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Systemic Delivery Technologies in Anti-Aging Medicine: Methods and Applications

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Rapidly changing demographics worldwide towards increased proportion of the elderly in the population and increased life-expectancy have brought the issues, such as "why we grow old", "how we grow old", "how long can we live", "how to maintain health", "how to prevent and treat diseases in old age", "what are the future perspectives for healthy ageing and longevity" and so on, in the centre stage of scientific, social, political, and economic arena. Although the descriptive aspects of ageing are now well established at the level of species, populations, individuals, and within an individual at the tissue, cell and molecular levels, the implications of such detailed understanding with respect to the aim of achieving healthy ageing and longevity are ever-changing and challenging issues. This continuing success of gerontology, and especially of biogerontology, is attracting the attention of both the well established academicians and the younger generation of students and researchers in biology, medicine, bioinformatics, bioeconomy, sports science, and nutritional sciences, along with sociologists, psychologists, politicians, public health experts, and health-care industry including cosmeceutical-, food-, and lifestyle-industry. Books in this series will cover the topics related to the issues of healthy ageing and longevity. This series will provide not only the exhaustive reviews of the established body of knowledge, but also will give a critical evaluation of the ongoing research and development with respect to theoretical and evidence-based practical and ethical aspects of interventions towards maintaining, recovering and enhancing health and longevity.

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Preface

Aging is a process associated with progressive accumulation of deleterious changes (ranging from cell senescence to a decline in immune functioning and hormonal secretion) over time, leading to a loss of function in multiple tissues and an increase in the probability of death. Over the years, extensive efforts have been paid by researchers to deciphering the mechanisms of aging and treating different types of age-associated diseases. The possible link between aging and diverse physiological processes (including telomere shortening, accumulation of free-radical mediated tissue damage, and random cross-linking among biomolecules) has also begun to be recognized. Such an understanding of aging has been further enhanced recently by rapid advances in computational technologies, which make the attainment of genome-wide expression profiles of diverse tissues from individuals of different ages possible. This greatly enhances the efficiency of the identification of age-related genes, and has led to the discovery of copious potential targets that may subsequently be used for manipulation of the aging network via both genetic and non-genetic means.

The gap between basic aging research and intervention development is a major obstacle that has to be overcome before biogerontological interventions can be put into practice. Regarding the fact that aging is a systemic degenerative process, the availability of technologies that enable cells and tissues in a fully developed adult body to be manipulated systemically are in dire need. As far as cell and tissue manipulation is concerned, delivery technologies find great importance because they are the ones that enable the initiators of biological effects to get to the proper site of action. Since the turn of the last century, significant advances have been achieved in the design of delivery technologies. Advances in the development of delivery systems, along with the possibility of achieving spatialtemporal confinement of intervention execution via proper carrier design, have opened up new possibilities for the attainment of interventions to tackle a range of diseases, ranging from cancer and cardiovascular diseases to neurodegeneration and diabetes

mellitus. Despite this, the delivery efficiency of most of the existing technologies varies from tissue to tissue. This impedes the successful implementation of interventions that require cells or tissues to be manipulated bodywide.

Regarding the importance of systemic delivery technologies in the development and execution of anti-aging interventions, and the lack of books and serious discussions currently available in the field on this important topic, this edited book intends to fill this gap by comprehensively revisiting the latest advances in the chemistry and engineering of technologies for systemic therapeutics delivery, with the strengths and limitations of those technologies being explored in the context of anti-aging medicine. The content of this book is separated into six part. Part I offers an overview of the need of systemic delivery technologies to the development of anti-aging therapies, and also provides an introduction to representative experimental approaches that will be required when a technology is designed and characterized to meet the need of systemic therapeutics delivery. In Part II, III, and IV, recent advances in different strategies that may enable systemic delivery to tackle aging and related diseases will be presented. Representative practical strategies to engineer and optimize the performance of delivery technologies for applications in systemic delivery, along with their working principles, will be discussed in Part V; whereas in the last part, major technical and biological barriers that have to be overcome will be presented for the transition of delivery technologies from the laboratory to reality for applications in systemic delivery to tackle aging and age-associated diseases.

