivery via oral, transdermal and parenteral routes
- Can accommodate small and large molecules
- Multi-drug therapy using one particle
- Stable delivery system for bioactive molecules
- Easy manufacturing and scale-up
- Eliminated cold chain requirements

3 Advantages of Particulate Vaccines

The use of micro- and nanoparticles as a vehicle to deliver vaccine antigens has attracted interest in conferring protection against infectious diseases. The unique morphology of particles mimics pathogen and thus facilitates ease of recognition and uptake by the body's immune system.

1. Encapsulation of antigen in the core of particulate system emerged as a promising approach for the oral delivery of vaccines. Particulate vaccines facilitate the oral delivery of antigen by protecting from harsh gastric pH, bile juices and digestive enzymes of the gastrointestinal (GI) tract. Entrapped antigens release slowly from the particulate matrix by virtue of their slow degradation rate. It eliminates the need for booster vaccination and thus enables the development of a potential single dose vaccine formulation.
2. Antigens can be either adsorbed, encapsulated or entrapped in the particulates. The location of antigen in the particulates can influence the immune response. Antigens bound to the surface are more likely to undergo proteolytic degradation and may result in premature degradation before reaching the APCs. However, it provides the benefit of interacting directly with B cells and can induce stronger antibody immune response. Multivalent presentation of antigens on the surface of particles promotes crosslinking of B cell receptor resulting in an enhanced humoral immune response. Moreover, antigen adsorbed onto the particles are cross-presented at 1000–10,000 fold lower antigen concentration compared to its soluble form due to enhanced antigen uptake upon particularization [28, 29]. Studies have shown that there is up to 30% increase in cellular uptake of vaccine antigen when present in a particulate form over a soluble form [28]. Particularization also facilitates the co-localization of antigen and adjuvant to the same APCs, thus limits the systemic exposure to adjuvant and its side effects. Moreover, uptake of particulate matter by the APCs generates inflammatory response and thus reinforces the adjuvanticity [28].
3. Particulates are the choice of vehicle for vaccine antigens due to their ease of preparation and potential modification of their physicochemical properties such as surface charge, hydrodynamic size and the solubility of particles.

4. Amongst the particulates, virus-like particles (VLPs) provide protection not only against the virus of origin but also against heterologous antigens. They are typically in the size range of 20–150 nm and consist of a self-assembled viral envelope, generated from a single protein to form a multimeric complex [30]. VLPs possess the antigenic properties of a virus but are not infective since it does not contain any genetic material. Engineering of VLPs allows expressing additional proteins either by fusing these proteins to the particle or by expressing multiple antigens. In addition, VLPs can be chemically coupled to non-protein antigens such as polysaccharides or small organic molecules to produce bioconjugate VLPs [31].
5. Mucosal surfaces provide a first line of defense from external invaders. Therefore, oral delivery of particulate vaccines have more access to M cells than its soluble counterpart. M cells can actively transport the particles to the underlying Mucosal Associate Lymphoid Tissues (MALT) to initiate an immune reaction [32].
6. Peptides derived from extracellular proteins are loaded onto the MHC class II molecules whereas endogenously synthesized peptides are loaded on MHC class I molecules. Cross-presentation refers to the loading of exogenous antigens on MHC I molecules and thus activates $CD8^+$ T cells. Cross-presentation is critical for priming $CD8^+$ T cells response to viruses, intracellular pathogens or tumors. Particularization of vaccine promotes cross-presentation due to internalization of particles *via* phagocytosis into phagosomes. Phagosomes is known to be an important organelle known to play a pivotal role in cross-presentation of antigen. PLGA nanoparticles possess phagosome disruptive properties resulting in enhanced delivery of antigen to cytosol [1].
7. Lack of cold chain for vaccine storage poses a major challenge for disease control and prevention in many developing and underdeveloped countries. According to a report from the Department of Health and Human Services, 76% of vaccines for children were stored at inappropriate temperature conditions. Exposure to inappropriate temperatures can reduce vaccine potency and efficacy. Studies have shown that particulate vaccines can withstand extreme temperature conditions and thereby enhance the shelf life of vaccines [33].

4 Particulate Drug Delivery Systems

Microparticles are generally defined as particles with diameter ranging from 1000 nm up to a few microns, whereas nanoparticles are usually under 1000 nm. Particulate drug delivery systems are a class of formulations where drugs are incorporated into polymer matrices. The application of drug loaded particles in clinical treatment range from improving patient compliance, keeping constant therapeutic drug levels in the systemic circulation (sustained release), maintaining higher concentration of the drug at the pathogenic site and lower concentration in normal tissue (targeting), and reducing adverse effects (less toxicity) [34]. With the development in nanotechnology, it is now possible to produce drug containing

nanoparticles that can be utilized in a variety of innovative ways. New drug delivery pathways can be used that increase drug efficacy and reduce side effects [35]. Nanoparticle drug delivery could potentially be one of the safe and effective approaches to overcome these issues based on their advantages over conventional solution-based drug delivery systems.

Micro-and-nanoparticles can be divided into two categories: the first is called the homogenous particles where the drug is dissolved or dispersed throughout the polymer matrix; the second is called the encapsulated microcapsules where the drug is surrounded by the polymer matrix. The encapsulation technique is used in the pharmaceutical industry in the areas of sustained release of drugs, taste-masking of unpalatable drugs, masking of unpleasant odors, stabilization of drugs sensitive to atmospheric conditions, modification of physical properties, altering the solubility of drugs, elimination of incompatibilities between two or more drugs and a multitude of other uses [36]. However, the primary focus of particulate research in the pharmaceutical field has been the sustained release of drugs and drug targeting.

4.1 Physiologic and Biological Characteristics of Nanoparticles

Chemotherapeutic drugs reach tumors with poor specificity and leads to dose limited toxicity. Conventional drug delivery methods include oral and intravenous routes. Oral administration of drugs suffers from drawbacks such as the exposure to several metabolic pathways resulting in disorderly pharmacokinetics. This can then require the administration of a higher dose of the drug which might result in toxicity issues. Further, low specificity of drugs to the tumor is seen when administered intravenously. As a result, tumor targeted drug delivery has gained a lot of attention in the recent years to overcome the disadvantages of conventional chemotherapy. Tumor targeted drug delivery can be achieved by either passive targeting or active targeting. The mechanisms used by passive and active targeting is illustrated in Fig. 1.

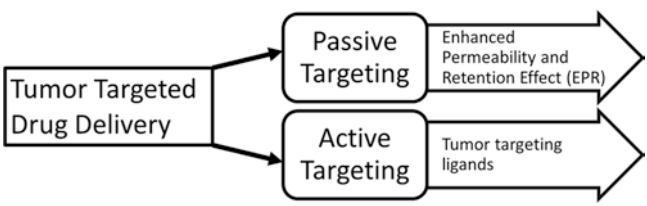

Fig. 1 Classification of tumor targeted drug delivery

5 Different Strategies of Micro-, Nanoparticles for Targeting

The downsides of conventional drug delivery systems such as low efficacy, poor bio distribution, toxicity and lack of sensitivity, can potentially be overcome by using controlled drug delivery systems. By means of controlled drug delivery systems, the drug is delivered to the site of action that increases its concentration and effect on the target organ or tissues and reduces the side effects at non-target organs. This leads to lowering the dose of drug required to perform the same action [37]. Micro and nanoparticulate drug delivery systems have the above mentioned benefits in comparison to conventional dosage forms [38]. Nanoparticles can be formulated to deliver drugs across several biological barriers. Anti-neoplastics, anti-viral drugs, and several other types of drugs are markedly hindered because of the inability of these molecules to cross the blood-brain barrier (BBB). The application of nanoparticles to deliver across this barrier is extremely promising [39, 40]. Further, biodegradable nanoparticles appear to be a promising drug delivery carrier system because of their versatile formulation, sustained release properties, sub cellular size and biocompatibility with various cells and tissue in the body [41].

Polymeric nanoparticles have been used for oral anticancer drug delivery and has gained significant attention lately due to the advantages summarized in Fig. 2. Anticancer drug entrapment within polymeric nanoparticles guards them from efflux transporters and the nano-sized range accelerates its entrance through biological membranes [42].

Passive targeting can be achieved by including the therapeutic agent into a macromolecule or nanoparticle which passively reaches the target organ. Drugs encapsulated in nanoparticles or drugs coupled to macromolecules can passively target tumors through the enhanced permeability and retention (EPR) effect. Instead, catheters can be used to infuse nanoparticles to the target organ or tissues. For example, localized delivery of drug-bearing nanoparticles to sites of vascular restenosis may be useful for providing sustained drug release at specific sites on the arterial wall. Tween 80-coated nanoparticles also have been shown to cross the BBB [39] [43].

Conjugation of nanoparticles with ligands of tumor specific biomarkers is a potent therapeutic approach to treat cancer diseases with high efficacy. It has been shown that conjugation of nano-carriers with molecules such as antibodies and

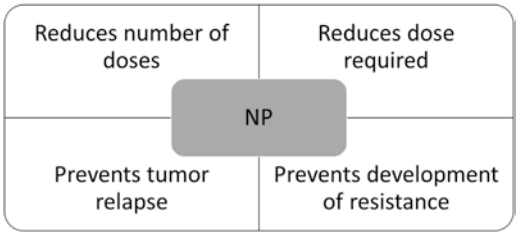

Fig. 2 Advantages of polymeric nanoparticles (NP) in cancer therapy

their variable fragments, peptides, nucleic aptamers, vitamins, and carbohydrates can lead to effective targeted drug delivery to cancer cells and thereby cancer attenuation [44].

One such approach to target brain delivery was to deliver oxytocin to brain using nanoparticles. Oxytocin is used for the treatment of social-deficit disorders such as autism spectrum disorder, but oxytocin cannot readily pass the BBB and requires frequent dosing because it is rapidly metabolized in blood. Polymeric nanoparticle formulations were made using poly(lactic-coglycolic acid) (PLGA) or bovine serum albumin (BSA) as the polymeric matrix. For brain targeting, these nanoparticles were conjugated with either transferrin or rabies virus glycoprotein (RVG) as targeting ligands. Release studies demonstrated that BSA nanoparticles exhibited a faster initial burst release compared to PLGA particles, in addition to later sustained release. This initial burst release would be favorable for clinical dosing as therapeutic effects could be quickly established, especially in combination with sustained release to maintain the therapeutic effects. The size and release profile data indicate that RVG-conjugated BSA nanoparticles is the most reliable formulation for brain delivery of oxytocin [45].

Other antigen delivery vehicles were formulated using polymers like hydroxypropyl methylcellulose acetate succinate,beta-cyclodextrin, ethyl cellulose etc. [24] Targeting ligand Aleuria Aurantia Lectin was used to deliver a particulate oral breast cancer vaccine. This particulate vaccine was formulated with enteric polymers to protect the antigens from the gastric environment and target the ligands to facilitate its uptake from M cells of the Peyer's patches (PPs) in the small intestine. M cells act as sampling ports for any foreign entities encountered in the small intestine. These M cells house various DCs and immune cells in them. Once the oral vaccine particle is sampled by M cells, it is processed by APCs and presented on MHC class I or MHC class II molecules. Thus, the particulate oral breast cancer vaccine was effective in providing protective humoral or antibody mediated immune response in a murine model [46].

In another study, poly (ethylene glycol) (PEG)-coated biodegradable nanoparticles were coupled to folic acid to target the folate-binding protein; this molecule is the soluble form of the folate receptor that is overexpressed on the surface of many tumor cells. For this purpose, a novel copolymer, poly [aminopoly (ethylene glycol) cyanoacrylate-co-hexadecyl cyanoacrylate] was synthesized and characterized. Nanoparticles were then conjugated to the activated folic acid via PEG terminal amino groups and purified from unreacted products. The specific interaction between the conjugate folate– nanoparticles and the folate-binding protein was evaluated by surface plasmon resonance. The analysis confirmed a specific binding of the folate–nanoparticles to the folate-binding protein. This interaction did not occur with non-conjugated nanoparticles used as control. Thus, folate-linked nanoparticles can be a potential new drug carrier for tumor cell-selective targeting [47].

6 Different Delivery Systems and Routes of Administration

Vaccines are by far the most effective and least costly way to offer protection against the debilitating infectious diseases. Vaccines are divided into two types: therapeutic vaccines and prophylactic vaccines. Prophylactic vaccines are primarily used for prevention of bacterial, viral or parasitic infectious diseases such ashuman immunodeficiency virus, influenza, malaria, tuberculosis, pneumonia, polio and small pox. At present, vaccines are administered intramuscularly, intravenously or subcutaneously. These routes have very low patient compliance and require intervention by trained personnel for their administration. Administering the vaccines via more patient compliant and easy to administer routes including oral, buccal, transdermal and pulmonary has been under investigation.

For current vaccines, some important points such as safety, effectiveness, ease of administration, time of preparation and cost need to be considered. The extent of success of vaccination depends on several factors such as nature of pathogen, delivery system, route of administration and immune system of the host. The following section discusses the various routes of vaccine administration.

7 Oral Route

The oral route is attractive primarily owing to its patient compliance and immune system activation. Oral administration of antigens has a potential to elicit mucosal and systemic immunity due to efficient antigen sampling by M cells in PPs. Although there are several advantages of oral administration, challenges such as the degradation of the antigen in harsh gastric and intestinal conditions is a significant concern [48].

Few of the oral vaccines that are currently in the market are, the Polio Sabin™ oral vaccine by Smithkline Beecham Biologicals, Dukoral™ oral vaccine for traveler's diarrhea and cholera manufactured by SBL AB, Stockholm, Sweden, Rota Teq oral vaccine by Merck vaccines, which is an oral vaccine to prevent rota virus infection and Vivotif (Typhoid Vaccine Live Oral Ty21a) manufactured by the Crucell Switzerland LTD for selective immunization against Typhoid fever for people travelling to endemic areas.

Apart from the above-mentioned advantages of the oral delivery in terms of patient compliance, ease of administration, lower cost of production and transportation (by avoiding cold chain), there is the added advantage of inducing both mucosal and systemic immunity. Recent studies have suggested that in order to produce a more robust immune response, both systemic and mucosal immunity must be induced. However, the major hurdle in oral vaccine delivery is the protection of the antigen from the acidic and enzymatic degradation in the gastrointestinal tract. Another obstacle to be considered while designing an oral vaccine is the probability

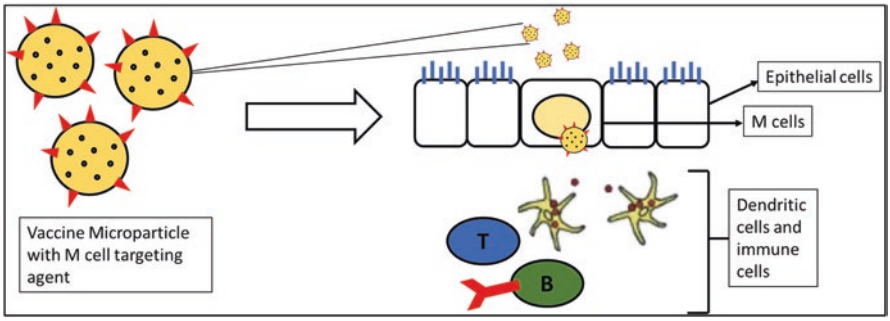

Fig. 3 Vaccine microparticle uptake by M cells of PPs in the small intestine after oral delivery

of oral tolerance. Low particle uptake and gastric degradation products of the antigens can cause oral tolerance [24].

Intestinal Peyer's patches (PPs) are the predominant site for uptake of such vaccine particles upon oral administration. The particle uptake at these sites depends on various factors such as size, charge and hydrophobicity. For oral delivery, particles of size less than 5 μm with a positive charge and hydrophobic nature can preferentially enter the PP of small intestine [31]. Orally delivered vaccines, especially particulate antigens are recognized and sampled by the M cells in PPs as illustrated in Fig. 3 [48]. This is followed by the transport of the particles to the underlying follicles that contain professional APCs such as DCs and macrophages. M cells house numerous APCs, which internalize the vaccine particles and express the antigen on its surface as MHC I or II complex.

8 Mucosal Immunity

The immune system is an intricate system in the human body that protects it from various types of pathogens. The mucosal surfaces of the respiratory, gastrointestinal and urogenital tract are the port of entry of pathogens into the body. This makes the mucosal system, which has a surface area of more than 300 m^2 susceptible to infections [49]. As a result, the mucosal system has developed its own mucosal immune system (MIS) to protect the body from onslaughts by external infections agents. The MIS is essentially the largest immune organ in the human body and it is noted that the intestinal lining contains the most immune cells and also secretes the highest amount of antibodies as compared to any other organ [50]. The MIS comprises of mucus covered epithelial layers, anti-microbial proteins and is augmented by the lymphoid tissues which encompasses the innate and adaptive immunity [51, 52]. The mucosal tissues are heavily populated with both innate and acquired immune cells, and their surfaces are the location of the secretory immune system, whose major immunoglobulin is secretory immunoglobulin A [49].

9 External Defense Mechanism by the Mucosal System

The mucosal surfaces provide the physical barrier which prevents the entry of the pathogens into the host. The cellular and chemical defenses differ from one mucosal surface to the other depending on the organ. The intestinal mucosa is covered by a single layer of epithelial lining, the respiratory tract is covered by epithelium lining which varies from pseudoestratified to simple epithelium and the oral cavity, pharynx, oesophagus, urethra and vagina are lined by a multilayered squamous epithelial lining [50]. These epithelial linings act as a first line of defense. In addition, the mechanical washing forces and cilial action create a current that rids the mucosal surface of organisms that enter the body and fail to bind early or well to the epithelium. The acidic pH of the stomach or some enzymes (lysozyme, lactoferrin, lactoperoxidase) secreted at mucosal surfaces or the anti- microbial peptides secreted by the epithelial cells such as defensins, cathelicidins and histatins can kill bacteria [49, 50].

10 Components of Mucosal Immune System

The MIS also comprises of symbiotic and commensal microorganisms which colonize the mucosal linings of the distal small intestine and colon, the skin, the nasal and respiratory tract, the oral cavity, and the female reproductive tract. These are also called as the natural microbiota [53]. The large intestine contains the largest number of microbes at approximately 10^{12} bacteria/ cm^3 [54]. This intestinal microbiota apart from helping to protect the body also helps in the digestion of food. The MIS can be separated into inductive and effector sites based upon their anatomical location. It is composed of the lymphoid tissues that are associated with mucosal surfaces (MALT) and can be separated into several components: gut associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), the mammary and salivary glands and the genitourinary organs [50, 55].

The GALT includes PPs, mesenteric lymph nodes (MLNs), and isolated lymphoid follicles (ILFs). The NALT includes tonsils/adenoids, inducible bronchus-associated lymphoid tissue, cervical lymph nodes, and hilar lymph nodes [49, 50, 56]. The MALT consists of the memory B cells and T cells which then move forward into the effector sites which include the lamina propria regions of the gastrointestinal, upper respiratory and reproductive tracts as well as the secretory glandular tissues. These sites contain antigen-specific mucosal effector cells such as IgA-producing plasma cells, and memory B and T cells [49]. The finger like projections in the intestine called villi play a major role in the absorption of nutrients as well as contain a large number of immune cells.

11 Mechanism of the Function of the Immune System in the Gut

The differentiation between the resident bacteria, pathogens and other antigens is mediated by three main types of immunosensory cell. First, surface enterocytes serve as afferent sensors of danger within the luminal microenvironment by secreting chemokines and cytokines that alert and direct innate and adaptive immune responses to the infected site [57]. The M cells that are present in the lymphoid follicles sample the environment and transport lumina antigens to sub-adjacent DCs and other APCs [51]. Finally, the intestinal DCs sense the gut contents by either entering or extending dendrites between surface enterocytes without disrupting tight junctions [58]. These DCs ingest and retain live commensal bacteria and travel to the mesenteric lymph node where immune responses to commensal bacteria are induced locally [59].

APCs, including DCs are responsible for the initiation of the specific immune reaction. The MALT is covered by a subset of differentiated M cells, epithelial cells, and underlying lymphoid cells that have a key function in the initiation of mucosal immune responses. M cells take up antigens from the lumen of the intestinal and nasal mucosa and transport them to the underlying DCs. The DCs take up the antigen, processes the antigen as peptides on their MHC class I or II molecules to underlying T cell region. The DCs migrate to the PPs or via draining lymphatics into the MLNs for initiation of mucosal T and B cell responses. The T cells migrate via the lymphatic system to the lamina propia of the villi, and then they secrete the immunosuppressive cytokine IL-10. The function of immune cells in the lamina propia and the epithelial layer is suppressed by the IL-10. The Wnt-β-catenin pathway in intestinal DCs also has a role in maintaining immunity in the gut. β-catenin expression in intestinal DCs induces the expression of anti-inflammatory mediators such as retinoic acid, IL-10 and transforming growth factor β [60]. Retinoic acid producing DCs enhance the expression of mucosal homing receptors (α4β7 and chemokine receptor 9 (CCR9)) on activated T cells for subsequent migration through the lymphatics, the bloodstream, and into the GI tract lamina propria. The T cell subsets Th1, Th2, Th17, and T_{regs} serve the crucial function of regulation within the MIS. Adaptive mucosal immune responses result from $CD4^+$ T cell help (provided by both $CD4^+$ Th2 or $CD4^+$ Th1 cells), which supports the development of IgA producing plasma cells.

12 Inducing Mucosal Immunity for Treating Disease

Many pathogens enter the body via the GIT, respiratory tract and reproductive tract therefore mucosal immunity can be targeted to achieve protection against these agents. Mucosal vaccines currently licensed for human use include oral vaccines against Vibrio cholera, Salmonella typhi, poliovirus and rotavirus, as well as nasal

vaccines for treating influenza [61]. The strategy used by these vaccines is to directly perturb the MIS thus providing immunity against the bacteria and viruses. The DCs then process the antigens and are presented to CD4 and CD8 T cells at inductive sites [62]. In case of orally administered antigens, the GALT induce effector or memory cells that express the integrin α4β7 [63]. These activated lymphocytes equipped with specific gut-homing molecules associate with the integrin and chemokine systems. Intranasally administered vaccine antigen sensitize the lymphocytes in the NALT and express the α4β1 integrin, a receptor for Vascular Cell Adhesion Molecule 1 [61]. These recent advances in the field of mucosal immunology has enabled us to glean a wealth of information about the intricate immune system. This has led to the development of vaccines that can be delivered through the mucosal route; the initiation of immune reactions at mucosal sites can provide both systemic and mucosal protection. Conventional parenteral immunization, however, produces only systemic protection without effective mucosal protection.

13 Case Study

13.1 Microparticulate Measles Vaccine in Oral Dissolving Film (ODF): Case Study

Measles is a highly contagious infectious disease caused by the measles virus. The advent of the vaccine, =significantly reduced the mortality due to measles [64].

Humoral as well as cell mediated immune responses are imperative for fighting against the measles virus infection. The humoral response is critical in controlling the viral replication and conferring protection, while the cell mediated immune response is necessary for overcoming acute measles infection by eliminating the infected cells [65].

An ODF formulation is a thin film prepared using hydrophilic polymers which dissolve rapidly in the mouth. The buccal cavity provides large surface area for rapid disintegration, release of the therapeutic entity, subsequent absorption and is a potentially good site for antigen delivery. The buccal cavity is rich in DCs like Langerhans cells, which are a type of APC. High density of T lymphocytes and MALT like tonsils, salivary glands, Waldeyer's rings and pharyngeal lymphoid tissue are present in the buccal mucosa. Hence, buccal immunization using an ODF can help to elicit both mucosal and systemic immunity. Microparticulate vaccine delivery via mucosal surfaces such as oral cavity has elicited a significantly higher immune response compared to an equivalent solution or suspension formulation. Consequently, the measles vaccine was encapsulated in the microparticles intended to be given via the buccal route. The microparticles were made from the biodegradable material that slowly releases the antigen, thereby having an antigen depot effect to enhance immunogenicity. Antigen presentation to the APCs is significantly improved when given in the microparticulate form. The measles vaccine

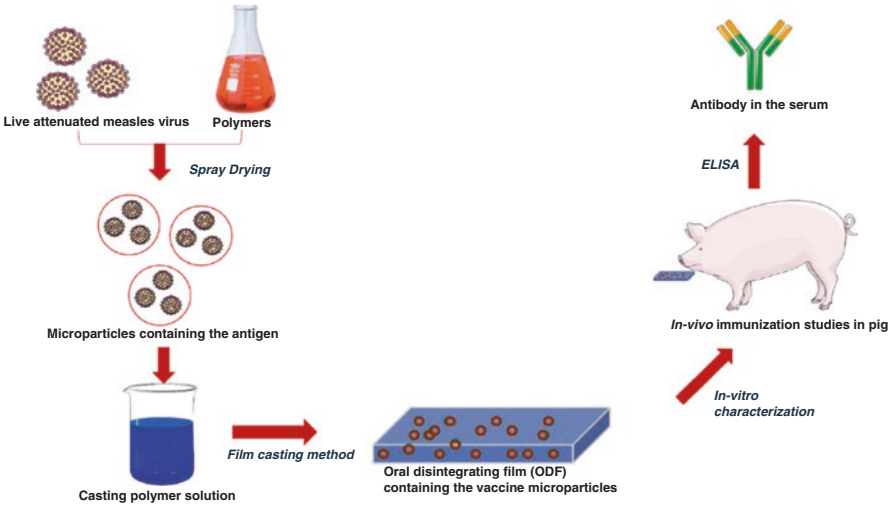

Fig. 4 Method of production of the ODF loaded with the microparticulate measles vaccine, and further immunization studies [66]

microparticles formulated using a spray drying technique were incorporated into an optimized ODF (Fig. 4).