Contrary to other edited books which are presented simply as a collection of reviews that target advanced researchers, this edited book contains several special features, making it suitable not only to those familiar with the field but also to readers who are relatively new to this research area. One feature is the “Glossary” section provided in each chapter. It intends to make the content of each chapter more accessible to readers who may not be that familiar with the terminology and abbreviations used in the field. Another feature is the “Important Notes”, which concisely convey to readers the take-home-messages and recent advances in the area to be covered by the chapter. Finally, at the end of each chapter, this is a “Questions for Future Research” section, which delineates some of the important yet unsolved questions to be addressed for future research. Because of the multidisciplinary nature of the topic covered by this edited book, our book is anticipated to be an appeal to advanced undergraduate- and graduate-level students training in pharmaceutical sciences and geriatric medicine, and those with an interest in the design and development of delivery technologies.

Here I would like to thank all of the authors who have contributed chapters to this publication. We are grateful to them not only because of their support and efforts, but also because of their responsiveness and patience to our editing. A number of people have reviewed chapters of this book. We want to acknowledge

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Part I
Fundamentals and Experimental Techniques in Systemic Delivery

Chapter 1
Systemic Delivery in Anti-aging Medicine: An Overview

Yi Wang and Wing-Fu Lai

Abstract Extension of longevity is no longer a science fiction and has been in progress of becoming scientifically achievable. The focus of current anti-aging strategies has shifted from geriatrics that is cost-effective but palliative to biogerontology that is fundamentally at the molecular level. Basic gerontological research has suggested that biological aging is closely associated with genetic/genomic factors, which has led to the development of gene therapies such as RNA-interference technology. This resulted in a subsequent need for developing reliable drug delivery systems. Numerous advanced systemic drug delivery systems have hitherto been developed but a number of challenges need to be conquered in order to make the systems practical, such as safety and effectiveness issues. For this reason, a considerable number of biogerontological intervention technologies have been taken to clinical trials but with limited success. As the first chapter of this book, this chapter will illustrate the role and limitations of technologies for systemic delivery in anti-aging medicine.

Keywords Anti-aging · Biogerontology · Drug delivery · Nanoparticles · RNA interference

1.1 Introduction

Prolongation of longevity has been a history-long desire of mankind since ancient time. Ancient Chinese emperors and European alchemists had been chasing after

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extension of longevity and even immortality (Lai 2013). In general cognition, however, lifespan is an impregnable natural trait and any attempts on intervention or reversion are doomed to fail. While scientists have shown enormous dedication and enthusiasm in the intervention of this natural process, and numerous encouraging progresses have been achieved (Lai 2011, 2013 ; Pazoki Toroudi et al. 2016), a practical approach to "stop the clock" is beyond reach, thus far. On the other hand, attributed to better living conditions and effective geriatric care, human lifespan was considerably extended over the last two centuries. The average life expectancy of babies bone in the twenty-first century was projected to be over a hundred years in countries such as Japan, Canada, France, etc. reported by Christensen et al. (2009).

Biological aging is often accompanied by an increasing range of age-related diseases (Guo et al. 2020). It is a time-dependent degenerative process of cells, tissues and organs, resulting in impairment of their structures and functions and eventually deterioration of health (Pazoki Toroudi et al. 2016; Kume et al. 2010). The aging process was found to be associated with extrinsic factors (such as environmental factors and oxidative stress) as well as intrinsic factors (such as metabolic pathways and genetic elements) (Herskind et al. 1996; Skytthe et al. 2003; Hjelmborg et al. 2006; Slagboom et al. 2011). Examples of these factors include, but not limited to, **mammalian target of rapamycin** (mTOR) which is a serine/threonine protein kinase that plays a role in controlling cell growth and proliferation (Lian et al. 2008; Sheaffer et al. 2008); insulin/insulin-like growth factor-1 signalling pathway which regulates the resistance to oxidative stress (Holzenberger et al. 2003); systemic chronic low-grade inflammation which contributes to age-related morbidities (Marengoni et al. 2009; Custodero et al. 2018). In addition, current aging research focuses extensively on improving health by developing novel therapeutic strategies against cellular senescence at molecular and cellular levels (Pazoki Toroudi et al. 2016).