The ODF vaccine formulation tested in the juvenile porcine model showed significantly increased antibody levels in contrast to naïve juvenile porcine controls. Thus, the novel particulate measles vaccine delivered in a flexible, ODF formulation can induce an efficient immune response that may be translated for global clinical applications [66].

ODFs containing measles vaccine microparticles dissolve on contact with the saliva and facilitate the coating of the buccal surface enhancing the delivery of the vaccine into the buccal mucosa. ODF also assures dosing accuracy and elicits the induction of an effective immune response. The current vaccine against the measles infection is invasive and requires medical professionals for its administration. This problem could be overcome using an ODF measles vaccine for which self-administration is possible. Thus, the cost associated with administration and cold chain storage could be minimized and large population could be immunized at an affordable cost.

The microparticles are made from the biocompatible and biodegradable polymer matrix which protects the antigen in the stable form and does not induce an immune response. The microparticles due to their size are easily taken up by the APCs and thus help in better antigen presentation to the immune system. The measles vaccine when formulated as the microparticles and incorporated into the ODF formulation induces significantly higher antigen presentation to the MHC I and MHC II molecules and corresponding co-stimulatory molecules CD40 and CD80 when compared to the blank formulation. This ensures that the microparticles are taken up by the APCs and stimulate both the arms of the immune system via Th1 and Th2

pathways. Gala R.P *et al* have reported a novel approach to formulate the microparticulate measles vaccine in an orally disintegrating film for delivery via the buccal route in a juvenile porcine model has revealed a promising mode of immunization strategy against measles in a commonly accepted surrogate model for human buccal delivery. In route is more patient compliant due to its ease of administration and can be given to populations of all ages from infants to adults [66].

14 Future Directions and Conclusion

Nanotechnology has been amply used in drug and vaccine delivery development. Various nanocarriers in the forms of emulsions, carbon-based materials, VLPs, liposomes, and polymeric particles have been utilized to formulate and deliver drugs and vaccines to combat a plethora of ailments. For vaccines, nanotechnology platforms have an enhanced potential to facilitate cell-mediated and humoral immune responses due to their nanoscale size, thereby improving the uptake of vaccine antigen resulting in enhanced antigen presentation to immune cells [67]. Polymeric micro- and nanoparticles are an attractive delivery system for vaccines as they can be optimized to produce desired sustained release profiles as well as specific surface properties for maximal vaccine efficacy. Most importantly, they have been shown to have good biodegradability and high biological safety in the body [68]. The antigen can either be encapsulated into the particle or incorporated into the surface morphology [69]. Additionally, adjuvants can be easily incorporated into these delivery systems to boost the innate immune response to the vaccine [67].

As reviewed in this chapter, micro- and nanoparticulate platforms can be integrated into various vaccines targeting diseases ranging from suspending microparticles in an oral-dissolving film for measles prevention to targeting various cancers. Larger microparticles, which can be more immunogenic, can be used to induce mucosal immunity. Particulate delivery can potentially achieve both passive and targeted delivery. Passive delivery can be achieved systemically, which passively reaches the target organ whereas targeted delivery can include conjugating the particle with a specific cell-targeting ligand [69]. As aforementioned, these microparticulate delivery systems have high versatility, which allow them to be administered through various routes including oral, buccal, intranasal, intramuscular, and subcutaneous delivery.

Nanomaterials in particulate delivery systems can function to have enhanced biocompatibility, biosafety, biodegradability, and mucosal absorption. Additionally, these materials can be relatively easy to modify in regards to shape, surface properties, release profiles, and protection of the antigen from degradation [70]. For vaccines, in addition to serving as delivery systems for the antigen, these materials can potentially confer certain adjuvant-like properties. Over the last few years, there has been a push to not only use microparticles as a delivery system for the antigen but also for them to promote immunomodulatory effects by serving as adjuvants [67].

Additionally, the future of micro- or nanoparticulate vaccines is dependent on the novel approaches and innovation of this technology based on the materials used for making the particles, the antigen/antigen dose, method of antigen loading, particle size distribution and uniformity, and routes of administration. Typically, polymeric micro- or nanoparticulate systems are delivered orally, subcutaneously, or intramuscularly. However, due to the rigidity and wide scale application of these particular systems, they can also be administered via less conventional routes of delivery such as buccal, nasal, pulmonary and transdermal routes. In this chapter, a novel polymeric buccal-absorbed microparticle-loaded oral dissolving film measles vaccine was discussed. Next, these particulate delivery systems can be optimized through various formulation methods involving specific surface and coating modifications for specific targeting in the body [71].These particles can then be applied to treat a wide array of diseases and infections prophylactically or therapeutically through less invasive routes of delivery to improve the lives of millions of people worldwide.

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Nanoparticle Vaccines for Immunotherapy: From Design to Clinical Trials

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Abstract Nanoparticles have the capacity to activate the immune system, based on both intrinsic particle characteristics and through the delivery of immune activating cargo. Co-delivery of antigens with adjuvants, such as cytokines, cytotoxic agents or pathogen-associated molecular patterns, presents opportunities for stimulating antigen-specific immune responses. This chapter highlights the immunogenic benefits of select chemotherapeutics and the use of nanoparticles to deliver immunogenic molecules and antigens. The influence of intrinsic nanoparticle properties and biological barriers on immune responses to nanoparticles is also discussed. In closing, a summary of nanoparticles approved for clinical use in the United States and examples of those approved in other countries are presented to highlight successes in nano-immunotherapy.

Keywords Immunotherapy · Nanoparticle vaccine · Delivery · Cancer · Adjuvant Nanomedicine

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1 Rational Design of Vaccines

Among the successes of worldwide vaccination programs are global eradication of smallpox [1] and a 50% reduction in the death rate of children under the age of five [2]. While these and other benefits of prophylactic vaccines against infectious diseases are undisputed [3], the application of vaccines to post-exposure prophylaxis, such as anthrax and Ebola, and non-infectious targets, such as cancer cells, has been challenging [4]. Live attenuated, inactivated microbial vaccines are intrinsically endowed with immune stimulating pathogen-associated molecular patterns (PAMPs) [5]. In contrast, vaccines against mutated or otherwise modified cells, such as those associated with cancer and neurological disorders, require the presence of components that activate immune cells, with PAMPs and damage-associated molecular patterns (DAMPs) being obvious candidates. The application of nanotechnology to vaccine development benefits from the ability to co-load antigens and immune stimulants in nanoparticles, modifiable release profiles and natural targeting of immune cells [6], giving rise to hopes of future successes in creating therapeutic vaccines with enduring cell-mediated and humoral immunity.

While early vaccine components were identified by trial and error, current technologies and advances in our understanding of mechanisms underlying immune responses have moved us to an era of rational design of vaccines [7, 8]. The first vaccine formulations included starch and oils, aimed at slowing antigen release. Later formulations used aluminum salts to precipitate and purify antigen. The inclusion of biological adjuvants, such as squalene (oil extracted from shark liver supporting both MF59 and AS03 formulations) began in the late 1990s [7]. Adjuvants are agents added to vaccines to enhance the immunogenicity of antigens that otherwise have insufficient immunostimulatory properties [9]. AS01, a liposomal formulation containing pathogen-derived monophosphoryl lipid A (MPL) and saponin, and AS02, an oil and water emulsion containing MPL and saponin, are currently in clinical testing. MPL adsorbed on aluminum hydroxide salt (i.e. alum, AS04) is currently being used in the human papillomavirus vaccine Cervarix® (GlaxoSmithKline) [10]. Adjuvants approved for human use also include virus-like particles (VLPs) [11]. VLPs are nano-platforms that resemble viruses in organization and conformation and are thereby able to trigger strong humoral and cellular immune responses, but lack the viral genetic material, to replicate. The historical use of vaccines with and without adjuvants is shown in Fig. 1. In 2014, the proportion of vaccines with adjuvants in clinical trials was greater than 50%; however, licensed vaccines lagged behind, with less than half containing adjuvants [7].

MPL is a detoxified version of the gram-negative bacterial endotoxin lipopolysaccharide (LPS). It triggers innate immunity through the engagement of receptors on the surface of antigen presenting cells (APC). Other examples of pathogenic molecules include Poly(I:C), a synthetic double-stranded RNA that mimics viral components, and CpG DNA, commonly found in bacteria [12]. PAMPs signal the innate immune system through Toll-like receptors (TLRs), NOD-like receptors (NLRs) and other pathogen-related receptors (PRRs). Cytokines and chemokines

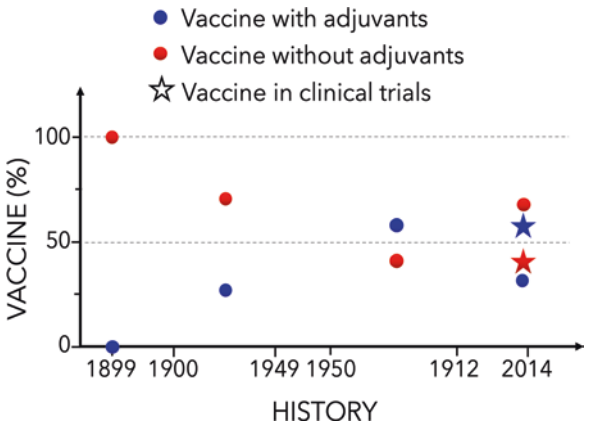

Fig. 1 The historical use of vaccines with and without adjuvants. Estimated vaccine percentage in history based on the fraction proportional to the log of the number of different vaccines [7]. The data points plotted for 2014 represent U.S. licensed vaccines listed by FDA. The clinical trial data points for 2014 (star-like) represent vaccines listed by HuVax (http://www.violinet.org) in clinical testing

are also candidates for inclusion in vaccines. IL-12, for example, helps to tailor immune responses towards pro-inflammatory T helper 1 (Th-1) responses. Our team has demonstrated that liposomal formulations containing MPL and IL-12 stimulate Th-1 responses and block tumor growth in mice bearing 4 T1 breast adenocarcinoma tumors [13]. Combinations of adjuvants and vaccines are an attractive means of eliciting better and long-lasting protective immune responses.

Cell mediators of sustained immune responses are APCs, with dendritic cells (DCs) being recognized as potent activators of adaptive immunity [14]. DCs engulf foreign objects (e.g. nanoparticles) by fluid-phase pinocytosis, receptor-mediated endocytosis, and phagocytosis, and then secrete cytokines and chemokines. Endogenous and exogenous proteins are degraded into peptides and assembled with major histocompatibility complex (MHC) class I and II molecules. The classical path of MHC class II loading with peptides includes processing of internalized (exogenous) antigens within the endosomal pathway. Conversely, processing of endogenous proteins occurs within the proteasome, with antigens transported to the endoplasmic reticulum for loading of MHC class I molecules [15, 16]. Models proposed to explain MHC class I loading of exogenous protein (i.e. cross-presentation) include a unique intercellular compartment known as an ergosome, which is a fusion compartment involving the phagosome and the ER [17, 18]. It has also been proposed that DCs contain a unique endocytic trafficking pathway that facilitates peptide loading into recycled MHC class I molecules within the endosomal compartments [19]. The ability of DCs to process endosomally-trapped antigens along both MHC class I and II pathways facilitates the use of nanoparticles as vaccine delivery platforms since the majority of nanoparticles are internalized into the endolysosomal pathway.

2 Development of Nanoparticle Vaccines: The Size Dilemma

The successful design of vaccines needs to consider two overarching goals: [1] efficient delivery of antigens to APCs; and [2] the capacity of APCs to process internalized antigens and present them to T-cells in association with MHC and costimulatory molecules [6]. Nanomaterials, the pillar of the nanotechnology [20, 21], provide opportunities for the rational design of potent vaccine platforms [22]. Owing to diverse features, such as tunable size (in the same range as pathogens), surface functionalization and hybrid nanoparticle platforms, antigen and adjuvant [23–25] can be loaded within or on the surface of the same nanoparticle and delivered to APCs to initiate a cascade of immune responses. In the last twenty years, particles of various nature have been used for the delivery of antigens, including (co)polymers [26], liposomes [27], mesoporous silica [28], chitosan [29], and others [30].

Amidst physicochemical characteristics dictating the immunological fate of particles, size plays a key role in their biodistribution, pharmacokinetics, efficacy, cellular internalization [31] in addition to their immunogenic behavior. Interestingly, no consensus has been established thus far on the optimal nanoparticle size for modulating immune responses, with the literature reporting opposing trends, sometimes even within the same study. In addition to size, surface potential, chemical composition, and selective opsonization, the site of administration of nanoparticle vaccines also influences outcomes. Common metrics for evaluating nanoparticle-mediated immune responses *in vitro* and *in vivo* are antibody titers and cytokines profiles. In this section, we will summarize immunological trends dictated by the size of nano/microparticles.

2.1 Benefits of Large Nanoparticles for Vaccine Design

Early work by Pedraz and colleagues [32] studied the influence of size and administration route of poly(lactic-co-glycolic) acid (PLGA) nanoparticles encapsulating bovine serum albumin (BSA) on vaccine efficacy. PLGA nanoparticles (200, 500 and 1000 nm) were administered via three injection routes in BALB/c mice: subcutaneous (1 μg antigen, one dose) intranasally (200 μg BSA, 3 doses), and orally (500 μg BSA, 3 doses). Serum anti-BSA immunoglobulin IgG was evaluated and compared with that resulting from administration of free BSA alone or adjuvanted with Freund's complete adjuvant (FCA) or alum. For all the administration routes, 1000 nm particles elicited the highest serum IgG titer. PLGA nanoparticles (500 nm) elicited superior antibody responses compared to 200 nm only when intranasally administered whereas similar responses resulted from nanoparticle injections through other routes. The difference in the relationship between particle size and antigen titer highlights the importance, sometimes overlooked, of the vaccine administration route which could show even opposed trend if the administration routes are different (*vide infra*). Overall,

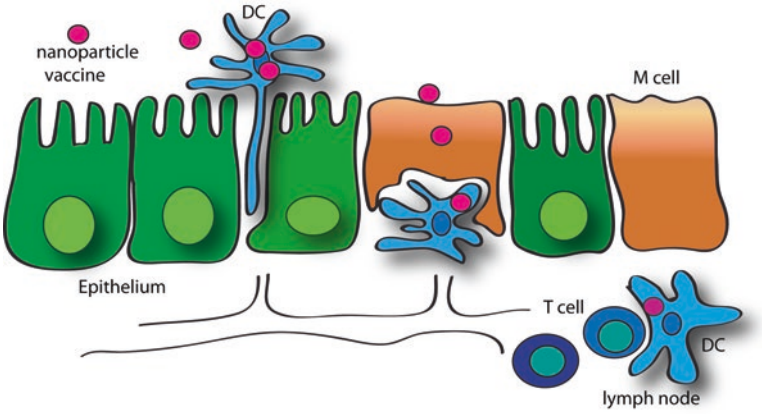

Fig. 2 Schematic of a mucosal membrane showing either direct DC internalization of nanoparticle vaccines or transport of nanoparticles across M cells to DC. DC then traffic through lymphatic vessels to lymph nodes where they present antigen in association with MHC to T cells

particle size predominated over antigen dose for influence on IgG titers. The authors attributed the higher immunogenicity of larger particles to the different distribution of differently sized particles in the lymphoid tissue but also to a lack of uptake of smaller particles by Peyer's patch microfold (M) cells, in agreement with other reports [33, 34]. M cells are epithelial cells of mucosa-associated lymphoid tissues that internalize antigens and transport them to lymphocytes and APCs in mucosal tissues (Fig. 2). Specialized DCs, also present on mucosal surfaces, are critical for recognizing pathogens and initiating and regulating immune responses [35].

In a more recent study, OVA-loaded polypropylene sulfide nanoparticles adjuvanted with soluble CpG with sizes of 30 (NP30) and 200 nm (NP200) were intranasally administered to C57BL/6 mice [36]. Here again, larger nanoparticles showed superior delivery of OVA into the MHC II presentation pathway as determined by inflammatory cytokine expression by OVA-specific $CD4^+$ T cells. Interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) cytokine levels were similar in the lungs and spleen. In all cases, negligible cytokine changes were detected from $CD8^+$ T cells. In addition, serum IgG2c antibody and proximal and distal mucosal IgA secretion were more pronounced following injection of NP200 compared to NP30 nanoparticles, suggesting that larger particles induce more efficient systemic and mucosal humoral responses and that a Th1-biased immune response was achieved. Interestingly, earlier reports by the same group showed that smaller nanoparticles (of the same nature) were more efficient when intradermally administered (*vide infra*) [37]. Therefore, understanding the interplay between size and administration route is critical for designing nanoparticle vaccines for optimal outcomes.

2.2 *Benefits of Small Nanoparticles and Shape for Vaccine Design*

The following studies support the opposite impact of particle size on elicitation of immune responses compared to the previous section. Cui and colleagues [38] demonstrated that nanoparticles obtained from a lecithin/glyceryl stearate emulsion in water (200 nm) conjugated with BSA induced strong immune responses when injected subcutaneously in mice compared to free BSA adjuvanted with alum or IFA (Incomplete Freund's adjuvant). Responses to *Bacillus anthracis* protective antigen (BAPA) using the same vaccine platform induced strong and long-lasting protection of mice exposed to a lethal dose of anthrax toxin (up to 473 days). Interestingly, when nanoparticles of two different hydrodynamic sizes (230 and 708 nm), presenting the same surface charge and conjugated with OVA (93 ± 4 μg/mg NPs) [39], were compared *in vitro*, internalization of the smaller (230 nm) nanoparticles by APCs was superior to that of the 708 nm nanoparticles. In addition, *in vivo* subcutaneous immunization (50 μg OVA/mouse/week) showed significantly higher anti-OVA IgG production with the smaller nanoparticles. Small (230 nm) nanoparticles inhibited the growth of B16-OVA tumors in C57BL/6 mice (18 days post tumor cell injection) to a larger extent than the following formulations: (1) 708 nm nanoparticles (300% volume increase); (2) an equimolar mixture of small and large NPs (160%); (3) pure OVA (700%); (4) OVA supplemented with IFA (100%), or PBS only control (1000%). It is noteworthy that nanoparticles induced significant immune responses only when OVA was covalently conjugated onto their surface, as opposed to adsorption [39].

Mitragotri's team recently evaluated antibody and cytokine responses to spherical and rod-shaped polystyrene (PS) nanoparticles of two diameter (193 and 521 nm) or aspect ratio (190 x 376 and 520 x 1530 nm) [40]. Nanoparticles, conjugated with ovalbumin (50 ± 3 μg/mg PS), were subcutaneously injected into female BALB/c mice. After the boost injection (21 days, 100 μg OVA), production of anti-OVA titers and cytokines were evaluated in splenocytes using IgG1 and IL-4 or IgG2a and IFN-γ as markers for Th-2 and Th-1 responses, respectively. They found that the smallest nanospheres induced the highest immune responses. With respect to Th-1 cytokine production, the smallest spheres induced significantly higher levels of INF-γ (800%) than all other particles, but no statistical differences in IL-4 production (Th-2) were observed amongst all the tested nanoparticles. With respect to antibodies, the smaller spheres produced higher IgG titers, however, the trend was reversed for rods. In addition to size effects, this work highlights the influence of particle shape on eliciting an immune response. Finally, the obtained ratios of IgG1/IgG2a indicated that the small spheres stimulated a cell-mediated Th1-biased response in contrast to the large rods, which stimulated a humoral Th2-biased response.

2.3 Impact of Nanoparticle Size on Dendritic Cells Targeting

APCs in peripheral tissues function mainly as sentinels. They readily internalize and process antigens, leading to maturation and migration to lymph nodes where they present the processed antigens to T cells [41]. Generally, nanoparticle vaccines target peripheral DCs, however, it has also been proposed that targeting lymph nodes with APCs uptake being secondary has the benefit of overcoming premature antigen presentation [42].

In 2006, Hubbel and Swartz demonstrated that interstitially injected small polypropylene sulfide (PPS) nanoparticles trafficked directly to lymph nodes without the use of targeting agents [43]. This occurred at a higher frequency for 20 and 45 nm particles compared to 100 nm particles, which is consistent with reports for liposomal or polymeric nanoparticles [27, 44]. They also demonstrated that the nanoparticles were predominately internalized by DC (amongst other APCs). In a subsequent study [37], they took advantage of the interstitial-to-lymphatic flow to transport small nanoparticles to the lymphatic vasculature bed where they induced maturation of lymph node-resident DCs leading to *in situ* activation of complement. In this study, 25 and 100 nm PPS nanoparticles, stabilized with hydroxylated or methoxylated pluronic (F127), were tested for adjuvant activity. The smaller 25 nm particles were much more efficient in the rate of accumulation in lymph node DCs 24 h post injection (50% compared to 6% for 100 nm) as well as their residency time (120 h minimum vs 24 h maximum for 100 nm). In addition, costimulatory molecules (CD40, CD80 and CD86) were found to be upregulated in the presence of hydroxylated 25 nm nanoparticles and the maturation of DCs, in this case, was comparable to that induced by the adjuvant LPS. To study antigen processing and presentation, mice were intradermally immunized (tail tip or dorsal foot skin) with OVA conjugated 25 nm nanoparticles. INF-γ expression, characteristic of T cell activation, was found to be 250% higher for nanoparticle-presented OVA compared to free OVA or methoxylated 25 nm OVA nanoparticles, but 83% lower than OVA+LPS. Interestingly, the same group reported in a subsequent study [36] the opposed trend when the nanoparticles were administered by a different route (intranasally) [36] and hence, once again, highlighting that vaccination route plays a key role in the fate of the nanoparticles. More recently, the group of DeSimone [45] subcutaneously injected PEG hydrogel nanoparticles (180 x 80 nm) and microparticles (1 μm) with OVA conjugated with or without PEG linkers (0.5 K and 5 K) to OT-II transgenic mice. In line with Hubbel study, they have clearly observed that small particles reached the APCs in the popliteal lymph node (PLN) more efficiently than larger ones. While less than 2% APCs were reached by microparticles, nanoparticles were uptaken and delivered antigen to up to 20% DC and 35% macrophages residing in the PLN.

2.4 Perspectives and Challenges

These studies demonstrate that within the panel of studied particle sizes, there is no universal particle size that supports optimal immune responses. While nano platform size is highly relevant and crucial, other factors need to be considered. For instance, the group of Plebanski [46] tested polystyrene nanoparticles (20–2000 nm) loaded with OVA on C57BL/6 mice bearing EG7-OVA tumors. Mice were injected intradermally with 100 μg OVA/mouse. Amidst the tested sizes, 40 nm nanoparticles stimulated the best T-cell responses (based on the production of INF-γ) following 1 and 2 injections, as well as the highest serum anti-OVA IgG titers 10 days after the second immunization (after the first immunization, 100 nm nanoparticles showed comparable responses to 40 nm nanoparticles). In addition, a comparison of 40 and 1000 nm nanoparticles, containing equivalent OVA doses, supported that the aforementioned behavior was not dose- but size-dependent. It was also discovered that 40 nm nanoparticles exhibited higher uptake by lymph node-resident DCs compared to either 1000 nm or 20 nm particles [43, 47]. DCs housing 40 nm nanoparticles expressed higher levels of DEC205, CD40 and CD86, while 1000 nm particles were more localized in macrophage-like APCs expressing F4/80 and CD80. It is also noteworthy that 40 nm nanoparticles displayed a greater adjuvant effect than commonly used adjuvants such as alum, MPL, Quil-A® and IFA. Finally, 40 nm particles also demonstrated the best performance in terms of hindering tumor growth. Hence, while the size of the nanoparticle is important for vaccine design, size thresholds most likely exist for all particle types, with a relevant influence by administration route.

The effect of size on immunogenicity was also studied using a library of OVA-conjugated polystyrene beads (approximately 20, 40, 50, 70, 90, 100, 120 nm) [48]. Nanoparticles were intradermally injected into mice (50 μg OVA/mouse) and cytokine secretion was evaluated in splenocytes 10 days after immunization. IFN-γ induction from $CD8^+$ T cells was mainly caused by 40–50 nm nanoparticles (~400% compared to other sizes) whereas IL-4, which mediates $CD4^+$ T cell activation, was mainly produced following stimulation with 90–120 nm particles (~150% higher). In addition, 40 nm nanoparticles were more efficiently internalized by lymph node-resident cells than their larger counterparts (70 nm), which is consistent with previous reports [37, 43]. It is noteworthy that the smallest nanoparticles (~20 nm) in this study were not outstanding in any aspect, in contrast to other reports [37, 43].

Four size ranges (50–150, 10–70, 2–8 and < 2 μm) of polylactic acid (PLA) microparticles encapsulating tetanus toxoid (TT), were injected intramuscularly in mice and anti-TT serum titers were evaluated [49]. Fifty days post single-point immunization, the 2–8 μm particles elicited 60% more antibody titers than their smaller counterparts and up to 120% and 320% than two larger groups of particles. The higher antibody response for the same group were sustained for 250 days post-immunization. Interestingly, in the presence of alum, the highest increase in titers was found for the 10–70 μm group of particles (+130%). One possible reason for low immunogenicity by 10–150 μm was suggested to be low macrophage

phagocytosis, which considerably diminishes for PLGA particles with sizes higher than 5–10 μm [49].

In agreement with previous studies on anti-hepatitis B particulate vaccines [50, 51], the Ahsan group demonstrated that PLGA particles (5 μm) loaded with hepatitis B surface antigen (HBsAg) were more immunogenic than their smaller (2 μm) and larger (12 μm) counterparts when administered using a pulmonary route [52]. Interestingly, the anti-HBsAg production was comparable for the three sizes at 21 days after vaccine administration, however, the 5 μm-particles showed an increase in immune response after 28 days (200% more antibodies than 2 μm- and 12 μm-particles).

Nano immunotherapy depends on the capacity of APCs to internalize nanoparticles, which is partly dependent on size, but also on shape and other physicochemical characteristics of nanoparticles. It is clear that a consensus on the effect of the size of vaccine nanoparticles is far from being established as numerous factors are involved within the reported works, including administration route and the chemical nature of the nanoparticles. A summary of findings on the effect of particle size on immune efficacy is presented in Table 1. In the majority of these studies, colloidal size measurements were omitted, and the reported size was the commercially provided size or that measured by electron microscopy. Significant differences in colloidal size may exist and other measurements should be considered. Thoughtful consideration of the mechanisms underlying vaccine efficacy will benefit the development of smarter and more adaptable nanosystems. Future studies would benefit from using different nanoparticle sizes with fixed administration routes and antigen doses in preclinical studies.

3 Biological Barriers to Nanoparticle Vaccine Delivery

Advantages of nanoparticles for immunotherapy include co-loading of biologically active compounds, natural APC targeting, sustained release of antigens, and multivalent presentation of immune stimulants for a heightened immune response [6, 25, 53]. Figure 3 illustrates DC internalization of a nanoparticle vaccine and presentation of antigens to T cells in association with upregulated co-stimulatory molecules. The confocal micrograph in Fig. 3b is a bone marrow-derived murine DC following internalization of fluorescent immunogenic lipid coated mesoporous silica nanoparticles (Serda laboratory at the University of New Mexico, image by Karen Sanchez; unpublished). The DC actin and microtubules, shown in red and green, respectively, are labeled with Alexa Fluor 647 phalloidin and anti-tubulin Alexa Fluor 488, while RITC-labeled nanoparticles are shown in white and the cell nucleus, labeled with DAPI, is shown in blue).

Potentially, every particle formulation has a unique behavior based on its physico-chemical properties [54]. Nanoparticle properties, including size, shape and chemical composition can be tailored to benefit the desired therapeutic effect. Such properties also include the particle surface charge and hydrophilic/hydropho-

Table 1 Summary of the main works reporting a systematic comparison of sizes of particles on immune response

Material	Size (nm)	Antigen (μg)	Administration route (adjuvant)	Immunization	Mouse model	Ref
PLGA	200; 500; 1 k*	BSA (1)	Subcutaneous (FCA or Alum)	Single dose	Balb/C	[32]
	200; 500; 1 k*	BSA (200)	Intranasal (FCA or Alum)	3 doses (d, d + 1, d + 2)	200–500 -1 k*	
	200; 500; 1 k*	BSA (500)	Oral (FCA or Alum)			
PPS	30; 200*	OVA (25)	Intranasal, CpG 4 μg	3 doses (d, d + 14, d + 28)	C57BL/6 OTI/OTII OVA transgenic	[36]
PPS stabilized with Pluronic F127	25*; 100	OVA (15)	Intradermal in tail tip or dorsal foot skin	Single dose	C57BL/6	[37]
Lecithin/stearate	200	BSA (5)	Subcutaneous	3 doses (d, d + 14, d + 28)	Balb/C	[38]
	200	BAPA (1–10)				
	230*; 708	OVA (50)		3 doses (d, d + 7, d + 14)	C57BL/6	[39]
PS	193*; 521; 193 x 176; 521 x 1530	OVA (100)	Subcutaneous	2 doses (d, d + 21)	Balb/C	[40]
PS	20; 40*; 100; 200; 500; 1 k; 2 k	OVA (100)	Intradermal	2 doses (d, d + 14)	C57BL/6	[46]
PS	20; 40*; 50*; 70, 90, 100, 120	OVA (50)	Intradermal in hind footpad	2 doses, (d, d + 14)	C57BL/6 OTI/OTII OVA transgenic	[48]
	50	G88 and M2.1 RSV proteins (40)	Intranasal	2 doses, (d, d + 14)	BALB/c	
PLA	50 k–150 k; 10 k–70 k; 2 k–8 k*; 0 k–2 k	TT (13)	Intramuscular, Alum 50 ng	Single dose	Wistar Rat	[49]
PLGA	2 k; 5 k*; 12 k	HBsAg (10)	Pulmonary (Inhalation)	1 dose	Sprague-Dawly rat	[52]
PGA-co-PDL	300 nm	rPspA4Pro	Inhalation via nasal delivery	2 doses (d, d + 14)	BALB/c	[58]

*indicates the size within the studied panel with optimal immune response. Materials acronyms: PS (Polystyrene) PPS (Polypropylene Sulfide) PLA (Polylactic acid) PLGA [Poly(lactic/glyotic) acid], PGA-co-PDL [Poly(glycerol adipate)-co-pentadecalactone]. Antigens acronyms: Ovalbumin (OVA), Bovine Serum Albumin (BSA), Bacillus anthrasis Protective Agent (BAPA), Recombinant pneumococcal surface protein A (rPspA4Pro), Respiratory Virus (RSV), Hepatitis B surface Antigen (HBsAg)

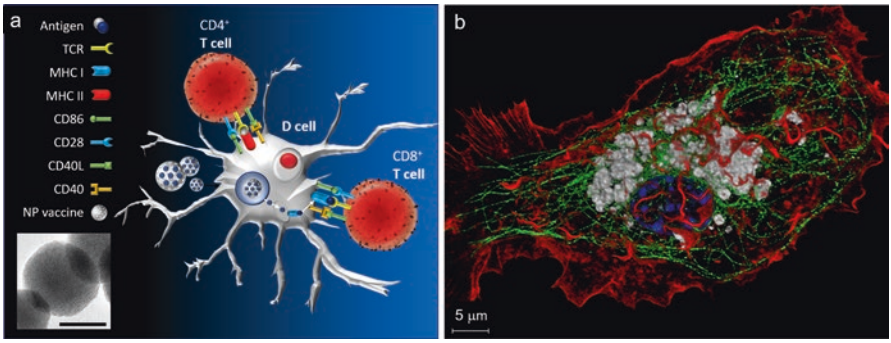

Fig. 3 Nano immunotherapy. (**a**) Artistic rendition of a DC interacting with two T cells following internalization of a nanoparticle vaccine (image by Jonas Croissant). Inset: transmission electron microscopy image of mesoporous silica nanoparticles (scale bar: 100 nm). (**b**) Confocal micrograph of a mouse DC following internalization of immunogenic lipid coated mesoporous silica nanoparticles (ILM: white, actin: red, microtubules: green, nuclei blue; image by Karen Sanchez)

bic balance, which can be challenging to tune for certain particle types (e.g. polymers) without extensive synthetic modifications [55]. The choice of route is determined by the desired target region, which may be preferentially accessible thorough lungs, gastrointestinal tract, brain, or central nervous system. Some of the routes of administration for mucosal delivery of nanotherapeutics are nasal and oral and are discussed below. We also address the challenges that face nanoparticle delivery in biological environments by chemical and materials nanoengineering.

3.1 Barriers to Nasal Delivery

Within the facet of nasal delivery, there are a wide variety of therapeutics and nanoparticle frameworks already available. Roux and colleagues [56] presented a potential nasal vaccine for respiratory syncytial virus (RSV). At the time of the article's publication in 2008, there was little discussion of using the nucleoprotein (N) from the RSV nucleocapsid as an antigen, making the team pioneers in the use of this antigen for intranasal delivery of the vaccine. They created a nanoparticle composed of a homogeneous ring of 10–11 N subunits enclosing bacterial RNA. Intranasal immunization of adult mice with the nanoparticles adjuvanted with detoxified bacterial endotoxin protected mice against RSV challenge.

Further, Yu and colleagues [57] engineered an RSV vaccine targeting an engineered G glycoprotein using an adenovirus nanoparticle platform (rAd/3xG). Strong mucosal IgA responses were elicited in the mice following a single intranasal immunization, but not following intramuscular or oral administrations. Interestingly, Th-1 and Th-2 $CD4^+$ T cell responses (IFN-γ and IL-4, respectively) were lacking following rAd/3xG vaccination, but restored following priming with vaccinia virus expressing RSV G. Other reports of intranasal nanoparticle delivery systems include poly(glycerol adipate-co-

omega-pentadecalactone) (PGA-co-PDL) polymeric nanoparticles loaded with a Pneumococcal surface protein A [58, 59] and mucoadhesive chitosan nanoparticles for delivery of inactivated influenza A virus [60]. While the data support the use of intranasal delivery of therapeutics, it also indicates opportunities for optimization of vaccine platforms to achieve diverse immunogenic responses.

3.2 Barriers to Oral Delivery

There is a large array of strategies being utilized for overcoming barriers facing oral delivery of nanoparticles. An example is a series of amphiphilic cetirizine-chitosan polymer (CTZ-CSs) cetirizine dihydrochloride (CedH) nanoparticles [61], which displayed both burst and sustained drug release profiles in the presence of lysozyme (cell-free). CedH showed a burst release during the first 6 h, after which the release rate slowed significantly and was sustained for 72 h. *Ex vivo* mucosal adhesion of CedH-CTZ-CS nanoparticles supported the potential for prolonged residence time for nanoparticles in the small intestinal mucosa. This strategy involves trafficking of nanoparticles through the digestive system while avoiding exposure of the drug to the stomach's acidic environment through encapsulation, retaining molecular integrity, and remaining unidentified as foreign material based on nature of the polymer throughout the journey. Their conclusion were that oral delivery has the potential to deliver drug without compromising their chemical integrity. In addition, the authors stated that timed release has the potential to deliver a drug/antigen over an extended period of time. Researchers have also used chitosan and PLGA based nanoparticles to effectively cross the epithelial layer of the intestinal epithelium [62, 63]. Mucosal immune cells, located in the basolateral domain of the intestinal mucosa [64], are able to internalize nanoparticles and initiate antigen-specific T cell responses in the body, supporting the potential use of orally-delivered nanoparticles to initiate T cell responses by targeting localized DCs.

3.3 Perspectives and Challenges

There are many challenges in designing nanoparticle delivery systems for mucosal delivery. At the forefront, the human body is extremely efficient when it comes to eliminating foreign materials from the body [65]. These challenges vary depending on the delivery route. Some of the challenges facing oral delivery include stability of the nanoparticles in the polarizing environments that exist throughout the human GI tract. Further, the GI contains a mucosal layer that is able to eliminate larger particles as part of the biological defense system. These barriers emphasize the importance of optimizing nanoparticle properties such as viscoelasticity, thickness, density and turnover time [65]. Ongoing research is aimed at manipulating nanoparticles and their loaded cargo to obtain the desired therapeutic effects.

4 Immunogenic Cell-Death Inducing Chemotherapeutics: Contribution of Nanoparticles

4.1 Immunogenic Cell Death (ICD)

Chemotherapeutic drugs have played a major role in the treatment of various types of cancer, yet the mechanisms of their inner workings are still being discovered. Historically, anti-cancer drugs have been used to elicit cytotoxic responses, such as DNA damage leading to apoptosis and inhibition of tumor growth. More recently, the ability of chemotherapeutic drugs to activate immune responses has surfaced, with a focus on their ability to trigger immunogenic cell death (ICD). The utilization of this immune response has led to the inception of chemotherapeutics as "anticancer vaccines [66].

During ICD, dying cancer cells transmit danger signals that activate the immune system. These signals are outlined by the emergence of danger associated molecular pattern (DAMP). These signals consist of three hallmarks: (1) the migration of calreticulin (CRT) from the endoplasmic reticulum to the surface of the cell, (2) release of ATP and (3) the dislocation of the high mobility post-apoptotic box 1 (HMGB-1). The first hallmark occurs soon after treatment and precedes other indications of apoptosis [67]. The presence of CRT is also accompanied by another corresponding disulfide isomerase protein, ERp57, without which, the translocation of calreticulin is not observed [68]. CRT functions as a beacon, enticing macrophages to consume the dying cells. Along with CRT, the presence of another "eat-me" signal is generated during early apoptosis, phospholipid phosphatidylserine (PS) [69]. In contrast to CRT, PS is believed to have immunosuppressive effects. For example, transforming growth factor beta (TGF-β), essential for the establishment of anti-inflammatory responses, is only observed in the presence of PS [70]. Therefore, PS acts to stimulate the removal of apoptotic cells without eliciting proinflammatory immune responses. In addition to "eat-me" signals, another essential event in ICD is the production of ATP, which serves as a "find-me" signal to attract DCs. Furthermore, ATP is a ligand for P2RX7 purinergic receptors, leading to secretion of the inflammatory cytokine interleukin-1 beta (IL-1β) [71]. The pro-inflammatory immunogenic signals are essential for the immune efficacy of chemotherapeutics. The final requirement for ICD involves secretion of HMGB-1 which is also another immunogenic molecule that is a ligand for Toll-like receptor-4 (TLR-4) on APCs [71].

4.2 Nanoparticles and ICD

Although, immunogenic chemotherapeutics under their free form are theoretically sufficient to produce an ICD, reported works show how the encapsulation of drugs within nanoparticles drastically increases their efficacy. In the following section, we present some examples for oxaliplatin, doxorubicin and paclitaxel which nanoplatforms made significant improvements in their ICD-inducing effect.

Oxaliplatin (OXA) The Nie group [72] created diblock copolymer PLGA-mPEG nanoparticles loaded with oxaliplatin (OXA) or gemcitabine (GEM) which are respectively immunologically active and silent agents. Both free OXA and OXA-loaded NPs showed a significantly higher release of ATP, CRT and HMGB-1 than free and NP-loaded GEM for both Panc-1 and Pan02 human and murine pancreatic cell lines. Additionally, it was demonstrated that the encapsulation within NPs improved the ability of OXA to induce DAMPs exposure as well as immune responses of dendritic cells and T-cells (measured by amount of expressed CD80, CD83, and INF-γ). In vivo studies on immunocompetent C57BL/6 mice showed that, free and encapsulated OXA resulted in 20 to 60% mice survival 60 days after challenge whereas all mice were dead before 10 days when treated with free GEM, free NP or encapsulated GEM. These results indicate that NP encapsulation of ICD-inducing drugs enhances the immune response in terms of APC maturation, T-cell activation and tumor infiltration. The authors confirmed the same trend by comparing ICD-inducer doxorubicin and immunologically silent 5-fluoruracil. The groups of Nel and Meng [73] recently reported the possibility to induce immunogenic cell death (ICD) by using "Immunogenic LCMSN" on pancreatic ductal adenocarcinoma (PDAC). Those I-LCMSNs being loaded with ICD-inducing agent oxaliplatin (OX) and by engineering the lipid bilayer coating to incorporate indoximod moieties that interfere with the immunosuppressive indoleamine dioxygenase overexpressed on PDAC site. KPC cells-implanted pancreas in B6/129 mice were IV-injected by free or ILCMSN-coloaded drugs (5 mg/Kg OX; 50 mg/Kg IND, corresponding to about 110 mg/Kg ILCMSN). Mature dendritic cells were enhanced to 32% in the case dual-loaded nanocarrier while remains below 18% in all other treatments. This induced significantly higher tumor shrinkage (up to 8 times) than free or liposome-loaded drugs and at least twice than OX-loaded LCMSN (w/o IND) outlining the synergistic (somehow unexpected) effect of IND on ICD when used along with OX.

Doxorubicin (DOX) The group of Zhang [74] used the concept of a nanomachine [75–79] to load DOX into integrated MSNs (IMSNs) while using a double pH- and redox-sensitive β-cylodextrine (β-CD)/rotaxane gatekeeper that will prevent DOX leakage before endosomal internalization. After IV injection of DOX-free or -containing samples into 4-T1 tumor-bearing Balb/C mice ([DOX] = 5 mg/kg), the tumor volume decreased 5 to 2 times compared to free DOX and DOX@MSNs. The authors showed an increase in spleen, tumor and serum interleukin-1β (IL-1β), IL-12P40, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), CD4, CD8, and CD86 levels was also observed after DOX@IMSN treatment, when compared with other control groups including free DOX or gate-keeper-free MSNs consistent with that encapsulation of DOX into IMSN increased the ICD. More recently, the group of Moon [79] reported ~10 nm sHDL nanodiscs coated with pH-sensitive DPPC-DOX conjugate that successfully showed higher release of CRT and HMGB-1 compared to free DOX.(4 mg/kg) in colon carcinoma cells CT26 and MC38 in BALB/C and C57BL/6 subcutaneously injected mice. It is noteworthy that the reported DOX nanodiscs enhanced the efficiency of antiPD-1 alone or with free DOX by dramatically increasing the survival rate from 13% up to 90% r in BALB/C mice.

Paclitaxel (PAX) Reports about PAX as ICD-inducing drug are relatively earlier than other known drugs such as OXA and DOX. Bhaskar group [80, 81] [82]created PLGA nanoparticles containing Paclitaxel (PAX) and the non-toxic fragment of the adjuvant LPS (P-LPS) as a TLR-4 ligand. APC and T-cell activation and infiltration into tumor was proven to be higher on the PAX@PLGA nanoparticles rather than free PAX or PLGA alone. The same group also reported a micelle-like conjugate of paclitaxel and SP-LPS (a similar fragment to L-LPS) that showed higher activated immune cells within the tumor microenvironment than liposomal PAX (Taxol). Lim's group [82] reported a system for treatment of B16-F10 melanoma cells, in fact they co-loaded PAX and imiquimod (TLR-7 agonist) within polyglutamic acid nanoparticles and those were intratumorally injected (4 doses) into C57BL/6 (H-2b) mice previously inoculated with B16-F10 cells. This system induced enhancement of up to 250% proliferation of dendritic cells as well as Th1 cytokines. *In vivo* results showed 70% mice survival after 41 days whereas all mice from all other groups were dead.

4.3 Perspectives and Challenges

In summary, chemotherapeutic drugs have the potential to induce a dual therapeutic effect, death of highly replicating cancer cells and stimulation of anti-cancer immune responses. Nanoparticles provide an opportunity to concentrate and protect drugs and enhance their pharmacokinetics. Combination therapy is also made possible by nanoparticles where emerging complex systems induce photothermal and photodynamic immunotherapy in addition to conventional chemotherapy. Co-delivery of chemotherapeutics and adjuvants, help alleviating immune suppression in the tumor microenvironment and enhancing the immunogenicity of chemotherapeutics. Because the use of ICD-inducing chemotherapeutics may require numerous experiments and time-consuming characterization and data analysis, researchers encounter difficulties to find one optimal drug/nanoparticle complex as all the reported studies were carried out independently and a comparison on the same basis is not possible. In any case, we are undoubtedly witnessing the first advances in a field with tremendous potential for cancer therapy which will be full of enthusiastic discussions in the next years.

5 Nanoparticle Vaccines in Clinical Trials

In this section, nanoparticle vaccines currently in clinical trials will be discussed and considered in relation to the various diseases they attempt to cure, namely respiratory syncytial virus (RSV), Influenza viral infections, Ebola virus disease (EVD), and cancer.

5.1 Nanoparticle Vaccines Against Infectious Diseases (RSV, HPV, HIV)

RSV infection is the leading cause of hospitalization of infants under one-year-old in the United States and is the second leading cause of infant mortality worldwide after malaria. To date, no vaccine technology is FDA approved for RSV infections. Commercialized prophylactic therapeutics include the neutralizing monoclonal antibody (mAb) palivizumab. Preclinical trials of nanoparticle vaccine technologies abound in the literature. Agilvax, Inc., a biotechnology company located in New Mexico in the United States, is for instance developing an immunotherapy vaccine nanoplatform that supports high immunogenicity to both foreign and self-antigens. Their VLP vaccine candidate is cost-effective by virtue of using a bacteriophage MS2 virus-like particles [83]. The candidate enables the recovery and amplification of affinity-selected sequences from vast libraries (through a process similar to phage display) by using a single technology, hence accelerating significantly the identification and development of novel vaccines. One preclinical study involved AX14, which targets pre-fusion F-protein identified via VLP affinity selection. Mice immunized with AX14 generated an immune response that effectively neutralized RSV infection *in vitro, and in vivo* tests are underway. The company is also working towards clinical trials with a universal virus-like particle-based (~25 nm in diameter) vaccine to combat human papillomavirus (HPV) infection, which is estimated to cause 5% of human cancers worldwide [84–86].

All nanoparticle vaccine clinical trials currently under examination in the United States were developed by Novavax, Inc., a clinical-stage biotechnology company headquartered in Maryland [88]. The public company trades under the symbol NVAX and aims to commercialize products to prevent a broad range of infectious diseases based on recombinant nanoparticle vaccine technologies. The goal of the clinical trials is to treat patients suffering from RSV (using RSV-F Vaccines) [89–92], seasonal influenza (using NanoFlu) [93–98], and Ebola virus (EBOV) [87, 99]. To achieve this goal, the company constructed nanosized vaccines via assembly of recombinant protein in star-like nanostructures (Fig. 4). The Company partners with the Bill & Melinda Gates Foundation, the US Biomedical Advanced Research and Development Authority (BARDA), LG Life Sciences, and has a joint venture with Cadila Pharmaceuticals. Table 2 summaries all the available clinical trials on the clinicaltrials.gov database using the keywords “nanoparticle” and “vaccine” (Accessed May 2019, novavax.com) [121– 127].

Novavax has an extensive publication record substantiating the science behind the clinical trials and communicating the data to the public [90, 91, 93, 99–102]. All the reported clinical trials have been performed using immunogenic nanoparticles based on fusion (F) proteins via the IM administration route . The studies enrolled participant ranges for phase 1 (32 to 230), phase 2 (50 to 1330), and phase 3 (8618) and mostly concerning RSV Infections. Although in September 2016, the stocks of company collapsed after the failure of Phase III RSV F vaccine, the company is still actively advancing on other RSV clinical trials. Figure 5 summarizes the pipeline of

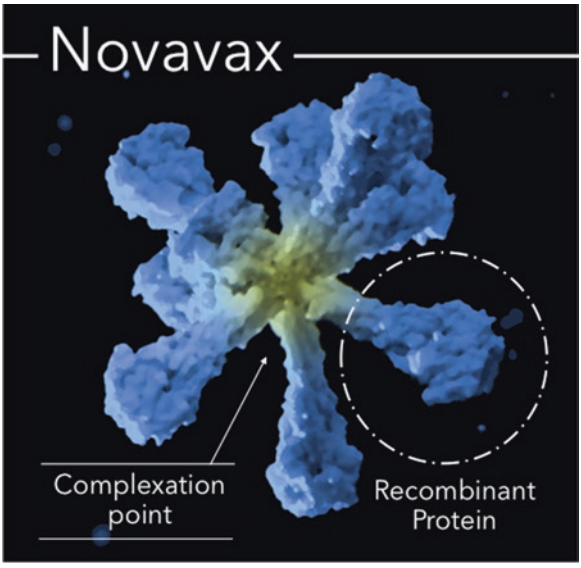

Fig. 4 Artistic rendition of Novavax nanoparticle vaccine based on negative staining electron microscopy — Hahn et al. [87]

all clinical trials revealed by the Novavax's website, as of June 2018. The company developed adjuvant formulations that enable the vaccine to induce potent immune responses that include enhanced production of antibodies and longer lasting protection against infections caused by various bacteria and viruses. Matrix™ and saponin-based Matrix-M™ proprietary adjuvants are also being used in the clinical trials.