1.1.1 A Three-Level Strategy for Anti-Aging Approaches

In order to achieve longevity extension, a three-level strategy for anti-aging has been proposed (as shown in Fig. 1.1) (Lai 2013). The first level is the pathological level which focuses on geriatric approaches. It aims to alleviate or eliminate geriatric symptoms after an age-related disease is diagnosed (Weinstein 1990). It is, therefore, a passive and palliative approach. In contrast, gerontological and engineering approaches that are preventive and proactive are more cost-effective and humane (Weinstein 1990). Therefore, they are comparatively desirable and draw more research interests. Nevertheless, the basic **biogerontology** had been progressing rather slowly in the last two decades due to an extensive focus on the development of geriatric over the last two centuries (Binstock 2003).

The second level is the molecular level, in which gerontological approaches are developed. The fundamental concept of the gerontological approach is established on genetical manipulation of metabolic pathways in order to retard aging and enhance longevity at a molecular level (Lai 2013). Techniques have been developed based on the establishment of a library of candidate genes associated with the aging

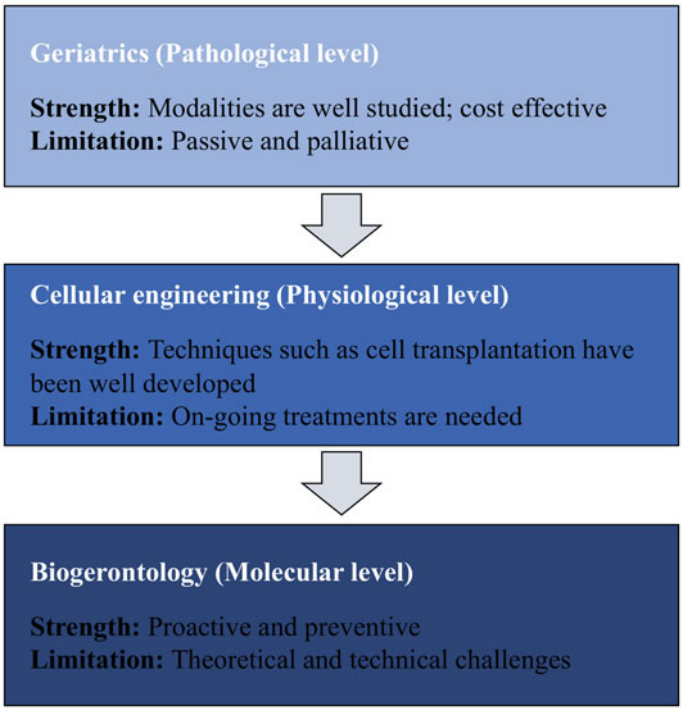

Fig. 1.1 A three-levelled anti-aging strategy

process (Swindell 2007; Kim et al. 2012). Animal models such as mice and fruit flies are often used in studies identifying candidate genes and developing intervention approaches due to their higher birth rate and shorter lifespan (as compared to humans). This makes the experiments comparatively affordable in terms of time, funds, and potential ethical concerns (Kaeberlein 2007). Moreover, eukaryote models (such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, etc.) are also popular experiment subjects due to their simplicity in biological structures (Kaeberlein 2007). For instance, the lifespan of fruit flies was successfully extended (while maintaining their fecundity and locomotor activity) by overexpression of the *D-GADD45* gene in their nervous system (which improves the efficiency of recognition and repairs damaged DNA) (Plyusnina et al. 2011). Recently, gene sirtuin 6 was found to be associated with an enhanced lifespan of mice due to its function in efficient DNA repairment (Tian et al. 2019). In these models, the feasibility of lifespan extension by transgenic manipulation has been corroborated, which has taken anti-aging research to the next level. However, due to the complexity of gerontological interventions, clinical applications are still yet to be feasible. Also, despite the practical plausibility of genetic manipulation, the physiological costs in genetic modulation in long term have not been completely revealed (Lai 2013).

The third level is the physiological level which involves cellular engineering approaches. In contrast to the gerontological approach that focuses on genetic manipulation, the cellular engineering approach is considerably straightforward and focuses on the physiological level. It aims at reversing diagnosed age-related damages, such as extracellular cross-links, mitochondrial mutations, nuclear mutations, cell senescence, lysosomal aggregates, etc. at a cellular level (de Grey et al. 2002). The ultimate goal of this approach is to ameliorate the damage to a threshold where pathological events can be controlled or eliminated (de Grey et al. 2002).

One of the cellular engineering approaches that has made significant progress clinically over the years is the cell transplantation technique (Fratino et al. 2013). For instance, replacement of disease-causing stem cells with normal ones by hematopoietic cell transplantation technology has been a successful clinical practice over the last four decades (Czechowicz and Weissman 2011). Cell transplantation has also been used to treat Parkinson's disease (a nervous system disease involving a progressive loss of dopamine neurons in the substantia nigra pars compacta) by inducing pluripotent cells derived from somatic cells (Chen et al. 2011). These examples illustrated the feasibility of the application of cellular engineering approaches in treating cell loss and tissue atrophy. Moreover, it was reported by Wang et al. that the phenotype of senescence (such as lowered basal cyclin-dependent kinase activity and ^{3}H-thymidine incorporation, and increased senescence-associated β-galactosidase activity) of IDH4 human fibroblasts could be rejuvenated by overexpression of *HuR* (an ubiquitously expressed *Elav*-like RNA-binding protein) (Wang et al. 2001). This study evidenced that reversion of aging and senescent phenotypes are potentially achievable at the physiological level.

Despite the possibility of manipulating the aging process at different levels, debates and secular challenges have been raised for various concerns. The primary argument concerns the necessity of developing biogerontology and engineering technologies when geriatric approaches are deemed to be more feasible and practical (Lai 2013). This resulted in the hesitancy of funding agencies to invest in anti-aging research projects (de Grey 2003). Secondly, political and society concerns would further impact funding opportunities due to society issues such as overpopulation and population aging (de Grey 2003). In aspects of biomedicine, for some genetic diseases/disorders such as Hutchinson–Gilford progeria syndrome and Huntington's disease, there is no actual curative therapy other than gene editing for embryos, which, however, is unacceptable ethically and legislatively (Li et al. 2019). For these reasons, some argued that anti-aging research is considered unjustified.

1.2 Translation of Basic Research: From Bench to Clinic

In addition to the difficulties in basic research for aging intervention, the translation of basic research into clinical practice has been another major challenge. Multiple steps are involved in the translation process (Lai 2013), including (1) basic

genetic/genomic research for identification of aging-associated genes and their functions; (2) mechanistic research on genetic/genomic manipulation of aging-associated genes; (3) development of delivery systems for therapeutic agents; and (4) clinical verifications of the previous steps. The entire process requires enormous efforts and time and involves numerous challenges which will be discussed in detail.

1.2.1 Basic Genetic/Genomic Research

The association of genetic elements and longevity was first revealed in 1988 by Friedman and Johnson (Friedman and Johnson 1988) who discovered the first gene that alters the lifespan of *Caenorhabditis elegans*. The study demonstrated a life extension of up to 65% due to the mutation of this gene. The gene was named *age-1* in the expectation that more genes affecting longevity would be found. Other examples of lifespan-associated genes include *AGTR1*, *sir-2.1*, *hcf-1*, *smk-1*, *daf-16*, etc.(Wolff et al. 2006; Rizki et al. 2011; Benigni et al. 2013), and some of which have been extensively studied for their functions and manipulation strategies (Dali-Youcef et al. 2007; Landis and Murphy 2010; Satoh et al. 2011).

In the last two decades, the computational approach has been used extensively to screen longevity-associated genes. Genome-wide transcriptional and expressional profiles of different tissues such as brain (Hong et al. 2008), skeletal muscles (Zahn et al. 2006), kidney (Rodwell et al. 2004), etc. examined by gene expression profiling and DNA microarray have been reported on individuals at various ages (Magic et al. 2007; Subramanian et al. 2005). The development of genome-wide analysis has taken the basic research on aging-associated genes to the next level. Seong et al. examined the lifespan of fruit flies undergone radiation-induced alterations in genomic expression and analyzed the genes underlying longevity extension (Seong et al. 2011). A group of genes were recognized to be responsible for the prolongation of lifespan, such as cytochrome-related genes (*Cyp1*, *Cyp4d21*, *Cyp4p3*, *Cyp6a9*, *Cyp6g1* and *Cyp318a1*), genes relating to protein turnover and ubiquitination pathways (*CG2924*, *CG7220*, *crl*, *neur*, *Roc1b*, *Ubc84D* and *Ubc-E2H*), and genes responding to oxidative stress (*GstS1*, *Jon65Ai*, *Jon65Aiv*, *Jon66Ci*, *Jon99Ci*, *Jon99Cii*, *mmd*, *Trxr-1* and *Trxr-2*).These candidate genes are worth further investigation for their roles in metabolic pathways and potential intervention approaches.