5.2 Influenza Nanoparticle Vaccines

Novavax Inc. is currently using NanoFlu™ to treat seasonal influenza in humans [93–98]. The phase 1/2 clinical trials include randomized and observer-blinded studies enrolling three groups of 110 patients who received IM administration of either one of two dose levels of NanoFlu™ nanoparticle vaccines subjects or Fluzone HD controls. Trial follow-ups were to last one full year and are still continuing as of June 2018.

In another study, completed in 2014, RSV-F vaccine and influenza vaccine were co-administrated to elderly people [103]. This phase I randomized, observer-blinded, and dose-ranging clinical trial aims to assess the immunogenic therapeutic efficacy RSV-F protein nanoparticle vaccine. The trials were conducted with or without aluminum adjuvants injected in combination with the inactivated influenza vaccines. The study enrolled 220 patients in elderly populations stratified by two age categories: 60 to <75 years and ≥ 75 years. On day zero, immunizations were performed with single IM dose injections of placebos or RSV-F protein nanoparticle vaccines, with concurrent IM immunization with an inactivated pathogen vaccine. On the 28th day, rescue doses of the licensed seasonal trivalent inactivated influenza

Table 2 Current US clinical trials on nanoparticle vaccine candidates

Ref	Phase	Disease type	Treatment type	Dosing time and number	Injection type	Particle type	Patients enrolled	Start/End dates
[121]	2	Respiratory Syncytial Virus (RSV)	RSV-F Vaccine	RSV-F Vaccine phosphate buffer placebo	IM	F Protein	1330	Oct 2015/ Nov 2016
[122]	1	Ebola	EBOV GP Vaccine	Base Dose EBOV GP Vaccine 2x Base Dose EBOV GP Vaccine 4x Base Dose EBOV GP Vaccine 8x Base Dose EBOV GP Vaccine Placebo Matrix-M Adjuvant	IM	Glyco-protein	230	Feb 2015/ Apr 2016
[123]	1/2	Influenza	NanoFlu	NanoFlu Fluzone HD - Day 0 Fluzone HD - Day 21 Saline - Day 21	IM	F Protein	330	Sep 2017/ Mar 2018
[124]	1	Respiratory Synctial Virus	RSV F vaccine	RSV F Vaccine with adjuvant RSV F Vaccine Hepatitis A Vaccine Placebo	IM	F Protein	32	Nov 2014/ Apr 2016
[125]	3	Respiratory Syncytial Virus Infections	RSV F vaccine	RSV F vaccine with adjuvant Formulation buffer	IM	F Protein	8618	Dec 2015/ Jun 2018
[103]	1	Respiratory Syncytial Virus (RSV)	RSV F vaccine	Low dose RSV-F Vaccine with Adjuvant (Day 0); Seasonal TIV (Day 0 & Day 28) Low dose RSV-F Vaccine without Adjuvant (Day 0); Seasonal TIV (Day 0 & day 28) High dose RSV-F Vaccine with Adjuvant (Day 0); Seasonal TIV (Day 0 & Day 28) High dose RSV-F Vaccine Without Adjuvant (Day 0); Seasonal TIV (Day 0 & Day 28) Placebo (Day 0 & Day 28); Seasonal TIV (Day 0)	IM	F Protein	220	Oct 2012/ Mar 2014

[126]	2	Respiratory Syncytial Virus Infections	RSV-F protein nanoparticle vaccine	Drug: Saline Placebo (0.5 mL injection) Drug: RSV F vaccine (0.5 mL injection)	IM	Protein	50	Sep 2014/ May 2017
[127]	2	Respiratory Syncytial Virus (RSV)	RSV-F protein nanoparticle vaccine	Low dose RSV-F Vaccine with Adjuvant Low dose RSV-F Vaccine Without Adjuvant High dose RSV-F Vaccine with Adjuvant High dose RSV-F Vaccine without Adjuvant Low dose RSV-F Vaccine with Adjuvant [Bedside Mixing] Placebo	IM	Protein	330	Oct 2012/ May 2013

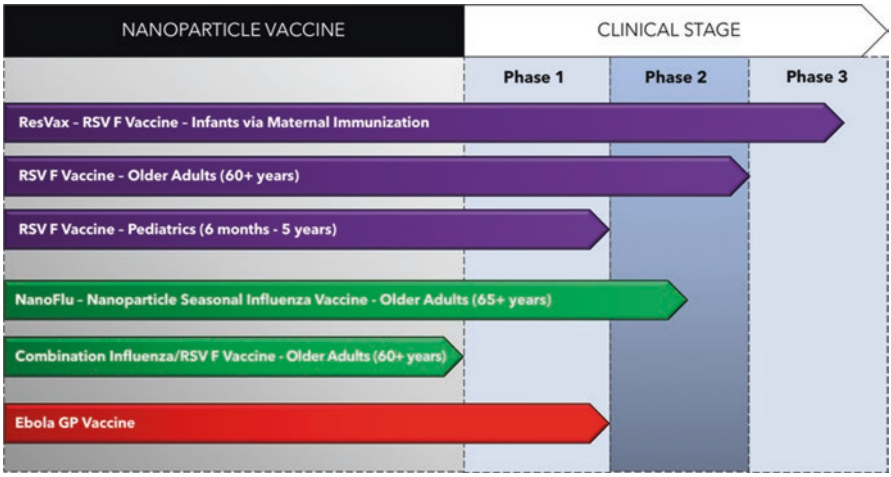

Fig. 5 Clinical trial stages of various Novavax nanoparticle-based vaccine technologies

vaccine were injected into healthy volunteers, with the placebo patient group receiving saline injections. Maximal anti-F IgG antibody titers were attained within 28 or 56 days' post-vaccination for adjuvanted and unadjuvanted treatments, respectively. The immune responses (IgG) persisted for 12 months after the vaccination [89].

Novavax also recently reported improved titers against influenza drift variants using an adjuvanted recombinant hemagglutinin trivalent nanoparticle vaccine [98]. In this clinical trial, trivalent nanoparticle influenza vaccine induced significantly higher hemagglutinin inhibition efficacies than the high-dose trivalent Fluzone vaccine against the A/Singapore strain. The possible implications of this work include more effective and long-lasting influenza vaccinations through avoiding the mismatch resulting from egg adaptive mutations.

5.3 Ebola Nanoparticle Vaccines

The Ebola virus (EBOV) exploded in West and Central Africa a few years ago without effective medical responses. Research efforts to combat the disease are still ongoing [104]. Novavax began clinical trials in February 2015 using its EBOV glycoprotein (GP) vaccine [87, 99]. The EBOV GP gene was cloned into a baculovirus vector and recombinant protein was produced in Sf9 insect cells. The resulting glycosylated trimmers formed spherical 30–40 nm particles. In mice [99], EBOV GP injected with the saponin adjuvant Matrix-M was significantly more immunogenic (based on virus neutralization titers and anti-EBOV GP IgG) compared to immunization with EBOV GP with alum or no adjuvant. Further, immunization of mice with EBOV GP with Matrix-M was 100% protective when mice were challenged

with the lethal virus. In support of the use of saponin-based adjuvants in vaccine, no protection was observed with the alum adjuvant and only 10% o mice were protected in the EBOV/Mak GP antigen alone group.

Results from the above Phase 1 clinical trial of the EBOV GP vaccine was presented at the World Health Organization (WHO) fifth Teleconference on Ebola vaccine trial. The dose escalation immunogenicity and safety trial included approximately 230 healthy subjects between the ages of 18 to 49. Immunizations included one or two IM doses of 6.5 to 50 μg antigen into the deltoid muscle in alternate arms. Adjuvanted EBOV GP was highly immunogenic at all dose levels. Single and two-dose regimens induced a 21–27 and 500–750-fold increase in antibody titer levels over baseline on day 35 after immunization. Local and systemic reactions were mild to moderate, and only slightly higher in the adjuvant group. Overall the clinical data indicates that the EBOV GP nanoparticle vaccine will be protective in humans.

5.4 Cancer Nanoparticle Vaccines

A wide variety of translational nanoparticle vaccines have been investigated by clinical trials to treat various cancers at a variety of stages [88, 105]. Liposomes have been used extensively due to their multifunctionality and biocompatibility [106–109]. Grippin and co-workers recently reviewed the subject in an excellent work chronicling nanoparticle-based cancer vaccines for antigen delivery used in human clinical studies [105], including Tecemotide™ liposomes [110], AS15™ lipids [111], DepoVax™ liposomes [112, 113], Cholesteryl pullulan (CHP) nanogels [114, 115], ISCOMATRIX™ liposomes [116], virus-like nanoparticles [117], OncoVAX-id/IL-2 liposomes [118], Lipovaxin™ liposomes [119], and Lipo-MERIT™ liposomes [120]. Two of the above examples will be discussed hereafter.

Tecemotide™ is a nanoliposomal cancer vaccine comprised of a mixture of cholesterol, dimyristoyl phosphatidylglycerol (DMPG), and dipalmitoyl phosphatidylcholine (DPPC) and loaded with MUC1 glycoproteins. The diameters of the liposomal vesicles ranged from 150 to 580 nm. The delivery of MUC1 glycoproteins was performed as they are overexpressed on the apical surfaces of epithelia in many mucosal cancers. In combination of immunostimulatory lipid BLP25 and TLR4 agonist MPL, the delivery induced a shift toward Th1 polarization and $CD8^+$ T-cell response [105]. However, phases II and III trials did not succeed in generating sufficient survival benefits for cancer patients except for patients with stage IIIB locoregional disease and patients treated concurrently with cyclophosphamide. Tecemotide™ is now being investigated in multinational phase III trials of colorectal cancers [105].

CHP nanogels containing cholesteryl pullulan complexed with truncated HER2 protein 1–146 were created to target the HER2 antigen [114]. The clinical trial aimed to evaluate the safety of the nanoparticle vaccine and HER2-specific T-cell immune responses, which were determined by an enzyme-linked immunospot assay

with mRNA-transduced phytohemagglutinin-stimulated $CD4^+$ T cells in HLA-A2402-positive patients with therapy-refractory HER2-expressing cancers. The study demonstrated the safe use of the vaccine as well as the induction of HER2-specific $CD8^+$ and/or $CD4^+$ T cell immune responses [114].

6 Conclusions and Perspectives

Preclinical and clinical studies support better vaccine efficacy in the presence of adjuvants. Benefits of nanoparticle vaccines include the ability to package adjuvant and antigen in the same construct and achieve potent, multivalent presentation of molecules to APCs. Here we have shown that the development of therapeutic cancer vaccines may benefit from the use of chemotherapeutics as adjuvants through induction of immunogenic cell death. The ability to co-load chemotherapeutics and other adjuvants into nanoparticles may enhance ICD-induced anti-cancer immune responses. Advantages of nanoparticle vaccines also include the ability to tailor nanoparticles for optimal performance based on cargo, route of administration and desired tissue targeting. While nanoparticle size clearly has a strong impact on nanoparticle efficacy for eliciting immune responses, other physico-chemical traits, such as shape, surface potential, and particle degradation/cargo release kinetics also impact therapeutic efficacy. While early nanoparticle vaccine clinical studies are promising, thoughtful consideration of the mechanisms underlying vaccine efficacy when delivered using nanoparticles will benefit the development of future vaccine nanosystems.

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Part III
Host Interactions with Nanoparticles

Engineered Nanomaterial Interaction with Epithelial and Immune Cells upon Mucosal Drug Delivery

Valerie C. Minarchick and Jared M. Brown

Abstract The mucosal membranes (i.e. oral, and nasal cavities, gut and female reproductive tract) throughout the body are ideal locations for drug delivery as they result in rapid uptake of the pharmaceutical into the blood system; however, these membranes are protected by the mucosal immune system (MIS). This intricate network of epithelial cells, microbiota, immune cells and mucus present a unique challenge for drug delivery due to their barrier functions and responses to foreign pathogens. There is a constant need to improve drug delivery methods, particularly in regard to mucosal drug delivery. The application of engineered nanomaterials (ENM), due to their unique physicochemical properties, have significant potential to improve mucosal drug delivery. This chapter will discuss key physicochemical properties (i.e. size, shape, surface functionalization, and solubility) and how modification of these properties can alter the biological impact of ENMs. Finally, a brief overview of the normal function of key MIS cells (i.e. epithelial cells, goblet cells, Paneth cells, dendritic cells, macrophages, T and B lymphocytes, mast cells, and endothelial cells) and how ENM exposure alters their function will be presented.

Keywords Mucosal immunity · Engineered nanomaterials · Drug delivery Physicochemical properties · Epithelial cells · Macrophages · Mast cells

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1 Introduction

Engineered nanomaterials (ENMs) hold great potential for the improvement of many biomedical applications. These applications range from refining diagnostic imaging, drug delivery, and even use as therapeutics. However, in order for these biomedical advances to be fully realized the toxicity and immunological alterations associated with them needs to be understood.

By definition, ENMs are less than 100 nm in at least one dimension, manufactured with a specific intent, and are designed to take advantage of unique physicochemical properties (i.e. size, shape, charge, surface area) that are different from their larger counterparts [1]. The ability to modify these properties for specific needs/applications is one of the reasons ENMs are being investigated for improved drug delivery. ENMs have been shown to cross cellular barriers (i.e. blood-brain and epithelial-endothelial cell barriers); can be directed to specific cell types or tissues within the body. Depending on the intended function of the ENM (i.e. drug delivery), these interactions could be beneficial; however, if these cellular disruptions are unintended the results could lead to increased and unwanted toxicity.

In basic terms, the mucosal immune system (MIS) is made up of an intricate network of immune cells, epithelial cells, mucus, and microbiota that aid in the protection of mucosal membranes (i.e. oral, and nasal cavities, gut, and female reproductive tract) from foreign pathogens [2]. Due to their protective function, the MIS also results in complications when developing drug delivery systems. Currently, speculation exists that ENMs may help overcome some of the barriers and challenges associated with mucosal drug delivery. However, to date, there is very limited research on the toxicity and health outcomes associated with mucosal drug delivery using ENMs.

Therefore, the objective of this chapter will be two-fold. First, a few of the major physicochemical properties of ENMs will be addressed and how changes in these properties may affect mucosal drug delivery. Second, ENM interactions with epithelial and the major immune cells of the MIS and their common toxicological/biological outcomes will be discussed.

2 Engineered Nanomaterials and Drug Delivery

The development of mucosal drug delivery is hindered by a myriad of factors, including physical barrier challenges. The mucosal immune system is uniquely designed to keep foreign xenobiotics out of the body, which includes pharmaceutical agents. Although ENMs are a xenobiotic, they also have great promise to improve the efficacy and efficiency of mucosal drug delivery. This is largely due to the ability of altering ENMs physicochemical properties (Fig. 1). The alteration of these properties may aid ENMs in avoiding interaction with physical barriers

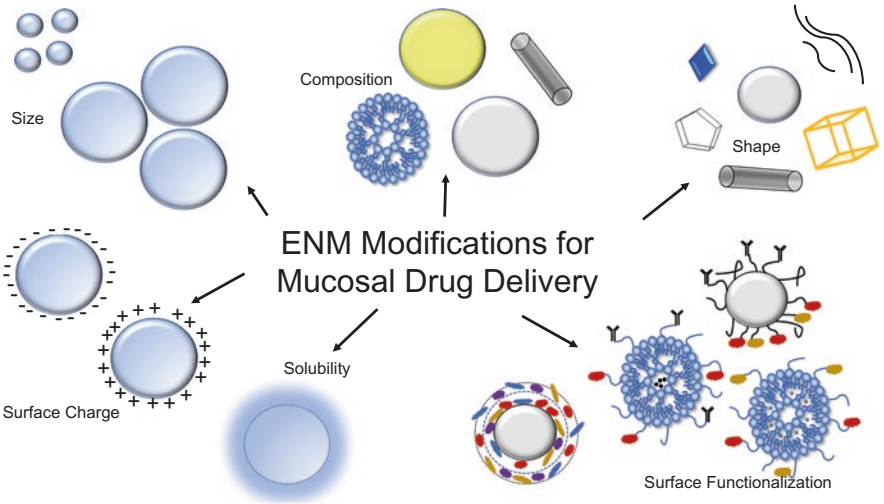

Fig. 1 An overview of some of the physicochemcial properties of engineered nanomaterials (ENMs). These modifications include size, composition (i.e. elemental, oxides, and phospholipids), shape, surface charge, solubility, and functionalization (i.e. protein corona, proteins, pharmaceutical drugs, antibodies, etc.)

(i.e. microbes, mucus, and surfactant), crossing the epithelial cell layer, and evading immune responses.

The modification of physicochemical properties is why ENMs hold so much potential for drug delivery [3]. ENMs are developed using a variety of compounds (i.e. lipids, carbon, metals, polymers etc.). Each material has its own unique drawbacks and advantages that make them appealing for drug delivery applications. Currently, lipids are increasing in popularity for drug delivery applications. Lipids are used to create hollow spheres called liposomes [4]. Liposomes are of great interest because theoretically they are biologically compatible, can be used to encapsulate various pharmaceutical agents including hydrophilic and hydrophobic compounds, and can be coated with other excipients (i.e. citrate, polyethylene glycol) to improve their circulation in vivo [5].

Other ENMs are less biologically compatible but have novel effects in vivo. Cerium dioxide nanoparticles have been shown to act as an anti-oxidant and may be developed to treat illnesses associated with high levels of oxidative stress (i.e. hypertension, stroke) [6, 7]. Finally, iron oxide nanoparticles have been investigated for years due to their magnetic properties [8]. By taking advantage of their magnetic properties, the nanoparticles can be directed to specific tissues and improve imaging compared to current magnetic resonance imaging [8]. Based on these few examples, it is clear that ENMs are being used to deliver drugs in a variety of ways. The following section will further highlight how modification of various physicochemical properties can enable and potentially enhance ENMs use for drug delivery.

2.1 Physicochemical Properties

The modulation of physicochemical properties (i.e. size, shape, charge, etc.) can have a huge impact on ENMs' in vivo behavior and toxicity (Fig. 1). It should be noted that the physicochemical properties described in this chapter are only an example of the many potential alterations that can be achieved with ENM manufacturing.

2.1.1 Size and Shape

Two of the most basic parameters that are modified during ENM drug development are size and shape. The size of the ENM used for mucosal drug delivery is an important parameter that needs consideration during development. Current research indicates that larger ENMs may be less toxic than their smaller counterparts; however, there is likely a need for smaller ENM in order to overcome the physical barrier function of epithelial cells [3]. The toxicity of silica nanoparticles has been assessed with a variety of sizes (20–200 nm), where 60 nm had the highest level of endocytosis as well as toxicity [9]. Although the ideal ENM size may vary depending on application, it is reasonable to speculate that smaller nanoparticles (~60 nm) would be engulfed by immune cells easier but also have a higher toxicity and risk for inflammation. This toxicity paradigm is important to consider for drug delivery with ENMs since the ultimate goal is for the ENM to reach the site of action within the body, while avoiding high levels of toxicity.

ENMs are also developed in variety of shapes. Different ENM shapes have been shown to be more biologically compatible (i.e. spheres, cubical) compared to other types (i.e. rods and wires) [3]. Several studies have shown that rod shaped ENMs penetrate cells, like macrophages, instead of being engulf and digested [10]; this penetration led to increased inflammation and fibrosis [10]. Furthermore, if these high aspect ratio ENMs penetrate epithelial cells they may cause the cells to become necrotic and release alarmins thus activating the MIS (see **Epithelial Cells**) [1]. Ideally, this response needs to be avoided in order for ENM based mucosal drug delivery to be successful. Therefore, ENMs with lower aspect ratios, like spheres, are more biologically compatible and may be better suited for mucosal drug delivery.

Although spheres are likely the most common ENM shape used for drug delivery, other shapes can be used for specific applications but these alternate shapes may present toxicity issues. ENMs made of carbon are developed in a variety of shapes, including rods, spheres, cubes and sheets, which impact toxicity. Carbon nanocubes were unable to be engulfed by cells and caused significant damage to the intestinal epithelial cell layer [11]. Whereas the spheres and tubes, while still causing epithelial damage and autophagy, was less severe than the carbon nanocubes [11].

For mucosal drug delivery, the size and shape of the ENM is even more critical. Adhesion molecules and tight junctions are essential for cell-cell communication

and optimal epithelial cell function [12]. In order for mucosal drug delivery with ENMs to be successful, they need to bypass the epithelial barrier without damaging the cells. Experimentally, ENMs have been shown in vitro to disrupt epithelial cell function; however, the size and shape of the ENM is likely to play a critical role in this disruption [11, 13]. Furthermore, the epithelial cell barrier of the MIS may be overcome by taking advantage of endogenous transport mechanisms via either passive or active transport [14]. For active transport, the drug needs to cross the epithelial cell barrier either using carrier-mediated transport or trans-epithelial phagocytosis [14]. Therefore, it is reasonable to speculate that the size and shape of an ENM may be exploited to take advantage of active transport. By taking advantage of biological processes to introduce ENMs to the body, restricted epithelial cell damage and distress signaling would occur, which in turn would result in limited immune cell activation.

2.1.2 Surface Functionalization

One of the main advantages of using ENMs for mucosal drug delivery is the ability to attach various compounds, including the drug of interest or targeting ligands, to the surface of the ENM. These surface modifications in theory would increase biocompatibility and allow the ENM to be targeted to a specific cell type. Hypothetically, this will decrease the amount of drug needed to treat a disease and improve bioavailability. These surface modifications are particularly appealing for the diagnosis and treatment of cancer. One advantage of using liposomes for cancer drug delivery is that they can be used to increase intracellular uptake and can be modified in variety of ways [15]. For example, chemotherapy drugs are encapsulated in liposomes, and the outer surface of the liposome can be modified with targeting peptides [15]. Taken together, these attachments help decrease the off-target toxicity associated with chemotherapy [15].

The addition of polymers to the surface of ENM can also help solve some common complications (i.e. protein corona, agglomeration) that are often encountered when used for drug delivery. When ENMs are exposed to biological environments they attract macromolecules (i.e. proteins, lipids, peptides) due to a variety of thermodynamic principles such as hydrogen bonding, van der Waals forces, and hydrophobic interactions [16]. The development of this protein corona has been shown to have a profound effect of the toxicity of the ENM often resulting in decreased levels of circulating pro-inflammatory cytokines, but may also effect the efficacy of the ENM when used for drug delivery [16, 17]. There is evidence that this ENM complication can be avoided by attaching “stealth” polymers [i.e. polyethylene glycol (PEG)], to help ensure the ENM does not interact with other proteins [16]. Another example is the addition of clusterin to the ENM surface to help evade macrophage uptake [16]. Both these surface changes help ensure the ENM reaches a target tissue for optimal drug delivery. It should be noted that the addition of these “stealth” molecules present a new set of challenges for ENM based drug delivery. For exam-

ple, PEG is not biodegradable, so accumulation over time may be an issue if the ENM is being developed for the delivery of a drug that needs to be given daily [16].

Various coatings may also help the ENMs evade the physical barriers of the MIS. One of the first barriers encountered during mucosal drug delivery is mucus. The purpose of mucus is to keep all foreign materials outside of the body by trapping them due to various glycoproteins. For mucosal drug delivery, ENMs need to avoid interaction with these glycoproteins and by treating the ENM with unique coatings; it may be possible to bypass this interaction. Finally, ENMs can be treated with chemical compounds to make them more lipophilic, which could assist with passive transport of the ENM through the mucus and across the epithelial cells.

2.1.3 Surface Charge

The previous section described ENM modifications focused on physical properties, it is also possible to alter the ENM's chemical properties. One of the most common chemical changes associated with ENM is altering the surface charge. The biodistribution and cellular uptake of charged ENMs (positive, negative, and neutral) were assessed following oral exposure [18]. There was an increase in epithelial cell particle uptake and improved bioavailability with the positively charged ENM, indicating that charge plays an important role as ENMs are developed for mucosal drug delivery [18]. Furthermore, there is evidence that the development of a protein corona is affected by surface charge which in turn can effect delivery and biodistribution of ENMs [19].

The surface charge of ENMs can also influence the mechanism of toxicity (i.e. alterations in ROS, ATP, and/or inflammasome activation). Cellular toxicity from positively charged cellulose nanocrystals was associated with increased inflammation and a decreased intracellular ATP levels [20]. Whereas, the toxicity with negatively charged nanocrystals was due to inflammasome development and increased reactive oxygen species (ROS) [20]. It is important to understand these different mechanisms of toxicity because they are likely to influence the development of ENMs for drug delivery. Overall, as ENMs are developed for mucosal drug delivery a balance between cellular uptake and toxicity due to surface charge needs to be established and understood.

2.1.4 Solubility.

Solubility can be modified via the ENM itself or the pharmaceutical agent. The main goal of improving solubility is to increase bioavailability and tissue distribution while decreasing the amount of drug needed to treat a particular disease thereby reducing toxicity regardless of exposure route [21]. In terms of ENM mucosal drug delivery, improving the solubility of the ENM and target drugs could potentially allow them to move into the body via passive transport across the epithelial cell barrier.

Many pharmaceutical agents are not water soluble, thus making mucosal drug delivery difficult. The creation of nanosuspensions with the drug may improve their solubility [22]. Nanosuspensions are dispersions of nanosized drugs that are stabilized by surfactants, and can then be delivered to the body by various exposure routes [22]. In addition to these nanosuspensions, liposomes, a type of biologic ENM, are also used to improve solubility and drug delivery. In the case of liposomes, pharmaceutical agents can be loaded into the center of the vesicles, thus protecting them during drug delivery. Since the liposomes are made of lipids, it is speculated that they will be biologically inert, lipophilic, and allow the incorporation of both hydrophilic and lipophilic compounds. These parameters make this ENM a prime candidate for mucosal drug delivery.

3 Engineered Nanomaterial Derived Mucosal Immunity Modulation

The greatest challenge to ENM drug development is evading host immune responses and off-target toxicity. ENMs have been shown to initiate an inflammatory response after inhalation, injection, and even oral exposure. These inflammatory responses are often associated with increases in ROS, circulating pro-inflammatory cytokines [interleukin-1β (IL-1β), interleukin-33 (IL-33), tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), and leucocyte recruitment]. Furthermore, these exposure routes have caused off-target organ toxicity in the kidneys, liver, and spleen. To date, limited studies have specifically focused on ENM based mucosal drug delivery and the potential for inflammation and toxicity. ENMs that interact with the MIS will interact with a variety of cells associated with barrier functions as well as both innate and adaptive immune responses. This section will focus on common cell types associated with a variety of mucosal surfaces, which have a high probability of directly interacting with ENMs during mucosal drug delivery.

3.1 Common Mucosal Barrier Cells

In addition to epithelial cells, several other important cell types (i.e. goblet cells, Paneth cells, alveolar epithelium) contribute to the integrity and function of epithelial cell barrier [2]. These cells provide initial protection against xenobiotics, and would be the first cells that ENMs interact with upon a mucosal-based exposure. ENMs developed for mucosal drug delivery should circumnavigate these barriers for optimal drug delivery [2].

3.1.1 Generalized Epithelial Cells

The mucosal barrier is a single cell layer of epithelial cells connected by tight junctions and adherens, which allow the cells to communicate and act as a functional unit. Ideally, the ENMs would cause minimal damage to epithelial cells and avoid triggering an immune response. If damaged, the epithelial cells may undergo apoptosis or become necrotic and release mediators that trigger the recruitment and activation of various mucosal immune cells (Fig. 2) [23].

One important mediator expressed in epithelial cells which influences immune function is interleukin-33 (IL-33) [23]. IL-33 is a unique cytokine that is termed an alarmin. Alarmins are a class of cytokines that are rapidly released from necrotic cells and initiate an adaptive or innate immune response [24]. Specifically, IL-33 binds to the ST2 receptor that is found on multiple immune cells (i.e. mast cells, dendritic cells, and macrophages), thus activating the cell and triggering an immune response [23, 25]. Epithelial cells released IL-33 after pulmonary exposure to multi-walled carbon nanotubes, which led to the development of inflammation, mast cell degranulation, and damage to lung tissue [26]. Furthermore, epithelial cells are also critical in the regulation of IL-33. These cells express a soluble form of ST2, which prevents interaction with ST2 on other cell types [23]. This regulation is important to understand, since ENMs could be modified with soluble ST2, which may limit IL-33 driven immune activation.

Epithelial cell damage from ENM exposure can also lead to an increase in ROS generation [27]. As the levels of ROS increase, the cellular responses change. Low levels of ROS are associated with induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein [27]. This induction leads to the transcription of antioxidant

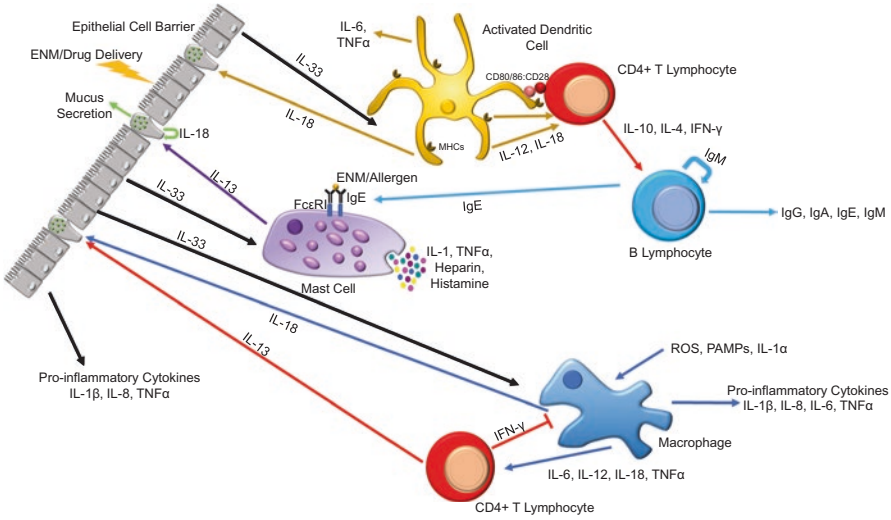

Fig. 2 A generalize schematic of a potential mucosal immune response following ENM exposure

enzymes such as heme-oxygenase 1, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [27]. It has been speculated that if ENMs activate this pathway they might be used in vivo as an antioxidant. Biogenic nanoselenium has been shown to activate this pathway leading to the induction of anti-oxidants in intestinal epithelial cells [28]. This activity was associated with decreased cellular apoptosis and oxidative stress [28]. In this capacity, it is speculated that ENM could be used to treat intestinal diseases with high levels of ROS.

However, if the level of ROS continues to increase after ENM exposure then redox-sensitive mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) are activated, which leads to a pro-inflammatory responses [27]. Many metal oxide nanoparticles have led to the activation of this pathway and the release of IL-1β, IL-8, and TNFα from macrophages and epithelial cells [27]. Additionally, these cytokines are known to activate additional macrophages and dendritic cells, which further stimulate an inflammatory response. Another pro-inflammatory mediator, toll-like receptor 2 (TLR2) was up-regulated due to activation of NF-κB in epithelial cells following in vitro gold nanoparticle exposure [29]. It should also be noted that if ROS is not controlled by either of these pathways then mitochondrial damage occurs and ultimately cell death [27]. Taken together, these responses to ROS indicated the dynamics of using ENMs for drug delivery.

3.1.2 Goblet Cells

The epithelial cell layer can be easily damaged due to physical and mechanical stresses. Therefore, goblet cells are specialized cells located in the intestinal and respiratory tracts, which secrete mucus to protect the epithelium [30]. Goblet cells contain granules of MUC2 polymers/mucin, which are regularly secreted into the intestine or airway. The secretion of mucus is influenced by a number of factors including autophagy, ROS production, and inflammasome activation [30].

Interestingly, these parameters are often altered upon ENM exposure therefore it is reasonable to assume that goblet cell and mucus production may be affected by ENM based mucosal drug delivery. There has been limited research on goblet cell function and ENM exposure. Pulmonary exposure to nanoparticle-size particulate matter was associated with an increase in goblet cell number and potential mucus secretions [31]. Although this study was not performed with ENMs, there are similar pulmonary responses between particulate matter and ENM exposure; therefore, it is possible that mucosal ENM exposure could alter goblet cell function in a similar way.

Historically, the inflammasome has been associated with the production of mature cytokines and macrophage function (see **Macrophages**); however, recent studies have shown inflammasomes are also important for goblet cell function. The inflammasome is a multiprotein structure created in the cytosol of multiple cell types in response to stress and cellular damage [1]. Inflammasomes are composed of NOD-like receptor proteins (NLRP) of which the NLRP6 has been shown be

critical in maintaining gut homeostasis and microbiota due to the secretion of IL-18 [32]. IL-18 is important for the synthesis of antimicrobial peptides and mucus secretion [32]. Although there are no studies investigating the role of inflammasomes and goblet cells specifically, ENMs have been shown to promote inflammasome formation in macrophages (see **Macrophages**) [33, 34]. Therefore, it is reasonable to assume that ENM exposure could lead to an increase in IL-18 production via inflammasome activation. This ultimately would lead to an increase in mucus secretion and antimicrobial peptides, which could affect the efficacy of ENM drug delivery.

As previously discussed, ENM are capable of triggering the release of IL-33. The release of this cytokine has been shown indirectly to affect goblet cell formation and function. IL-33 can induce the release of IL-13 from CD4+ T helper cells and mast cells [35]. This cytokine influences gene expression (i.e. *Muc2*) and goblet cell hyperplasia, indicating that IL-13 has influence on goblet cell function, which may be modified upon ENM exposure [35]. Finally, if IL-33 and IL-13 modifications from ENM exposure continue to increase various bowel diseases could develop (i.e. inflammatory bowel disease, acute colitis) [35].

3.1.3 Paneth Cells

Paneth cells are important immune cells that are located in the intestinal epithelial cell layer and may play a role in the development of intestinal inflammatory diseases (i.e. Crohn's disease and ulcerative colitis) [36]. Paneth cells produce antimicrobial peptides, known as α-defensins, chemokines, and cytokines [36]. Defensins released by Paneth cells are important in the regulation of T cell, dendritic cell, macrophage, and epithelial cell function. Alpha-defensins have been shown to be a chemoattractant for immature dendritic cells and T cells by acting through receptors (i.e. CCR6) that are expressed on these cells [36]. Furthermore, defensins can also promote dendritic cell maturation via pattern recognition receptors (PRRs) and Toll-like receptors (TLRs) [36].

From a disease progression perspective, patients with inflammatory disease had impaired defensin secretion from Paneth cells [36]. Inflammatory intestinal diseases are associated with damage to epithelial cells and impaired immune function following an active disease state. It has been showed that after this inflammatory episode, there is a significant increase in Paneth cells number, which potentially serve to restore mucosal immunity [36]. To date, there is little to no research done on the influence of ENM exposure and Paneth cell secretions. Despite this lack of research, it is reasonable to speculate that due to the nature of mucosal drug delivery, ENM would interact with Paneth cells and may trigger an innate inflammatory response and potentially lead to inflammatory pathologies.

3.1.4 Alveolar Epithelial Type I and II Cells

Alveolar epithelial cells are important in modulating pulmonary immune responses. In the lungs, two types of epithelial cells exist: Type I and Type II. Type I epithelial cells are integral for gas exchange and maintaining barrier function; whereas Type II are responsible for the production and secretion of surfactant.

Type 1 alveolar epithelial cells have only recently been shown to be involved in initiating inflammatory responses following ENM exposure. Multiple ENMs such as carbon nanotubes and zinc oxide nanoparticles cause damage to epithelial cells [10]. This damage leads to cellular apoptosis and oxidative stress. The increase in oxidative stress can lead to the activation of macrophages, which can then trigger release of pro-inflammatory cytokines and recruitment of other lymphocytes (see **Macrophages**). Additionally, research indicates that damaged epithelial cells when undergoing apoptosis, may trigger inflammatory responses within the lungs but the exact mechanism is unknown [37].

Surfactant is naturally produced and secreted from Type II alveolar epithelial cells; however, it can also be artificially manufactured, which may be important for mucosal drug delivery. From a physiological standpoint, the production of pulmonary surfactant is critical for the reduction of surface tension in the lungs and preventing alveoli from collapsing. In terms of mucosal drug delivery, artificial surfactant has been used to prevent ENM agglomeration [38]. ENM agglomeration can have a huge impact on distribution and the drug delivery efficacy and therefore needs to be assessed when developing ENM for novel delivery methods. Furthermore, research with silver nanowires showed that Type II epithelial cells maybe more susceptible to ENM exposure as indicated by an increase in IL-8 production and ROS generation [39]. This research also showed that artificial surfactant was able to protect Type I epithelial cells from the toxic effects of silver nanowire exposure [39]. This research highlights the potential application of artificial surfactant for optimal mucosal/pulmonary drug delivery. Additionally, surfactant may alter epithelial cell toxicity of a given ENM, which also needs to be further investigated when developing novel ENMs for mucosal drug delivery.

3.2 Common Mucosal Immune Cells

Regardless of mucosal location, once ENMs penetrate the epithelial cell layer, they may interact with numerous lymphoid cells. To date, there is little research on mucosal drug delivery using ENMs, and their potential toxicity. Therefore, the immune cell effects discussed in this chapter will be based primarily on in vitro ENM studies.

3.2.1 Macrophages

Macrophages are phagocytic cells that have multiple functions depending on activation status and location. These cells are responsible for the clearance of cell debris during wound healing and the removal of bacteria from the gut and the lung [40]. In relation to ENM exposure, macrophages have a critical role in the removal of particles from tissue (i.e. lungs, gut, and skin). The function and activation of this key immune cell is vital as ENMs are developed for mucosal drug delivery and therefore this section will focus primarily on macrophages ability to augment immune responses due to ENM exposure. The majority of research with ENMs and macrophages has focused on pulmonary exposures. This focus is largely due to the occupational inhalation exposure risk associated with the ENM manufacturing; however, similar responses are likely as ENM are also developed for pulmonary mucosal drug delivery.

Macrophages can be activated by multiple mechanisms including pattern recognition receptors (PRRs), ROS, and alarmins. These factors employ a variety of surface receptors (i.e. complement receptors, scavenger receptors, and Toll-like receptors) leading to the rapid production of pro-inflammatory cytokines (i.e. IL-1β, IL-6, and TNFα), ROS, and inflammasome activation [1, 41]. In response to pathogens, macrophages can detect antigens based on PRRs and pathogen-associated molecular patterns (PAMPs) [1]. The detection of PRRs and PAMPs lead to phagocytosis by the macrophage. This activation pathway is especially important for ENMs as they could be modified to express specific PRRs, which could allow macrophages to be employed to initiate a specific immune response thus aiding in drug delivery.

ROS have also been shown to play a role in macrophage activation. Although low levels of ROS produced by macrophages are beneficial in combating foreign pathogens; high levels have been associated with toxicity, inflammasome activation, and immune cell death [1]. It has been well documented that exposure to multiple ENMs (i.e. carbon, silica, and metal oxides) leads to increased cellular ROS and macrophage activation [42–45].

In addition to ROS and PRRs, macrophages can also be activated by alarmins and other cytokines. As previously mentioned, alarmins play a key role in the activation of mucosal immune responses. The alarmins, IL-1α and IL-33 been shown to be a key contributors to macrophage activation following pulmonary exposure of silica [46]. Another cytokine that has been linked with macrophage activation is interferon (IFN)-γ. IFN-γ is secreted by T lymphocytes and has been shown to increase following exposure to copper nanoparticles [47]. Taken together it is clear that macrophages can be activated via multiple stimuli in response to ENM. Once activated these phagocytic cells are important mediators in a mucosal immune response. Briefly, upon detection of a xenobiotic material (such as ENMs), it is engulfed and processed by macrophages. This digestion leads to the release of multiple pro-inflammatory cytokines (i.e. IL-1β, IL-6, and TNFα) and ROS production, which initiate a variety of host immune responses (i.e. macrophage recruitment, leukocyte recruitment and inflammasome activation).

Macrophage cytokine release is essential for the recruitment of other immune cells and the propagation of an inflammatory response. Silica nanoparticles and graphene sheets have been shown to increase lipid peroxidation and TNFα secretion, which are considered markers of inflammation and cytotoxicity [42, 48]. Pulmonary exposure to carbon nanotubes has been well studied, particularly in regards to macrophage influences. These studies have shown associations between exposure and increases in cyclooxygenase-2 (COX2) production, IL-6, and IL-1β secretion [33, 34, 43, 44, 49, 50].

Inflammatory mediator production has been associated with the modulation of other immune cells. The induction of COX2 is a hallmark of inflammation and its downstream mediators influence mast cell, endothelial cell, and macrophage function [51]. Furthermore, IL-6, IL-12, and TNFα have been shown to activate T lymphocytes [52]. Additionally, IL-1β secretion and ROS changes influence inflammasome activation. The inflammasome is critical for the processing of mature cytokines such as IL-1β. Titanium dioxide (TiO_2), silica, and silver nanoparticles have all been shown to activate NOD-like receptor 3 inflammasomes and this activation was independent of phagocytosis [33, 34]. Upon formation, the inflammasome cleaves inactive pro-IL-1β through a caspase-1 mechanism to form biologically active IL-1β. Once secreted, IL-1β acts as an alarmin and recruits other inflammatory cells thus prolonging local and systemic inflammation [53].

Finally, it should be noted the macrophages could be deactivated by IL-10, an anti-inflammatory cytokine. IL-10 is produced by CD4+ T lymphocytes and has been shown to inhibit IFN-y production in T lymphocytes and thus prevent macrophage activation [54]. Carbon nanotubes and liposomes have both been shown to cause an increase in IL-10, which indicates they may cause immunosuppression upon exposure [55]. Although some level of immunosuppression may be beneficial for ENM based drug delivery, uncontrolled suppression may result in fibrosis or cancer due the lack of particle clearance and other essential immune mediators (i.e. T and B lymphocytes, phagocyte activity, and mast cells) thus the need for more research of ENM and immune modifications is essential [55].

3.2.2 Dendritic Cells

Dendritic cells are phagocytic cells, which are important messengers between the innate and adaptive immune systems. The activation of dendritic cells is currently of great interest for ENM based drug delivery, specifically with cancer and vaccine research [56, 57]. This is due to their ability to present peptides on major histocompatibility complexes (MHC) which theoretically can be exploited to modify T lymphocyte function [1].

These cells are located in the skin and express a variety of PRRs (i.e. TLRs, NOD-like receptors, and C-type lectins), which bind molecular motifs of pathogens, and cellular damage, thus activating phagocytosis [41, 58]. Under normal conditions, antigens bind to these surface receptors; however, research has shown that ENMs may not utilize the previously mentioned specific receptors to promote

phagocytosis. Quantum dots are phagocytized by dendritic cells through a clathrin-mediated mechanism and scavenger receptors, which are regulated by F-actin and phospholipase C compared to the normal actin-dependent uptake [59].

Normally, once antigens are engulfed by dendritic cells, unique peptides are presented on MHCs (class I or II), which leads to T lymphocyte activation [60]. In addition to increased MHC expression, activated dendritic cells also undergo morphological changes, increased immune markers, and increased cytokine secretion. Long dendrites develop and cluster of differentiation (CD) 80 and CD86 expression increase upon activation, which all aid in antigen presentation for T lymphocyte recruitment and activation [61]. Typically, T cells mature in lymph nodes but activated dendritic cells are capable of activating naïve T cells due to the secretion of IL-12 and expression of surface CD80/86, which interacts with CD28 on T cells [1]. However, CD80 and CD86, and MHC expression are altered upon treatment with various ENMs (i.e. TiO_2, silicon dioxide, and zinc oxide) suggesting the ability of ENMs to alter antigen presentation and T cell activation [62–64]. Finally, in addition to T lymphocyte activation, dendritic cells secrete pro-inflammatory cytokines (i.e. IL-12, IL-6, and TNFα), whose secretion was altered following zinc oxide nanoparticle exposure [1, 62].

Ultimately, ENM based mucosal drug delivery will lead to interactions with dendritic cells but the activation of an immune response will largely depend on the design of ENM itself. Ideally, ENMs could be used to enhance the immune response of dendritic cells. The ENMs are targeted to dendritic cells and have unique peptides attached or encapsulated inside a nanostructure. In theory, once phagocytosis occurs, the antigen of choice will be expressed on MHC, T cell activation and expansion will occur, and the T lymphocytes will attack cells that express the antigen. This response has been shown to be achievable especially with biodegradable and lipid based nanostructures (56; 65). However, other ENMs (particularly elemental based nanoparticles) have not had the same success. Gold nanoparticles labeled with unique antigens were engulfed; however, dendritic cell activation did not occur [66]. Additionally, TiO_2 nanoparticles were unable to activate dendritic cells despite clear nanoparticle uptake by the cells [67]. This lack of activation may be due to engulfment via macropinocytosis versus receptor-driven uptake [67].

Although ENM-based immune modulation through dendritic cells is promising, additional research will be needed to determine if these alterations cause off target effects and the long-term consequences of these immune manipulations.

3.2.3 Mast Cells

Mast cells are present in multiple tissues (i.e. skin, airways, gut mucosa) and contain mediators that are important in angiogenesis, homeostasis, and immunity [68, 69]. Mast cells contain large granules containing inflammatory mediators (i.e. histamine, heparin, cytokines, and proteases), which upon degranulation release these mediators triggering leukocyte recruitment (i.e. eosinophils and neutrophils) and immune responses [1].

Mast cell degranulation is initiated by a variety of surface receptors. Traditionally, mast cell activation occurs through an IgE-mediated pathway. Mature B lymphocytes produce IgE antibodies, which bind to the FcεRI receptor on the surface of mast cells. This promotes crosslinking between the receptors and increases intra- and extra-cellular calcium flux and degranulation [68]. Exposure to TiO_2 nanoparticles can lead to mast cell activation because TiO_2 nanoparticle interacts with L-type calcium channels leading to a calcium influx [70]. Furthermore, other research has shown that silver nanoparticles can interact with mast cells leading to degranulation and pro-inflammatory cytokine release [17]. ENM derived mast cell degranulation seems to be IgE independent and likely acting through other receptors than FcεR1. For example, silver nanoparticles cause robust mast cell degranulation and may act through a unique receptor or may enter the mast cells and cause degranulation through other signaling mechanisms [71, 72]. Furthermore, research has shown carbon nanotubes activate mast cells by prompting the release of IL-33 from epithelial cells [26, 73]. IL-33 acts on the mast cell ST2 surface receptor and initiates mast cell activation [26, 74].

Once activated, the mediators released from mast cells have multiple downstream effects influencing immune, pulmonary, and vascular function. Pulmonary exposure to cerium dioxide nanoparticles resulted in vascular dysfunction, and increases in IL-6, TGF-β and macrophage inflammatory protein-1 α secretion [75]. These inflammatory and vascular changes were not observed in mice that were mast cell deficient thus highlighting the systemic impact of mast cell activation [76]. Histamine is another major mediator released from mast cells and is important for allergic responses. Both TiO_2 nanoparticles and silver nanoparticle exposure resulted in the release of histamine [70, 77]. Histamine release also has systemic effects resulting in bronchoconstriction, vasodilation, and nasal mucus production. Taken together, the influences ENM have on mast cell activation highlight a need to understand not only the downstream effects of mast cells, but also the initial mechanism(s) of activation.

Mast cells are also capable of modulating immune function by influencing dendritic cell, T cell activation, and leukocyte recruitment. Mast cells express TLRs and when stimulated secrete IL-1 and TNFα [68]. These pro-inflammatory cytokines promote the migration and activation of dendritic cells, which promotes T lymphocyte activation [68]. Furthermore, TNFα has been shown to directly activate cytotoxic T cells [68]. Finally, secreted cytokines have a key role in the recruitment of leukocytes (i.e. eosinophils and neutrophils), which contribute to initiating and/or maintain a mucosal immune response [31, 78, 79]. Although, it is not known if ENMs influence cytokine release from mast cells via direct interaction with surface receptors (i.e. ST2 and FcεRI), it is reasonable to speculate mast cell function may be modulated this way since ENM interact with dendritic cells, T lymphocytes, and other leukocytes.

Finally, despite multiple studies showing ENMs are capable of triggering mast cell activation, other ENMs have been shown to inhibit degranulation. Fullerenes are able to prevent degranulation by blocking cross-linking to the FcεRI, which inhibited intracellular calcium release and ROS production [80]. The ability to

potentially block mast cell activation is important because allergies and anaphylaxis are associated with the activation of mast cells and ENMs could potentially be developed to block mast cell activation, thus providing a treatment for allergies and prevention of anaphylaxis. Overall, as ENM-based mucosal drug delivery is developed, the influences and modulation of mast cells needs to be thoroughly understood due to the ubiquitous role mast cells have on allergic and immune responses, as well as pulmonary and vascular function.

3.2.4 T Lymphocytes

Although there is currently limited research on the influence of ENMs on T lymphocyte maturation and activation, it remains a critical area of research. This is largely due to the idea of using ENMs and the MIS to manipulate T cells and dendritic cells (see **Dendritic Cells**) to target specific cell types for the treatment of diseases like cancer while decreasing off target toxicity [81].

Briefly, T cells originate in the bone marrow, and migrate to the thymus gland to fully mature. Mature and activated T helper (T_H) cells express CD4+ and recognize major histocompatibility complex (MHC) class II; whereas, mature cytotoxic T cells express CD8+ and recognize MHC class I [1]. Once active, T cells have an important role in adaptive immunity and influence the activity of other immune cells (i.e. mast cells, dendritic cells, and macrophages).

ENMs are capable of influencing T cell proliferation, which is required for proper immune function. TiO_2 nanoparticles have been shown to decrease T_H and cytotoxic T cell proliferation and was associated with immune inhibition; whereas, carbon nanotubes has the opposite effect [82, 83]. Intravenous exposure to carbon nanotubes resulted in increased T cell proliferation and a potential enhanced immune response [83]. Understanding the influence of ENMs on T cell proliferation is particularly important in regards to asthma. Asthma is associated with T_H2 inflammation and potentially IL-10 (an anti-inflammatory cytokine) secretion, which results in airway hyper-responsiveness and tissue remodeling to normally inert airborne particles [84]. Historically, pulmonary exposure to ENMs have elicited responses similar to airborne particles of comparable size [85]. Since ENMs can also alter T_H responses it is reasonable to speculate repeated pulmonary ENM exposure may result in an enhanced asthmatic response via increased airway hyper responsiveness in susceptible populations. Pulmonary exposure to graphene oxide nanoparticles when given in an asthmatic mouse model resulted in enhanced airway remodeling and hyper-responsiveness but simultaneously decreasing T_H2 immune responses [86]. This apparently divergent response could potentially be due to increases in macrophage specific enzymes known as mammalian chitinases, which have been associated with asthma [86].

Finally, like many immune cells, mature T_H cells secrete a variety of cytokines (i.e. IL-10, IL-4, IFN-γ) that are important in maintaining an immune response because they help maintain macrophage and dendritic cell activity [61]. Overall, if ENMs are to be developed for mucosal drug delivery further research needs to be

completed to fully understand the influences on T lymphocyte activation and potential role in autoimmune diseases, like asthma.

3.2.5 B Lymphocytes

Similar to T lymphocytes, limited research on the influence of ENM and B lymphocytes has been completed. Briefly, these bone marrow derived cells express unique membrane bound antigen-binding receptors. Normally, these antibodies interact with antigen from various pathogens, which result in the differentiation of memory B cells and effector B cells or plasma cells. Since ENMs are often seen as foreign, it is possible that they could interact with naïve B lymphocytes, which could result in a humoral immune response.

Titanium dioxide nanoparticle exposure resulted in a significant drop in circulating B cells following multiple exposure routes (i.e. intraperitoneal and intragastric), indicating a potential impairment in humoral immune response [82, 87, 88]. Furthermore, exposure to TiO_2 nanoparticles appears to influence B cell activation. B lymphocytes are activated via a thymus-dependent or -independent pathway. For thymus dependent activation, cross-linking between surface IgM needs to occur due to either interaction with an antigen, or CD40 and cytokines from T_H2 cells [1]. As previously discussed, ENMs are capable of modulating T lymphocytes responses and intragastrically delivered TiO_2 nanoparticles also decrease serum IgM, which when taken together could indicate that ENMs may delay B lymphocyte development [88]. Thymus independent activation of B lymphocytes requires IgM cross linking in addition to secondary activation signals through TLRs; however, the impact ENMs have on this activation pathway are largely unknown [1]. Although the interactions presented here are not due to mucosal delivery it is reasonable to speculate that mucosal delivery of ENMs could result in B cell activation. This could be due to the activation of other MIS cells (i.e. dendritic cells and T lymphocytes), which could lead to the downstream activation of B cells (Fig. 2) [1, 61, 62, 64, 84].

Upon activation, B cells secrete specific classes of antibodies. IgG is associated with enhanced phagocytosis, whereas IgA is secreted into mucus membranes [1]. Finally, IgE is secreted to combat parasitic infections but is also associated with allergic responses due to its role in the activation of mast cells [1]. Pulmonary exposure to TiO_2 nanoparticles and platinum nanoparticles resulted in increases in serum IgE levels as well as increases in circulating IL-4, IL-5, and IL-10 [89–91]. Taken together these circulating mediators indicate the potential activation of mast cells and the ability to trigger an allergic response (see **Mast Cells**). Furthermore, is should be noted that TiO_2 nanoparticles have different impacts on B cell development which are dependent on exposure route. Therefore, understanding the immune influences of mucosal exposure routes and ENM is critical for proper drug development.

Immunoglobin M is a pentamer antibody and has important roles in B lymphocyte development and mucosal immunity [92]. In regards to immunity, IgM is capa-

ble of activating complement and initiating a mucosal immune response [92]. There are limited data referencing changes in IgM upon ENM exposure and the current data have conflicting results. Zinc oxide nanoparticle exposure resulted in decreased IgM concentrations in weaned pigs indicating possible immune suppression. However, copper nanoparticle and multiwall carbon nanotube exposure resulted in increased IgM and an enhanced immune response [93, 94]. Although no mechanism for these immune differences currently exists, it may be due to the different materials used to manufacture the ENMs. Finally, there is evidence that IgM (along with IgA) are major contributors to the development of a protein corona in vivo [95]. The development of a protein corona with IgM could have major impacts on their ability to activate complement as well as activate naïve B lymphocytes. Overall, it is clear that future studies utilizing ENM based mucosal drug delivery need to focus on B cells modulation and antibody secretion both during acute and chronic exposures.

3.2.6 Endothelial Cells

Although not normally considered an immune cell, endothelial cells have a critical role in modulating and regulating inflammatory responses. Therefore, it is essential to understand the influences ENM exposure has on endothelial function. These cells line all the blood vessels in the body and express various integrins and adhesion proteins (i.e. VCAM-1, ICAM-1, E-selectin, and P-selectin) [96]. These proteins are important for the rolling, adhesion, and extravasation of leukocytes once an immune response is triggered. For example, mast cells express α4β7 integrins that bind to VCAM-1 and initiates movement of CD34+ progenitor cells out of the circulation, which is required for maturation and activation [68]. The extravasation of leukocytes and progenitor cells also alters vascular permeability and permeates the immune responses.

This extravasation of leukocytes is critical for a proper immune response and requires endothelial activation. Carbon and TiO_2 nanoparticle exposure are associated with increases in ICAM-1 and VCAM-1 expression as well as increasing circulating pro-inflammatory cytokines like IL-6 and TNFα [94, 97, 98]. Taken together, these results indicate ENMs are capable to altering integrin expression on endothelial cells in order to maintain an inflammatory response by promoting movement of leukocytes from the bloodstream into an extracellular compartment. A thorough understanding of ENMs influence on endothelial cell activation is needed because if these responses are blocked, a prolonged immune response may not occur and could therefore increase the efficacy of mucosal drug delivery.

ENMs are also capable of altering vascular function via endothelial dependent mechanisms. Proper vascular function is essential for the exchange of nutrients and prevention of diseases like hypertension and edema. TiO_2 nanoparticles, cerium dioxide nanoparticles, and carbon nanotubes have all been shown to impair vascular reactivity in vivo [99–101]. Following these exposures, arterioles were unable to dilate in response to increasing concentrations of nitric oxide via an endothelial-dependent mechanism [99]. Although the exact mechanism is unknown, this impair-

ment is likely due to changes in COX expression and ROS production [102, 103]. If this dysfunction is unresolved over time the arterioles inability to respond to vasoactive mediators could result in disease progression (i.e. hypertension).

Endothelial cells are also capable of secreting cytokines (i.e. IL-6, TNFα, and IFN-γ), which affect not only vascular function but also can activate other immune cells, specifically dendritic cells and macrophages. Multiple studies have indicated that ENM exposure can lead to increased pro-inflammatory cytokine expression and endothelial cell dysfunction [97, 104, 105]. Although neither initial activation and/or downstream recruitment of other immune cells was not assessed, it is reasonable to speculate that this endothelial impairment and cytokine release could alter the function of other immune cells (i.e. macrophages, dendritic cells, and mast cells) (Fig. 2); additional studies are needed to determine the order of cell activation.

Additionally, it should be noted that although these examples have primarily focused on the inflammatory effects of ENM exposure, other studies have found that ENMs can inhibit inflammatory responses. Gold nanoparticles decrease inflammation via inhibiting COX2 expression and TNFα secretion in a rheumatoid arthritis model [106]. Similarly, in a hypertension rat model cerium dioxide nanoparticles were capable of decreasing ROS and improving arteriole function [107]. These examples highlight the concept that ENM may respond differently in a diseased animal model compared to a healthy model and this paradigm is especially important for ENM based drug delivery applications. As ENMs continue to be developed and used for mucosal drug delivery, it is important to remember not only their potential toxic effects on immune cells but also on endothelial cells since they play a key role in regulation of immune responses.

4 Conclusion

In conclusion, it is clear that ENMs can be both beneficial and detrimental to the MIS. In order for mucosal drug delivery to be successful, impacts on immune function need to be recognized. Regardless of potential negative effects, there is evidence that some immune modifications could be beneficial for disease treatment (i.e. inflammatory bowel disease). This division between the positive and negative effects of ENMs needs to be fully understood for mucosal drug delivery to be optimized.

Additionally, investigations of acute and/or chronic immune modifications caused by ENM interactions are needed. Acute activation of the innate immune system would likely have few negative biological drawbacks and allow drug delivery improvement. On the contrary, chronic inflammation could lead to the development of autoimmune disease and organ toxicity, which may negate the purpose of using the ENM for drug delivery. As ENMs are developed for drug delivery the severity of these immune responses needs to be taken into consideration.

Finally, despite potential acute or chronic inflammatory responses to ENM, they may solve many of the barrier challenges that are associated with mucosal drug

delivery. This potential is due to the ability to modify their physicochemical properties. Overall, despite biological and engineering challenges of ENM development, they hold great potential for the advancement of mucosal drug delivery.

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Toxicity of Nanomaterials to the Host and the Environment

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Abstract Although silver nanoparticles (AgNPs) display excellent antibacterial, antifungal, and antiviral properties; the pervasiveness of AgNPs in occupational, medicinal, consumer, and environmental settings has raised concerns about the potential for adverse health effects and environmental risks. We provide herein an overview of the use of AgNPs, routes of AgNPS exposure, physicochemical properties and mechanisms responsible for toxicity, and strategies to establish the safety of AgNPs. The core of this book chapter is the notion that while AgNPs may be effective agents in exterminating various pathogens, they may also damage healthy cells, animals, humans, and ecosystems. Thus, the manufacture and usage of AgNPs should be closely monitored and regulated.

Keywords Silver nanoparticles · Properties · Toxicity · Dermal exposure · Oral exposure · Inhalation exposure

1 Introduction

Nanotechnology represents the merging of science, engineering, and technology expertise at the next great industrial revolution: control of matter at the nanoscale level. In recent years, nanoparticles have become widely used in electronics, agriculture, textile production, medicine, and many other industries and sciences. Nanotechnology involves imaging, measuring, modeling, and manipulating substances which have one dimension less than 100 nanometers (nm). At this scale, matter exhibits unique physical, chemical, and biological properties, which differ from the properties of bulk materials and single atoms/molecules. Some are stronger

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or have different magnetic properties compared to other forms or sizes of the same material; whereas others are better at conducting heat or electricity, or become more chemically reactive, reflect light better, or change color as their size or structure is altered. These unique properties enable novel applications, which promise great technological advances in chemistry, biology, physics, materials science, engineering, agriculture, and medicine. Nevertheless, many of the same properties that make nanomaterials (NMs) so promising from a technological standpoint make their interactions with biological systems and the environment difficult to predict, raising concerns for the safety of workers, consumers, and the environment. Depending on their physicochemical characteristics, NMs can enter the human body through blood, inhalation, dermal, and digestion exposures. Consequently, NMs can not only access, but also accumulate in vital organs and damage tissues and cells [1]. Significant gaps in knowledge remain for many factors to fully characterize the risk of NM such as dose-response relationships and differences across species. Moreover, complex and largely unknown properties of NMs together with a lack of toxicological and exposure data are currently among the major barriers for robust risk assessment, which is critical to formulation of rigorous policies around regulation of NMs. Thus, there is a recognized demand for robust assessment of NM toxicity using well-characterized NMs, standard protocols, and relevant route and doses of human exposure from fields of research ranging safety assessment to drug delivery [1, 2]. For the purpose of this chapter, we will study silver NMs as an example; although there may be a few properties that are specific to each NMs, many of the NM toxicity assessment will be similar between different types of NMs.

1.1 Silver Nanoparticles (AgNPs)

Among the NMs, silver nanoparticles (AgNPs) are considered one of the most important due to their excellent antibacterial, antifungal, and antiviral properties, relatively low cost of manufacturing, and unique properties and ability to form diverse nanostructures. AgNPs are used across a diverse range of commercial consumer products including food packaging materials, food storage containers, water purificants, odor-resistant socks and underwear, room sprays, laundry detergents, washing machines, lotions and soaps. Additionally, AgNPs are widely used in medical applications including wound dressings, female-hygiene products, surgical instruments, bone cements, and implantable devices [3]. More recently, AgNPs have become attractive materials for application as effective drug delivery vehicles and cancer therapeutic agents [4]. The primary concerns associated with such applications is the risk of accumulation of silver in the body causing heavy metal toxicity, for nanosilver surface oxidation releases Ag^+ ions which are known to be toxic. In fact, the smaller the AgNPs, the higher the surface area to volume ratio, and the more Ag^+ ions are released [5]. The ubiquity of AgNPs will likely result in repeated contact through multiple routes of exposure, which collectively may lead to health complications. As with many NMs, the usage of AgNPs should be closely monitored

and regulated. Of particular concern is the potential for unnecessary use of non-selective biocidal AgNPs in consumer products to contribute to the escalating problem of worldwide bacterial resistance.

1.2 Toxicity of AgNPs

Nanomaterials may enter the human body through different routes: inhalation (respiratory tract), ingestion (gastrointestinal tract), dermal contact (skin), injection (circulation), etc. as a result of intentional or accidental exposure. Although NMs have been on the market for several years, the full scope of their effects on the human body has not been discovered yet. Numerous *in vivo* studies have assessed the distribution of various NMs after inhalation, oral, dermal and intravenous delivery and consequently accumulation of NMs in different tissues and organs [6–17]. These studies show that NMs can cross the lung, gut, skin, and blood brain and placental barriers depending on the route of exposure, time, concentration, and characteristics of the NM. Even in cases of low absorption, chronic exposure may result in internal accumulation of the NMs potentially reaching levels that might give rise to adverse health concerns [18]. Many recent studies using animal models indicate that exposure to AgNPs via one of several routes (inhalation, oral, dermal, intravenous, etc.) results in genotoxicity and DNA damage [19–22], inflammatory responses in the liver, lung, and kidney [23–25], and adverse functional effects in the lungs, heart, intestine, and spleen [25–28]. Consistent with its role in detoxification, the liver appears to accumulate a disproportionate amount of AgNPs and may be especially susceptible to nanosilver exposures [29, 30]. While these *in vivo* studies provide unique information on the distribution of AgNPs in a whole organism, given the great number of and variety in different AgNPs, it is essential that we remain cautious with usage while further studies are conducted to determine their safety.

1.3 Inhalation Exposure

One of the primary target organs of AgNPs exposure is the lung, which is directly exposed after inhalation of airborne nanosilver—especially through consumer products such as disinfectant sprays. Inhaled AgNPs can interact with alveolar macrophages and airway epithelial cells, as well as pulmonary surfactant resulting in the formation of a biomolecular corona containing both proteins and lipids [29, 31]. After uptake of the particles, macrophages gradually move upward by the mucociliary escalator [32], are subsequently swallowed, and may enter the gastrointestinal tract. If not cleared by phagocytosis, AgNPs may also be taken up by the alveolar epithelium and reach the pulmonary interstitium from which they are transported to the local lymph nodes, or reach the blood circulation [33, 34]. Due to their size,

AgNPs distribute throughout the respiratory tract, can reach pulmonary alveoli, and even translocate beyond the respiratory tract to various other organs, including the diaphragm, brain, liver, and kidney [35–37]. The deposition of inhaled AgNPs depends on the morphology of the airways, the respiratory conditions, and the physicochemical properties of the particles. The most important physicochemical properties of inhaled particles that influence deposition are size (agglomerate), size distribution, density, shape, charge, and hygroscopicity [1]. Deposition of NM in the lungs results in an acute granulomatous inflammatory response which can progress to interstitial fibrosis, as well as systemic immune dysfunction. Thus, in addition to its importance as an environmentally-mediated lung disease, NM-induced injury serves as a paradigm for understanding the underlying cellular and molecular mechanisms responsible for pulmonary immune responses.

1.4 Oral Exposure

Given the growing use of NMs in consumer products, including food and packaging, the oral route of exposure has been poorly explored in nanotoxicology. Ingestion of NMs can occur through direct ingestion of food ingredients, additives, and supplements or by mucociliary escalator transport (e.g cough and swallow) [38]. Many factors are involved in controlling the absorption of NMs via the GI tract including size of particles, geometry, surface charge, ligand type, and attachment potential to ligand [39]. Following ingestion, translocation of NMs into and across the gastrointestinal mucosa can occur via four different means: (1) via endocytosis through enterocytes, (2) via the M cell-rich layer of Peyer's patches (small intestinal lymphoid aggregates), (3) via persorption, where particles can translocate through a 'hole' left in the epithelium when enterocytes shed from the villous tip, and (4) via the paracellular route, where NMs pass across tight junctions of the epithelial cell layer [40]. Animal models suggest that NMs can accumulate in the Peyer's patches of the small intestines, and although the absorbance of NMs is low in the healthy individual, damage to the intestinal wall or reduced intestinal barrier function (e.g. Crohn's disease or Ulcerative Colitis) may result in higher levels of NMs uptake. In rodents, AgNPs (5–20 nm) damaged the epithelial cell microvilli and intestinal glands after 21 days of administration at 20 mg/kg [41]. Moreover, oral administration of 30 mg/kg (60 nm AgNPs) for 28 days increased the frequency of goblet cells in the intestine that had released their mucus granules [42]. Lastly, abnormal pigmentation of the ileum was observed following the administration of 125 and 500 mg/kg (56 nm) AgNPs [43]. More recent studies which have begun to explore the potential size-dependent effects of AgNPs on the host microbiome, have provided conflicting data on the impacts of AgNPs on the microbiota [44, 45]. Again, many factors control the absorption of nanoparticles in the GI tract: size of particle, shape, surface charge, ligand, etc. Ingested nanoparticles may be excreted or agglomerated by physico-chemical changes resulting in potential intestinal blockages [46].

1.5 Skin Exposure

The exposure of human skin to NMs can occur via intentional and unintentional means. Intentional exposure to NMs could result from applications of cosmetics products such as creams, lotions, and sunscreen containing nanoparticles (e.g. TiO_2 and ZnO). Unintentional exposure of NMs to human skin occurs through directly generated nanoparticles during manufacturing, combustion, and disposal of used nanomaterial-based products. Two primary mechanisms exist for penetration of AgNPs into the skin: intercellular trans-epidermal transport or diffusion through skin pores and hair cavities. Concerns include cytotoxicity of skin, toxicity during accumulation in skin over long periods of time, metabolism with potential of toxicity, and photo-activation of NMs present in the skin. Human skin is an effective barrier toward NMs and most toxic chemicals' although penetration can occur when the protective layer of skin is removed, damaged, or wounded. Similarly, hair follicles and sweat glands increase the barrier susceptible by facilitating the penetration of nanoparticles. Moreover, the use of nanoparticles in treatment of wounds and damaged skin accelerates penetration [47]. The potent antimicrobial properties of AgNPs have made these nanoparticles one of the most frequently utilized NMs in skin care products [48]. Dermal toxicity of AgNPs has been proposed to be mediated by oxidative stress associated with decreased viability, inhibition of mitochondrial activity, and the induction of apoptosis and cell death [49]. A recent study showed hepatotoxicity and nephrotoxicity in mice following dermal absorption of AgNPs (40 nm) in a dose-dependent fashion [50]. Thus, it is essential to determine the amount of AgNPs in dermal tissue of model animals.

2 Factors Influencing the Toxicity of NMs

NMs can be broadly classified based upon their origin (natural vs. engineered NMs) and organized into carbon based, metal/metal oxides, dendrimers, or composites. On their own, NM display a vast array of characteristics in size, shape (tubes, films, rods, etc.), composition, and surface chemistry (metal ions, small molecules, surfactants or polymers), as well as agglomeration and aggregation when suspended in solution. Although it is often tempting to consider NMs as simple molecules, they are in fact complex mixtures. Interactions between NMs and biological molecules, cells, animal, humans, and the environment are incredibly complex. With these differing physicochemical properties, NMs exhibit different biocompatibilities when interacting with different cell types [51, 52]. Upon arrival at the cell surface, multiple processes exist by which NMs may be internalized by the cell (e.g. phagocytosis, endocytosis, direct trans-membrane transport, passive diffusion, etc.) and once inside the cell, many locations to which NMs may be routed [53–56]. Moreover, once NMs come into contact and interact with biological molecules and fluids, these properties may undergo significant surface modifications. After several decades of

research, scientists have only begun to understand how the inherent properties of NM dictate the ultimate impact of NM on human health. However, it has become clear that parameters such as surface area to mass, shape, purity and associated changes in surface parameters such as reactivity, charge and solubility are associated with toxicity [57, 58].

Change in size can greatly affect the physical and chemical properties of NMs, and thus the deposition and fate [59]. Although AgNPs provide many benefits, AgNPs induce size-dependent toxicity in the brain, lung, liver, and kidneys when delivered via systemic, oral, and inhalation exposures [60–64]. As AgNPs become smaller, the surface to volume ratio increases greatly—which makes these NMs more reactive and/or toxic. The increased bio-availability combined with a larger surface area may worsen interaction with cellular organelles, increasing reactive oxygen species production, inflammation and cytotoxicity [65]. Smaller NMs more easily cross cellular barriers by modulating specific uptake and endocytic processes [66]. Indeed small-sized (10–15 nm) AgNPs caused more toxicity by generating 10 times higher amounts of reactive oxygen species than larger sized (30 and 55 nm) AgNPs, independent of surface coating [67, 68]. Similarly, small-sized (1.4 nm) gold (Au)NPs induced 60 to 100 times more cytotoxicity to a variety of cells than large size (15 nm) NPs [69]. General consensus within the field is that smaller sized particles are more susceptible to cellular internalization and show more toxicity than larger ones.

Shape and charge on NMs also contributes to the toxic effects of NMs and can accelerate membrane translocation by up to 60 orders of magnitude [70]. For example, different shaped AgNPs affect human alveolar epithelial cells in different ways: Ag wires induced cytotoxicity and elevated intracellular calcium levels, whereas Ag spheres did not [71]. Furthermore, different functionalities can create different charges over NM surfaces: –COOH functionalized NMs are considered positively charged, whereas –NH2 functionalized NMs are considered negatively charged. The surface properties of NMs (e.g. hydrophobicity and hydrophilicity) affect many of the biological responses to NMs including interactions with plasma proteins, cellular uptake or phagocytosis, immune stimulation, and NM removal. Negatively charged AgNPs appears to be more potent in inducing adverse effects on the macrophages than positively charged AgNPs [72]. Similarly, studies of uncoated and coated AgNPs revealed that toxicity was highly dependent on surface charge [73]. Moreover, NMs adsorb proteins on their surface and form NP-protein coronas. Such adsorptions depend on particle size and interaction between the groups on nanoparticle surface and amine groups of proteins [74] and can alter the toxicity, fate, and stability of AgNPs [75]. Lastly, NMs have the ability to agglomerate in solutions depending on size, shape, concentration, charge, temperature, and type of NMs. Agglomerated NMs behave in a different way than the individual dispersed particles mainly because of changes in surface properties [1, 76, 77]—particularly with regards to cellular uptake and toxicity.

2.1 Environmental Impacts of AgNPs

In addition to the increasingly widespread application of AgNPs directly to the human body, the production, transport, erosion, washing or disposal of AgNP-containing products presents the potential for ecological harm [60]. At present, there are ~500 consumer products that contain AgNPs (e.g. textiles, toiletries, cosmetics, household appliances and some paints) whose production, usage and disposal will lead to environmental exposure and deleterious effects on the organisms that are exposed to these materials [29, 78–80]. Several reports indicate that AgNPs leach into water during laundering and into simulated perspiration fluids [81–83], and can be found at trace levels in aquatic environments [84, 85]. Given elemental silver's high toxicity to aquatic life—which is second only to mercury; there is a high risk associated with AgNPs in the aquatic environment. When released to the environment, AgNPs may undergo various transformations: aggregation, agglomeration, dissolution, and consequent formation of different chemical compounds (e.g. Ag sulfides and chlorides). Of these, Ag sulfide is of particular importance because it is insoluble, making it a stable compound in the environment [86]. Ag sulfides can be found in wastewater treatment plants and sometimes even in fresh water [86]. It is therefore essential to determine whether AgNPs eradicate those bacteria necessary to decompose solid wastes, and to understand the fate of AgNPs in soils and waterways when the sludge from sewage plants is utilized as fertilizer. Studies indicate that AgNPs cause developmental effects in embryonic zebrafish and size and dose dependent hemotoxicity in goldfish including hemolysis, membrane injury, lipid peroxidation, and antioxidant enzyme production [87, 88]. Other studies in fish suggest that AgNPs toxicity results from uptake into key organs and changes in cellular signaling pathways and gene expression [89, 90]. Although information on AgNPs effects in terrestrial organisms is scarce, adverse effects on growth and germination have been reported [91, 92]. In particular, crop quality and yield of wheat, corn, tobacco diminished with increasing exposures to AgNPs [91, 93, 94].

3 Strategies to Establish the Safety of NMs

Numerous studies have endeavored to evaluate the health impact of occupational and environmental exposure to select NMs. Unfortunately, toxicological analysis of new molecular entities is not a straightforward process. Because NM are relatively new, there are currently no comprehensive guidelines for assessing NMs toxicity within a given organ or system. Both 'classical' and novel toxicological assessments of NM require stepwise validation, standardization, and demonstration of their physiological relevance. The majority of the standard toxicological methods are applicable to NM; however, as NM represent physically and chemically diverse materials, the classical methods cannot always be applied without modification, and

novel approaches are often required to overcome challenges inherent to NM. For example, many NM absorb in the UV–Vis range and may even catalyze enzyme reactions or quench fluorescent dyes commonly used as detection reagents in various end-point or kinetic assays. Industry, academia, and federal agencies are cooperating to identify critical parameters in NM characterization and to establish criteria for NM-specific toxicological assays; however several challenges exist, including the importance of: (1) detection and prevention of potential particle contamination with such things as bacterial endotoxins and/or toxic synthesis by-products (e.g. heavy metals), (2) understanding how route of administration and biodistribution may result in either desirable and undesirable immunomodulation (e.g. complement activation on i.v. administration is not desirable, whereas on s.c. administration, it is beneficial for vaccinations), and (3) choosing an experimental approach that is free of false-positive or false-negative readouts. Those NM produced in the highest volume include carbon nanotubes, titanium dioxide, zinc oxide, and silver, and thus present the greatest potential for human exposure. However, human health risk is not entirely linked to production volume and probability of exposure, but also to reactivity and potency of impact on organ systems. Thus, NM produced in lower quantities that have potent and selective effects on the immune system (e.g. nickel, gold, and cobalt NM) may exhibit significant immunotoxicity. Consequently, it is necessary to assemble a more comprehensive understanding of the association between diverse NMs and the toxicological profiles.

4 Summary/Conclusions and Directions for Future Research

Over the last 30 years, the number of products containing engineered nanomaterials increased across diverse fields mainly due to our growing capacity to synthesize and manipulate such materials. Nanotechnology has generated, and will continue to generate, a wide spectrum of novel NMs that will revolutionize many fields. However, because of their unique and unpredictable physico-chemico-biological nature, it is necessary to adequately address the potential adverse health risks posed by NMs in occupational, medicinal, consumer, and environmental settings and identify those physicochemical properties and corresponding molecular mechanisms that are responsible for toxicity. Although toxicity is a significant concern for NMs exposure, the scientific literature remains quite limited given the plethora of combinations of NMs and potential surface modifications. Moreover, few studies have applied state of the art methodology regarding particle characterization and standardized study design with respect to toxicological testing. This lack of high-quality studies and insufficient, comprehensive data hinders risk assessment and grouping of NMs based on their physical, chemical and biological properties. Lastly, a comprehensive assessment of each NM will require considerable technical and financial efforts. Therefore, definitive conclusions on the toxicological risks of groups of NMs cannot be reached at this time.

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Pharmacokinetic Modelling to Study the Biodistribution of Nanoparticles

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Abstract Pharmacokinetics is a key component of pharmacology and is an essential aspect during drug discovery and development phases that evaluates the safety and efficacy profiles. Mathematical models mainly physiologically based pharmacokinetic (PBPK) models have been increasingly used that can help in drug screening and identification; dose optimisation prior to preclinical and clinical trials using *in vitro* data, thus saving time and resources. PBPK models describe the pharmacokinetic processes – absorption, distribution, metabolism and elimination (ADME) using various mathematical correlations including *in vitro – in vivo* extrapolations in humans. Nanoparticles are been increasingly used for drug delivery due to their advantages over conventional formulations such as enhanced absorption, longer half-life, good safety and efficacy, targeted delivery etc. However, studies using nanoparticles in humans can be associated with various obstacles including ethical and logistical, hindering the drug development process. PBPK models overcome the earlier mentioned problems and can evaluate various biological and molecular processes that define drug pharmacokinetics using *in vitro* data. This chapter summarises the approach of PBPK models, its challenges and possibilities to assess the key ADME mechanisms involved during various mucosal routes of administration using several allometric, anthropometric and rate equations to inform drug pharmacokinetics in humans.

Keywords PBPK · Pharmacokinetics · Mucosal · Compartmental model · ADME · Nanoparticles

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1 Introduction

Drug pharmacokinetics is an important aspect to assess the safety and efficacy of therapeutics. Pharmacokinetics is the study of drug disposition from the time of administration to its elimination in the body which is regulated by four key processes namely absorption, distribution, metabolism and elimination (ADME) [1]. Pharmacokinetic processes are identified with the help of biomarkers - substances used in the reliable measurement of any activity or a process [2]. Occasionally drug quantification at the site of action may not be practically feasible, in which case plasma concentrations can act as a biomarker to identify exposure-response relationships [3]. For most of the drugs to have a therapeutic effect, the drug concentration at the target site should be over the minimum effective concentration (MEC), however high concentrations can lead to drug accumulation and tissue toxicity (Fig. 1). Also, suboptimal adherence to the dosing regimen can lead to the drug concentration falling below the MEC level and this provides an opportunity for the pathogenic organism (virus, bacteria etc.) to develop resistance against existing therapy. Therefore, it is essential to administer optimal dosages such that the drug pharmacokinetics stay in the therapeutic range for the necessary dosing interval.

1.1 Modelling Approaches

Drug development of new chemical entities (NCEs) in humans often consumes lot of resources beginning with target identification, high-throughput screening, selection of lead candidates, preclinical experiments leading to high costs (average – USD 802 million) and long time periods (8–12 years) for the product to reach the market. Also, the average success rate of a drug in any therapeutic area reaching the market is abysmal, approximately 11% [4]. Therefore, pharmaceutical companies

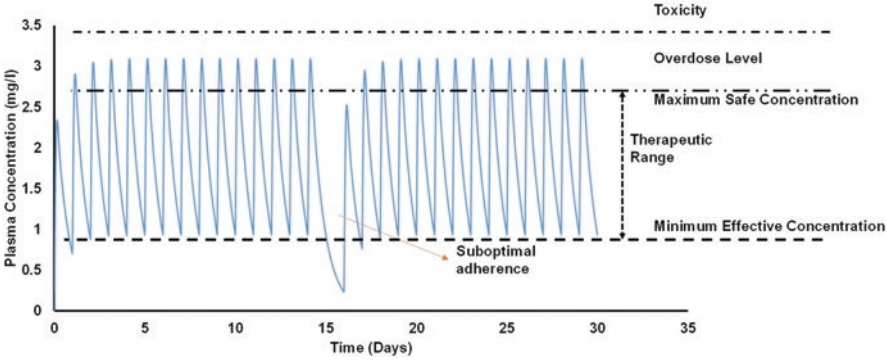

Fig. 1 A representative illustration of a once daily administered oral formulation. The blue curve represents the drug plasma concentration over time

look for an alternative solution that could accelerate this fundamental process of drug development. Pharmacokinetic computational modelling is a solution to this problem, an emerging area valuable to the field of drug discovery, drug development and the clinical study design.

Computational models can virtually identify the ADME characteristics of any given compound/formulation with physiochemical or drug-specific properties and formulation characteristics. Physicochemical and drug specific parameters can predict drug pharmacokinetic pattern, even among compounds with similar structures, pharmacokinetics can vary widely depending on a number of physiological interactions. For example, tenofovir alafenamide (TAF) and tenofovir disoproxil fumarate (TDF) are two prodrugs with the same parent molecule, tenofovir (TFV). However, the intracellular concentrations of TFV-diphosphate obtained with a 300 mg once daily (OD) TDF is comparable to an 8 mg OD TAF [5].

Models are broadly divided into two types: top-down approach and bottom-up approach, based on the nature of input data used in the models. Top-down approach involves the estimation of ADME parameters such as absorption rate, apparent volume of distribution, rate of elimination etc. based on the available pharmacokinetic data from clinical studies in human volunteers e.g. population pharmacokinetic modelling, whereas, bottom-up approach identifies drug pharmacokinetics based on the characteristics of an individual and *in vitro* data e.g. pharmacokinetic-pharmacodynamic modelling [6]. Other types of modelling also exist such as the quantitative structure activity relationship (QSAR) and quantitative structure property relationship (QSPR) models. These models inform ADME characteristics using various molecular descriptors. However, these types of models require extensive training and test data sets from similar chemical library, and they lack mechanistic description of physiological processes. Physiologically-based models that require minimal drug specific and *in vitro* data can be used to describe both ADME characteristics and also mechanistic description of the physiological processes [7].

2 PBPK Modelling

Physiologically based pharmacokinetic (PBPK) modelling is the integration of various mechanistic processes, using mathematical descriptions, in a virtual individual to simulate drug pharmacokinetics. The concept of PBPK was first introduced in the year 1920 by Teorell et al. [8], however it was not renowned back then due to the practical complexity of solving numerous mathematical equations, until the breakthrough of computers in early 1970s [9]. The use of PBPK models have been exponentially increasing and their importance was acknowledged by the U.S. Food and Drug Administration (U.S. FDA) and the European Medicines Agency (EMA) with their recent issue of guidelines for documentation of PBPK models in regulatory reports [10, 11]. Currently, regulatory approvals include a modelling component in NCE applications [12, 13]. Several PBPK software are already present in the market that are broadly divided into two different categories 1) commercial software such

as Simcyp, PK-Sim, GastroPlus etc. where the service is based on subscription at a nominal fee and 2) open software like Matlab, Berkeley Madonna etc. where the user is flexible to design their own PBPK models [14].

PBPK modelling is a bottom-up approach where various anthropometric, allometric and *in vitro* data are used to predict pharmacokinetics in humans. Models that define the whole anatomy and physiology to evaluate drug pharmacokinetics are termed as 'whole-body' PBPK models. The characteristics of an individual such as age, weight, height and body mass index (BMI) are defined initially. Using various anthropometric measurements, i.e. the earlier defined characteristics are used to describe individual organ and tissue weights such as liver, lungs, adipose, muscle etc., and blood and lymph flow rates connecting these tissues and organs [15, 16].

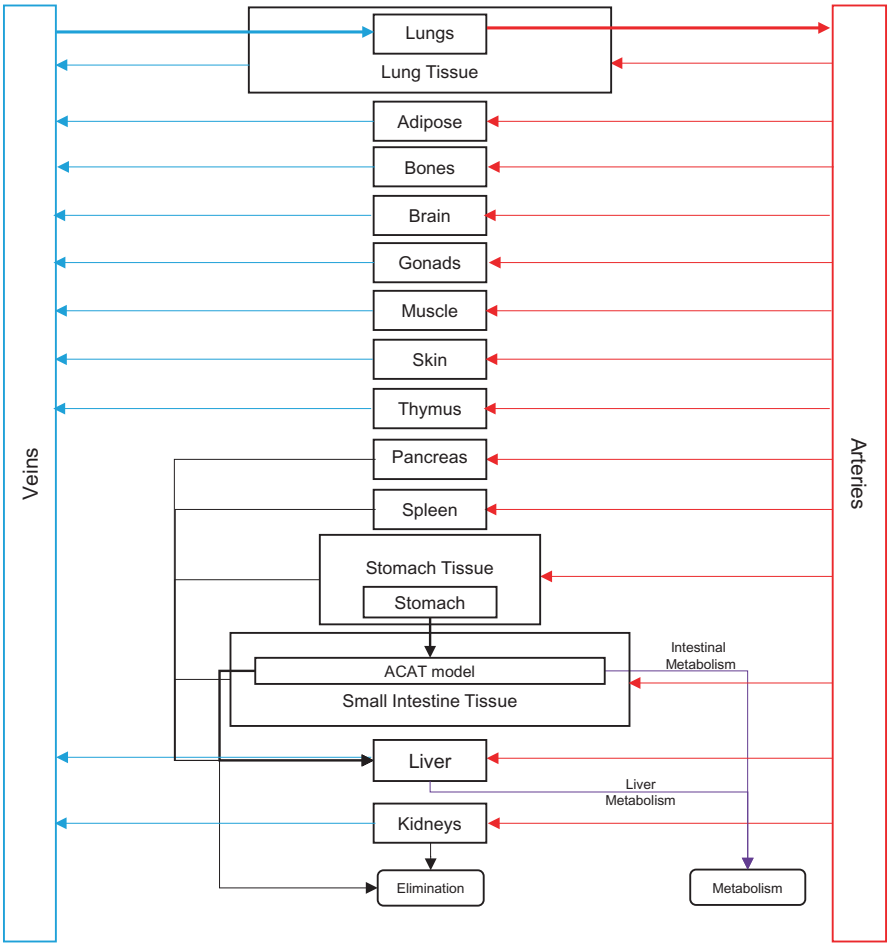

Fig. 2 Diagrammatic representation of a physiologically based pharmacokinetic model. Each box represents a tissue or an organ, the arrow mark represents the direction of drug disposition defined using first-order rate equations

The organs and tissues are represented as compartments in PBPK models, connected by first-order rate equations that describe drug disposition kinetics (Fig. 2).

PBPK models generally use *in vitro – in vivo* correlations to extrapolate data obtained from *in vitro* experiments such as Caco-2 permeability for computation of absorption rate, intrinsic clearance from baculosomes etc. to evaluate ADME parameters used in the computation of drug pharmacokinetics. Various disposition kinetics, *in vitro – in vivo* extrapolations (IVIVEs), partition coefficients used in the computation of PBPK model parameters are described in the following sections with a special focus on nanoparticles:

2.1 Absorption

Drugs can be administered through various routes including – mucosal routes such as oral, nasal, rectal, vaginal and topical; and parenteral routes e.g. intravenous, intramuscular and subcutaneous. Drugs not administered intravenously (i.e. straight into the blood stream) undergo absorption process through a passive or an active pathway. During these pathways, a fraction of the drug is unabsorbed due to numerous factors including drug solubility, dissolution rate, pH, permeability, physiological characteristics of the surrounding environment (e.g. oral, nasal, buccal etc.), food (low or high fat meal), route of administration, etc. [17, 18]. Further, in the case of nanoparticles, hydrophilic or lipophilic excipients/polymers entrapping drug molecules, size and surface charge of the nanoparticle, nanoparticle degradation, modified drug release etc. can affect drug absorption.

2.1.1 Oral Model

The small intestine is the primary site of absorption for orally administered drugs. Various mechanistic modelling approaches have been discussed in the literature for oral absorption that attempt to capture the absorption kinetics similar to a human gastrointestinal tract. Few of the compartmental models include, compartmental absorption and transit (CAT) model, advanced compartmental absorption and transit (ACAT) model, grass model, gastrointestinal transit absorption (GITA) model and advanced dissolution, absorption and metabolism (ADAM) model [19]. Each of these models have their limitations, for example, grass and ADAM models do not consider drug or nanoparticle degradation and active transport or first-pass metabolism; CAT model does not consider first-pass metabolism. The optimal models that considers most of the physiological processes are the GITA and ACAT model. The commonly used ACAT model captures detailed drug absorption from the small intestine by dividing it into seven compartments represented by the different parts of the small intestine i.e. the duodenum, jejunum and the ileum (as shown in Fig. 3). For an orally administered drug, beginning from the stomach, the drug traverses through the different sections of the small intestine depending on the residence

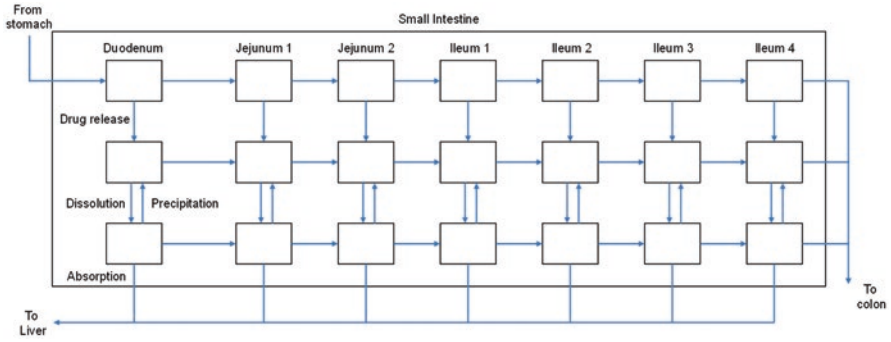

Fig. 3 Schematic of the advanced compartmental absorption and transit (ACAT) model

times in each section. The residence time largely varies in the absence or presence of food. Lateral drug disposition through the small intestine is described using first-order mass action kinetic equations (shown in eqs. 1–3) [20].

$$\frac{dM_s}{dt} = -K_s M_s \tag{1}$$

$$\frac{dM_n}{dt} = K_t M_{n-1} - K_t M_n \tag{2}$$

$$\frac{dM_c}{dt} = K_t M_n \tag{3}$$

Where M_s is the amount of drug present in the stomach at a given time t, M_n is the amount of drug in the *n*th compartment, K_s and K_t are the rate constants for stomach and small intestine transit. The rate constants are the inverse of the mean residence time in each section of the small intestine.

According to the U.S. FDA, drugs have been classified based on biopharmaceutical classification system (BCS) into four different combinations of high/low permeability/solubility [21]. ACAT model captures the drug release rate from the formulation and drug dissolution rate, which can be identified using *in vitro* experiments. Drug release rate from the formulation can be identified using dialysis experiments in rapid equilibrium dialysis systems [22]. Soluble and insoluble drug is in equilibrium, depending on the amount of water present in each compartment of the small intestine. It should be noted that drug dissolution from the formulation in the stomach and the small intestine can lead to variable drug absorption. This is described using additional compartments denoted for dissolution and precipitation as shown in Fig. 3. The rate of drug dissolution depends on a few factors as described in eq. 4 [23].

$$K_{(n)d} = \frac{3\gamma\left(C_s - C_{(n)L}\right)}{\rho r T} \tag{4}$$

where $K_{(n)d}$ is the dissolution rate constant for the nth compartment, γ is the molecular diffusion coefficient, Cs is the drug solubility in water, $C_{(n)L}$ is the lumen concentration for the nth compartment, ρ is the density, r is the effective radius of the molecule, T is the thickness of the diffusion layer.

The intestinal wall acts as a barrier to absorption of unwanted substances or pathogens from the gut and selectively absorbs essential nutrients. Passive diffusion of small and lipophilic molecules crossing the intestinal epithelial cells through lipid bilayers is evident; however, nanoparticle absorption is complicated. Mucus is the first layer encountered on all the epithelial surfaces not covered by the skin and heavily glycosylated mucins can block the absorption of certain nanoparticles [24]. Transport across intestinal epithelial cells depends on factors that include cell surface binding, endocytosis and exocytosis with M-cells receiving particular attention with their ability to allow nanoparticles through transcytosis [25].

Various methods have been defined to obtain an IVIVE to identify the effective permeability across the intestinal barrier [26]. Molecular descriptors such as polar surface area (PSA), molecular weight, number of hydrogen bond donors (HBD), octanol-to-water ratio ($P_{o:w}$) have been identified to describe permeability across the apical to basolateral membrane [21]. Winiwarter et al. have described an equation to identify the effective permeability (P_{eff}) using PSA and HBD values of 22 compounds and is represented in eq. 5 [27]. Other methods include the computation of P_{eff} from *in vitro* apparent permeability studies using Caco-2 (P_{app}) and MDCK cell monolayers, given in eq. 6 [28] and 7 [26].

$$\log P_{eff} = -2.546 - 0.011 \times PSA - 0.278 \times HBD \tag{5}$$

$$\log P_{eff} = 0.6836 \times \log P_{app} - 0.5579 \tag{6}$$

$$P_{app} = 0.746 \times P_{app,MDCK} \tag{7}$$

The rate of absorption from the small intestine (K_a) is directly proportional to the P_{eff} and inversely proportional to the radius of the small intestine (r_{si}), given as:

$$K_a = \frac{2 \times P_{eff}}{R} \tag{8}$$

The ACAT model has been further extended to include sublingual delivery to describe drug absorption from the oral cavity as shown in Fig. 4, named as the oral cavity compartmental absorption and transit (OCCAT) model [29]. Similar mass-transfer first-order kinetic equations described earlier in eqs. 1–3 can describe the drug disposition from one compartment to the other.

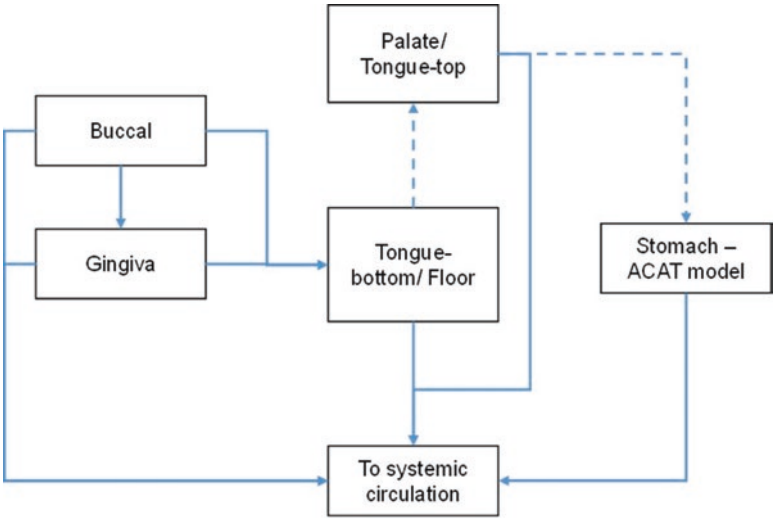

Fig. 4 Oral cavity compartmental absorption and transit model

2.1.2 Respiratory Model

Respiratory delivery is an attractive route of administration due to its ease of access to the systemic circulation and the large surface area available in the lungs for drug absorption. Physiological representation of drug transport through the respiratory system is complicated due to various complexities associated with the flow dynamics in lungs. Again, mucus plays a key role to trap any aerosolised particles. Nanoparticle size is the major determinant that dictates the fraction of delivered dose to the alveolar region [30]. Jaworski et al. has described the transport of air and particles through the respiratory tract using a ventilation-perfusion gradient that accounts for factors such as inhalation volume, fraction reaching the alveoli, permeation through alveolar-capillary membrane, cardiac output etc. to capture the kinetics in detail [31]. A detailed permeation map for an inhaled drug reaching the systemic circulation is represented in Fig. 5. The drug transport through these various layers can be a passive or an active process and is expressed as a mass transfer first-order equation simplified as follows [32]:

$$\frac{dM_i}{dt} = A_{i-1}J_{i-1} - A_iJ_i \tag{9}$$

where M is the amount of drug present in the current layer i, A is the surface area of the layer exposed to the drug, J is the flux (rate of drug transfer per unit area) from the upper to the lower layers. The above equation can be further modified to include efflux ratio from one layer to the other, thickness of the layer and drug affinity to each layer, based on the availability of the *in vitro* data and the desired accuracy in detail.

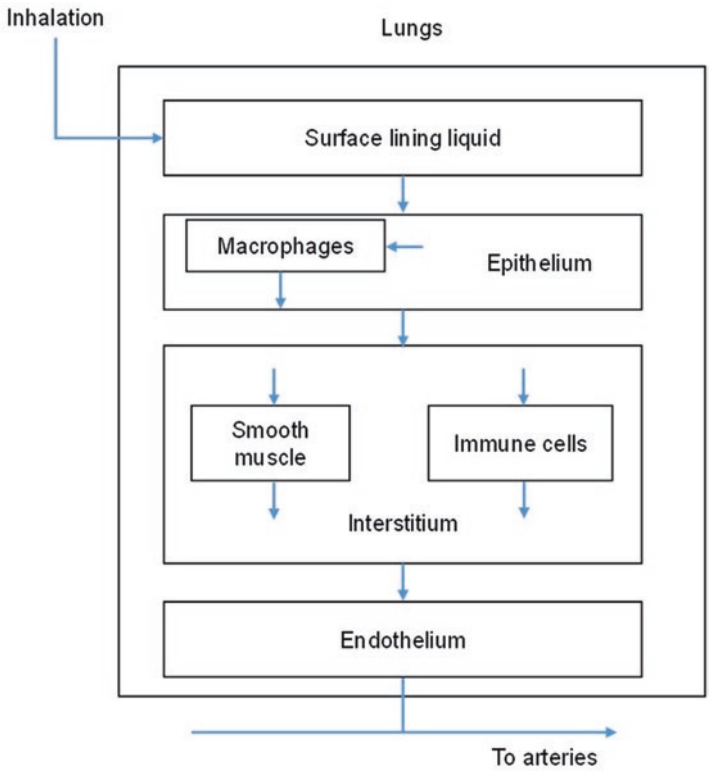

Fig. 5 Schematic representation of the physiological description of drug absorption from the lung tissue

2.1.3 Ocular Model

Ocular drug delivery is one of the most challenging routes of delivery due to the presence of unique barriers and a low surface area. Representation of drug administration through the ocular route necessitates the understanding of complex physiological processes that are encountered during drug distribution in the eye. A detailed ocular compartmental absorption and transit (OCAT) model was developed to inform the drug pharmacokinetics of fluorometholone in albino rabbits (Fig. 6) [33]. Various compartments were used to denote the cornea, aqueous and vitreous humor, conjunctiva, sclera, iris-ciliary body and distribution through the nasolacrimal route. The OCAT model can assess the drug concentrations in each layer and inform the disposition kinetics for local or systemic delivery. Since eye is a sensitive organ, *in vitro* data used in the model should be thoroughly evaluated to obtain relatively good predictions.

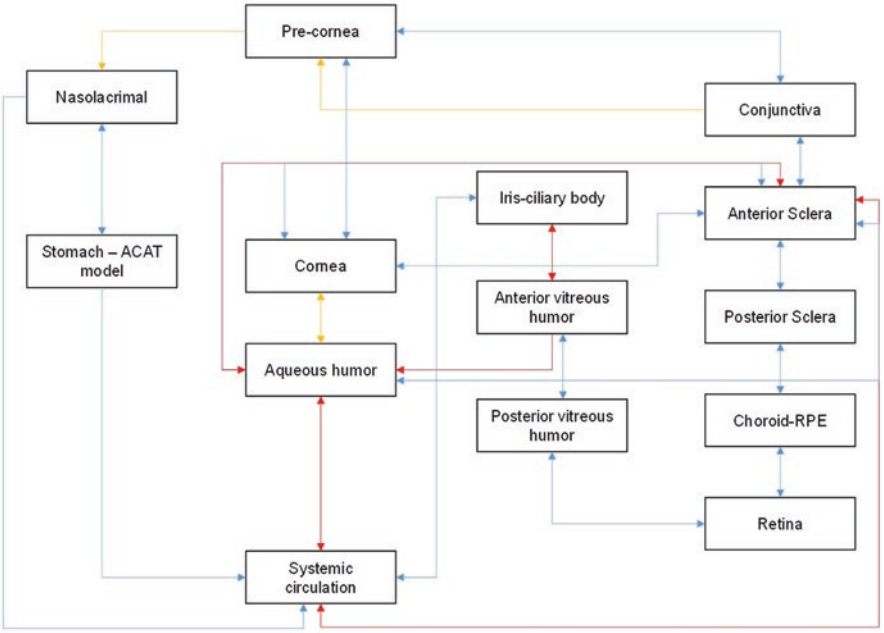

Fig. 6 Ocular compartmental absorption and transit model. Blue lines represent permeability through different compartments, red and orange lines represent the flow of intraocular fluid

2.1.4 Vaginal and Rectal Models

Vaginal and rectal drug delivery are relatively infrequent routes of administration, however they can be useful if the individual cannot be administered using alternative routes especially in paediatrics or geriatrics. Rectal models are still yet to be investigated, however, a vaginal PBPK model has been developed to identify dapivirine ring concentrations, which is used in prevention strategies for protection against HIV [34]. The model consists of compartments defining the drug permeation across the vaginal luminal fluid, vaginal epithelium, stroma tissue and stroma blood to the systemic circulation (Fig. 7). Apparent permeability and *in vitro* studies using Franz cell model were used to identify the effective permeability rates across different barriers to evaluate drug concentrations for local and systemic delivery.

2.2 Distribution

Drug absorbed through the various mucosal routes reaches the blood *i.e.* the circulatory system which plays a key role in drug transportation across the human body. Drug distribution describes the amount of drug disseminated to each organ or tissue.

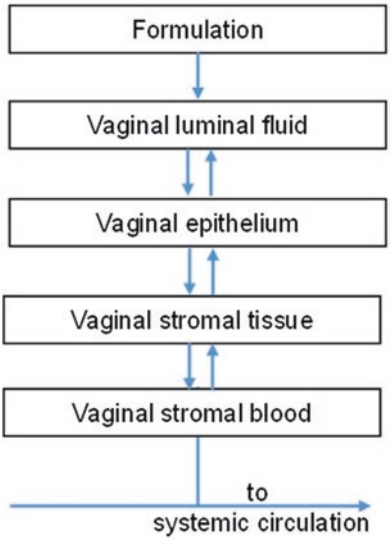

Fig. 7 Ocular compartmental absorption and transit model. Blue lines represent permeability through different compartments, red and orange lines represent the flow of intraocular fluid

This data is important to measure drug safety and toxicity and identify cases of drug accumulation in a particular organ or tissue. The composition of a tissue or organ varies from blood components depending on water content, amount of neutral lipids and phospholipids due to which drug partition and distribution differs across tissues. The tissue-to-plasma ratio of adipose and non-adipose tissues are given as follows [35]:

$$P_{t:p\,adipose} = \frac{\left[D_{vo:w} \times \left(V_{nlt} + 0.3 \times V_{pht}\right) + 1 \times \left(V_{wt} + 0.7 \times V_{pht}\right)\right]}{\left[D_{vo:w} \times \left(V_{nlp} + 0.3 \times V_{php}\right) + 1 \times \left(V_{wp} + 0.7 \times V_{php}\right)\right]} \times fu_p \tag{10}$$

$$P_{t:p\,nonadipose} = \frac{\left[P_{o:w} \times \left(V_{nlt} + 0.3 \times V_{pht}\right) + 1 \times \left(V_{wt} + 0.7 \times V_{pht}\right)\right]}{\left[P_{o:w} \times \left(V_{nlp} + 0.3 \times V_{php}\right) + 1 \times \left(V_{wp} + 0.7 \times V_{php}\right)\right]} \times \frac{fu_p}{fu_t} \tag{11}$$

where $D_{vo:w}$ is the olive oil: buffer partition coefficient of non-ionised or ionised drug at pH 7.4, V is the volume fraction of neutral lipids (nl), phospholipids (ph) and water (w) in tissue (t) and plasma (p), *fu* is the unbound fraction, $P_{o:w}$ is the n-octanol: buffer partition coefficient.

Nanoformulation distribution can vary based on the characteristics of the nanoparticle, the route of administration and the characteristics of the individual (weight, height, BMI *etc.*). Distribution can be affected by various physiological processes such as enhanced permeability and retention (EPR) effect, target-mediated disposition, lymphatic transport, affinity towards a specific cell/tissue type, *etc*. EPR effect, commonly observed in solid tumours or inflamed tissues can lead to tissue accumulation of nanoparticles depending on their size, blood-flow rate to the

tissue, tumour vasculature and intratumoral pressure [36]. Distribution can also be affected by the interaction of nanoparticles to various biomolecules and proteins with the formation of a protein corona [37]. Nanoparticle characteristics such as size, shape, surface area and charge can influence the formation of the protein corona. Specific proteins such as opsonins adsorbed by the nanoparticles inform their presence and assist their uptake by phagocytic cells. Opsonisation and protein corona formation can majorly influence the biodistribution of nanoparticles. Macrophages during opsonisation process can remove the targeting moieties thereby reducing the specificity of the nanoparticles [38]. For example, a solid drug nanoparticle designed by McDonald et al. enhances drug absorption, however this phenomenon does not affect distribution and clearance patterns as it only increases the amount of drug permeating the intestinal wall [39]. Drug distribution comparison studies of an antineoplastic drug, mitoxantrone as a free drug and drug encapsulated in liposomes and nanoparticles showed higher concentrations in liver and spleen for liposomes and in tumour, heart and spleen for nanoparticles compared to the free drug [40]. Since liposomes are high in lipids, they are more attracted to the fat tissue present in the liver, and nanoparticles due to their smaller size compared to liposomes are distributed more in the tumour due to EPR effect.

Distribution kinetic model for small molecules can be blood-flow limited or diffusion-limited. Blood-flow limited models as the name suggests limits the distribution of drug depending on the blood-flow rate. In a blood-flow limited model, the drug is assumed to have instant and uniform distribution once it reaches the tissue. In the case of diffusion-limited model, the rate limiting step is the diffusion component that controls drug perfusion across the different tissue layers and takes longer to reach equilibrium across the tissue [41].

In a blood-flow limited model, drug distribution depends on the rate of blood flow to the particular organ, drug protein binding fraction, blood-to-plasma ratio, octanol-to-water ratio (log P), pH, water and lipid content in the individual [35]. Drug escaping the first pass metabolism, through the pulmonary system, reaches the arteries. The drug concentration in lungs, arteries and veins is described as follows [42]:

$$\frac{dC_{lu}}{dt} = \frac{Q_{lu}}{V_{lu}}\left(C_{ve} - \frac{C_{lu} \times R}{fu_p \times P_{t:plu}}\right) \tag{12}$$

$$\frac{dC_{ar}}{dt} = \frac{1}{V_{ar}}\left[Q_{lu}\left(\frac{C_{lu} \times R}{fu_p \times P_{t:plu}} - C_{ar}\right)\right] \tag{13}$$

$$\frac{dC_{ve}}{dt} = \frac{1}{V_{ve}}\left[\sum \frac{Q_t \times C_t \times R}{fu_p \times P_{t:pt}} - Q_{lu} \times C_{ve}\right] \tag{14}$$

where C is the drug concentration in the non-eliminating organ or tissue (ne), arteries (ar) at time t, Q is the blood-flow rate to that organ, V is the volume of the organ, R is the blood-to-plasma ratio

Drug distribution from the arteries to non-eliminating organs or tissues is described in eq. (15) whereas from kidneys (ki) and liver (li) are described in eq. (16) and (17) respectively [42].

$$\frac{dC_{ne}}{dt} = \frac{Q_{ne}}{V_{ne}}\left(C_{ar} - \frac{C_{ne} \times R}{fu_p \times P_{t:pne}}\right) \tag{15}$$

$$\frac{dC_{ki}}{dt} = \frac{Q_{ki}}{V_{ki}}\left(C_{ar} - \frac{C_{ki} \times R}{fu_p \times P_{t:pki}}\right) - \frac{C_{ki} \times K_e}{P_{t:pki}} \tag{16}$$

$$\frac{dC_{li}}{dt} = \frac{1}{V_{li}}\left(Q_{ha} \times C_{ar} + \sum \frac{Q_i \times C_i \times R}{fu_p \times P_{t:pi}} - \frac{Q_{li} \times C_{li} \times R}{fu_p \times P_{t:pli}} - C_{li} \times CL_h\right) \tag{17}$$

where K_e is the renal elimination rate constant, ha is the hepatic artery, *i* refers to the gut, pancreas, spleen and stomach, CL_h is the hepatic clearance, *ar* stands for the arterial compartment, Q is the blood-flow rate to the tissue/organ, V is the volume of the tissue, $P_{t:p}$ is the tissue to plasma ratio.

In a tissue-limited model, the flow kinetics across a tissue are divided into two compartments that define the vascular and extravascular spaces [43] given by:

$$\frac{dC_i}{dt} = \frac{Q_i}{V_i} \times \left(C_{ar} - \frac{C_i}{P_{t:pi}}\right) - \frac{PS}{V_i} \times \left(C_i - \frac{C_j}{P_{t:pi}}\right) \tag{18}$$

$$\frac{dC_j}{dt} = \frac{PS}{V_j} \times \left(C_i - \frac{C_j}{P_{t:pi}}\right) \tag{19}$$

where C_i is the concentration, PS is the permeability-surface area product, *i* is the drug in the vascular compartment, *j* is the drug in the extravascular compartment.

Blood-flow limited model can be used for small molecules or nanoformulations where passive diffusion plays a major role whereas tissue-limited model can be used for large molecules or target-mediated nanoparticles where permeation can be restricted by specific factors such as size, shape or charge.

2.3 Metabolism and Elimination

Drug intrinsic clearance can be obtained from *in vitro* experiments in baculosomes or human liver microsomes. This is scaled up using the total abundance level of the specific isoform, amount of protein per gram of liver (MPPGL), and the liver weight (LW) [44] as:

$$TCL_{\text{int}} = \sum \frac{V_{\max,i}}{K_{m,i} \times fu_{mic,i}} \times ISEF_i \times Abundance_i \times MPPGL \times LW \tag{20}$$

where TCL_{int} is the combined intrinsic clearance from all the isoforms involved in the metabolism of the drug, V_{max} is the maximal rate of metabolism, K_m is the Michaelis-Menten constant, fu_{mic} is the fraction unbound drug in microsomal incubations, ISEF is the scaling factor to compensate the difference between recombinant and hepatic systems, *i* is the isoform involved in drug metabolism (CYP or UGT or any other isoform).

The hepatic clearance (CL_h) using the *in vivo* intrinsic clearance, TCL_{int} is expressed as [45]:

$$CL_h = \frac{Q_h \times fu_p \times TCL_{\text{int}}}{Q_h + fu_p \times TCL_{\text{int}}} \tag{21}$$

where Q_h is the blood flow rate through the hepatic vein

Renal elimination of drugs or nanoformulations through the process of glomerular filtration in the kidneys is given [42] as:

$$Renal\ clearance = \frac{C_{ki} \times K_e}{P_{t:p\,ki}} \tag{22}$$

Clearance of the free drug follows the traditional pathway; however, metabolism of nanoparticles can vary based on the physicochemical properties. Biodegradable nanoparticles can be metabolised by chemical degradation, enzymatic metabolism or renal and biliary elimination [46, 47]. However, non-biodegradable particles can take longer time periods before elimination. Clearance can also vary based on *in vivo* pH conditions, aerobic/ anaerobic processes, tissue specific enzyme activity. *In vitro* experiments can be used to investigate the relevant processes underpinning nanoparticle metabolism [36].

Nanoparticle size and charge can affect the clearance, with particle size less than 8 nm or < 5 KDa for polymeric nanoparticles rapidly cleared by the kidneys through glomerular filtration and negatively charged particles are not cleared through the negatively charged glomerular capillary membrane due to the repulsive force between the particles and filter [38]. Some nanoparticles can be solely drug carriers, where drug is entrapped in the nanoparticle and released over time with zero- or first-order kinetics. This prolongs the drug half-life with lower free drug concentrations in the blood plasma, in which case, the model should include the release rate of the drug from the nanoparticle and their clearance component simultaneously to simulate the combined effect of these processes. Pharmacokinetic comparison between six unformulated drugs (9-nitrocamptothecin, camptothecin, clozapine, and cyclosporine, epirubicin and vinpocetine,) and drugs encapsulated in nanoparticles significantly reduced the apparent drug clearance and enhanced the drug con-

centrations in studies conducted in rats and dogs [48]. Therefore, appropriate *in vitro* experiments should be performed to investigate the underlying clearance pathway of nanoformulations.

2.4 Bioavailability

Drugs metabolised by CYP3A are also cleared at the intestinal metabolic site and the fraction available after the intestinal metabolism (F_g) is given as:

$$F_g = \frac{Q_{gut}}{Q_{gut} + fu_{gut} \times CL_{\text{int},g}} \tag{23}$$

$$CL_{\text{int},g} = CL_{\text{int}} \times CYPabundance \tag{24}$$

Q_{gut} is the blood flow rate through the gut, fu_{gut} is the fraction unbound in the gut, $CL_{int,g}$ is the total intrinsic clearance of CYP3A in the liver.

Fraction of drug escaping the first-pass metabolism (F_h) is given as:

$$F_h = 1 - \frac{CL_h}{Q_h} \tag{25}$$

Absolute bioavailability (F) is the multiple of dose fraction absorbed from the intestine (F_a), fraction of absorbed dose escaping the intestinal metabolism (F_g) and the fraction of dose escaping first-pass metabolism through the liver (F_h) [49].

$$F = F_a \times F_g \times F_h = \frac{AUC_{roa} / Dose_{roa}}{AUC_{iv} / Dose_{iv}} \tag{26}$$

AUC is the area under the plasma concentration – time curve and dose is the amount administered, *roa* is any route of administration, *iv* is the intravenous administration.

Bioavailability of nanoparticles also depend on the values of F_a, F_g and F_h, which are further dependent on other parameters, as described in the equations above.

2.5 Model Qualification

Mathematical representation of physiological processes is one of the ways to identify trends across different compounds, populations and species, and this helps in building extrapolations that can be used for predictions of NCEs or novel formulations. The IVIVE equations should ensure that the extrapolated data predicted in

humans is comparable to the *in vivo* data with a good correlation coefficient ($R^2 > 0.8$). This ensures that the input data in the model is of high quality and reliable for good prediction accuracy. Since various IVIVE equations are used in the PBPK model simultaneously, the reliability of the model decreases as the R^2 value of IVIVE equations deviates from one [50]. Therefore, the simulated data obtained from the models are generally compared against observed data to check its reliability and this process is termed as 'qualification'.

Since modelling and simulation is a relatively recent approach to investigate drug disposition, in 2016, EMA has provided a few guidelines for model qualification [10]. However, there is still a dilemma in the way models are qualified. A summary of various PBPK models assessed in a study shows the broad acceptance criteria used for qualification varying from <30% to <two-fold from the mean observed pharmacokinetic data such as the AUC, maximum concentration and trough concentration [14]. This wide range in the acceptance criteria can question the integrity of the PBPK model and the reliability of the predictions especially at higher-fold difference from the observed values. Qualification of nanoparticles, although not standardised by either EMA or FDA, could presume to follow the same PBPK guidelines that exist for small molecules. However, for the qualification of PBPK models involving nanoparticles, further complexity is encountered that would require additional *in vitro* and *in vivo* data. Adequate data such as the description of the behaviour of nanoparticles in various organs and tissues, cellular penetration if any, drug release kinetics from the nanoparticles in different environments (various cell types, biological fluids), etc. would be necessary for model qualification and the lack of animal and human data can further complicate the model qualification. In order to avoid the problem with the requirement of extensive data for a detailed model, a minimal PBPK model with a few essential compartments that sufficiently addresses the problem at hand could be used instead [36].

Currently, there are numerous ways to obtain a certain parameter value from *in vitro* data and since these experiments are not standardised for PBPK models, high variability is observed across literature for the same set of parameters. As the modelling component in drug development is on the rise, it is essential to have appropriate regulations in place for reliable qualification such that there are standards in place that ensure confidence in the models. *In vitro* assessment of nanoparticles should follow the same and standardise *in vitro* methods that provide consistent outputs in order to minimise the variability thereby improving the quality of the input data for the PBPK models to generate superior predictions [51].

2.6 PBPK Modelling of Nanoparticles

PBPK modelling of nanoparticles follow the same principles as previously described that inform the ADME processes. However, a replica of the compartments as described in the sections above should be introduced to the various absorption and distribution models to include the description of drug release from the nanoparticle

in one set of compartments and the other for the dissolved drug that describe its transition through the different mucosal layers [52]. Only a handful of examples can be found in the literature that incorporate a PBPK model to describe absorption through the mucosal routes since additional *in vitro* data is required to compute parameters such as the rate of drug disposition from the different compartments during absorption, dissolution rate of the nanoparticle in various tissues and at different pH, tissue to plasma partition ratios, unbound fraction, accumulation etc. would be necessary to describe nanoparticle disposition.

A PBPK model describing administration of silver nanoparticles through dermal, inhalation and oral route in rats and humans was published where the PBPK models were divided into a replica of two compartment models – one, to describe the ionic silver and the other for nanoparticulate silver, where the nanoparticulate silver slowly dissolves to the ionic form over time. Several assumptions were made in the study and the model was minimised (due to the unavailability of physiochemical properties of silver nanoparticles essential for modelling) however, using the observed data and curve fitting, these parameters were estimated. The disposition of ionic silver was successfully predicted with varying sizes of silver nanoparticles [53]. Another study by the same group similar to the previous study describes the disposition of titanium dioxide nanoparticles in humans with a physiological description of its oral absorption and the nanoparticle uptake by the mononuclear phagocyte system for phagocytosis at various particle sizes using a PBPK model [54]. Kumar and Singh have developed sustained release silk fibroin-casein nanoparticles loaded with carvedilol and the pharmacokinetics were assessed in rats through the oral route using Gastroplus™ which showed an improved pharmacokinetic profile with an increase in C_{max} and bioavailability by 2.04-fold and 6.87-fold respectively in rats showing its superior absorption profile for potential use in humans [55]. A two-compartment (plasma and peripheral) absorption model was described by Jung et al. where drug release rate of nanocarriers from different *in vitro* dissolution experiments were used to predict the output from PBPK model, which was then compared against *in vivo* pharmacokinetic data [56]. This study informs the necessity of standardising *in vitro* experiments to obtain reliable input data for PBPK models, highlighting the importance of the quality of the in vitro data.

3 PBPK and Nanotechnology Possibilities and Challenges

Application of PBPK modelling can be broadly classified based on factors such as drug-drug interactions, genetics, population, target tissue penetration and type of formulation. Drug-drug interaction studies can be complicated in patients with co-infections and PBPK modelling can be useful to understand the pharmacokinetic behaviour of multiple scenarios that are difficult to evaluate clinically [57]. Genetics is another factor that can change the pharmacokinetic behaviour of drugs. Individuals with polymorphisms in N-acetyltransferase 2 (NAT2) enzyme have either rapid or slow clearance rate of isoniazid, an anti-tuberculosis agent [58]. Paediatric popula-

tion need a fraction of the adult dose and these dose optimisation studies can be computed based on the individual or target population. Drug penetration in tissues depend on the physicochemical properties, drug specific *in vitro* data, formulation characteristics etc. and variation in any of these parameters can alter drug pharmacokinetics which can be captured using PBPK models [57].

Reformulation strategies with nanoparticles often have a positive effect on drug pharmacokinetics. Problems associated with conventional formulations such as insufficient absorption or targeted diffusion in tissues can be addressed using nanoformulations, however, efficacy and toxicity can also have a negative effect on nanoformulations: high affinity or accumulation towards a specific cell type could lead to potential tissue-specific toxicity. The ADME mechanistic processes have to be thoroughly investigated to understand the interaction of nanoparticles with the human body, thus ensuring safe and optimal delivery of nanoformulations. Several nanoparticle characteristics can vary the absorption and distribution patterns depending on the composition of the nanoformulation [59]. Nanoformulations clearly have alternative ADME mechanisms compared to conventional formulations. Every nanoparticle is unique in its own way and their behaviour is unpredictable in the physiological environment which complicates the administration in humans due to their questionable safety and efficacy. It is quite essential to evaluate the safety and efficacy *in vitro,* identify potential ADME pathways prior to their modelling. PBPK modelling can be a useful tool to predict the ADME characteristics of nanoformulations *in vivo* through various administration routes. EMA and U.S. FDA have acknowledged the use of PBPK modelling in NCE applications which represents a positive step to modelling and simulation approaches supplementing drug development process [10, 11]

Since PBPK models are bottom-up approaches, the quality of the predicted output typically relies on the quality of the *in vitro* input data. Most of the IVIVEs derived in the literature have used a low number (<50) of compounds [26, 28, 35] and this questions the accuracy in the extrapolations for new compounds or compounds that are not similar to the test dataset. Use of several IVIVEs having low reliability on the extrapolated values can reduce the quality of the predicted outcomes. Nanoparticles add an additional layer of complexity to this fundamental problem since their behaviour can be unpredictable in biological matrices especially *in vivo* where there is simultaneous interaction with multiple proteins, tissue-specificity, EPR effect, macrophage uptake, degradation etc. Since no two nanoparticles are the same, deriving an IVIVE across various nanoformulations synthesized using diverse polymers can be complicated. Also repeated dosing of nanoformulations can raise concerns over the behaviour of nanoparticles that alter their ADME behaviour and this may not be captured using PBPK models [36]. Extensive *in vitro* tests that can understand the correlation between the utilization of different materials would be necessary to address the concerns of PBPK modelling of nanoparticles.

4 Conclusion

Pharmacokinetic modelling is an emerging application in the drug development process and is still in its initial phase for pharmacokinetic assessment of nanoparticles. Unlike conventional formulations, nanoparticle behaviour can change *in vivo*, therefore extensive *in vitro* studies have to be performed to understand the nanoparticle physical modifications and ADME behavioural changes prior to their implementation in PBPK models. PBPK model qualification primarily depends on the quality of the input data which underlines the importance of reliable IVIVEs. The use of PBPK modelling has been exponentially increasing, and with it the amount of *in vitro* data generated. This data from IVIVEs combined with the understanding of nanoparticle ADME processes can improve confidence in model predictions thus saving time and resources during drug development.

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Index

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