Another advanced technology, **genome-wide association studies** (GWAS), have also been widely used for genetic mapping. With the advances of single nucleotide polymorphism (SNP) genotyping technologies, GWAS are an effective approach genotyping hundreds of thousands of SNPs across the entire genome in one go and identifying new susceptibility genes with the phenotype of interest (Kronenberg 2008). This superiority makes GWAS a powerful tool to identify genetic contributors of aging-associated phenotypes. Deelen et al. applied GWAS on analyzing the genomic data of a large group of genetically independent longevous individuals and discovered that a gene *POT1* plays an important role in the telomere maintenance pathway and in turn the longevity (Deelen et al. 2013). They also found that the

influence of the insulin/insulin-like growth factor-1 signalling pathway on lifespan is associated with a group of genes including *AKT3*, *AKT1*, *FOXO4*, *IGF2*, *INS*, *PIK3CA*, *SGK*, *SGK2*, *YWHAG* and *POT1*. As compared to candidate gene studies, the application of GWAS could be limited by its current drawbacks such as poor reproducibility and false positive results. An unneglectable advantage of GWAS, on the other hand, is that it requires no prior assumption in respect to gene functions in contrast to the hypothesis-driven approaches (Kronenberg 2008). Despite the fact that GWAS have currently been used more often in studies of age-associated diseases (such as cancer, diabetes, atherosclerosis, etc.) rather than the process of aging itself (Kronenberg 2008), it is believed that GWAS have hitherto been one of the most effective tools for anti-aging research.

1.2.2 Mechanistic Research on Genetic/Genomic Manipulation

As the fact that biological aging is associated with genetic elements has been corroborated, mechanistic research on gerontological interventions should therefore not passively rely on palliative disease-oriented studies but on active and systemic approaches such as genetic/genomic manipulation. For example, the traditional treatments for lysosomal storage diseases (which are genetically determined metabolic disorders characterized by defective lysosomal enzymes causing an age-associated systemic accumulation of lysosomal aggregate) include enzyme replacement therapy (Lachmann 2011) and substrate reduction therapy (Cox 2005), which are essentially palliative. A novel gene therapy that regulates the expression of lysosomal enzymes by administrating encoding nucleic acids (Eto and Ohashi 2000; Sands and Davidson 2006), on the other hand, offers a rather curative approach.

Present genetic/genomic manipulation research for anti-aging strategies focus extensively on the **RNA-interference (RNAi) technology** (Xue et al. 2015). RNAi is a pivotal biomolecular means to regulate target gene expression (Xue et al. 2015). RNAi agents such as small-interfering RNA (siRNA), micro-RNA (miRNA) and PIWI interacting RNA (piRNA) are potential therapeutic agents for the treatment of a broad range of aging-related diseases that are associated with undesired gene expression, such as cancer, neurological diseases, autoimmune diseases, etc. (Fire et al. 1998). Small non-coding RNA molecules have been shown to mediate target gene expression in various approaches, most notably via the silencing of undesired genes by inducing mRNA degradation in cytoplasm, which subsequently supresses the expression of the corresponding proteins (Kumar and Clarke 2007). The mechanism of RNAi was first discovered by Fire et al. (1998) who reported a systemic suppression of *unc22* gene in *Caenorhabditis elegans* induced by an exogenous double-strand RNA molecule. This technology has later been used in longevity research. Bernardes

de Jesus et al. (2012) illuminated that the induction of mouse telomerase reverse transcriptase cDNA which regulates the telomerase gene has successfully prolonged the lifespan of mice by up to 24% without an increased risk of cancer.

Accounting the superior customizability of RNAi agents and the possibility of silencing almost any gene in a convenient and moderately specific manner (Xue et al. 2015), the biomedical application of RNAi has been extensively studied and reviewed by researchers and clinicians. Nowadays, RNAi technology has already become a standard experimental tool for validating gene functions (Mansoori et al. 2014). Currently, taking the advantage of numerous in vitro and in vivo data supporting the therapeutic potential of RNAi (Musacchio and Torchilin 2013; Gavrilov and Saltzman 2012), the focus of research has shifted to clinical applications. In recent years, a number of clinical trials of RNAi therapeutics especially siRNA have been conducted, with, however, limited success so far (Xue et al. 2015).

Another promising gene therapy technology is site-specific genomic integration. An example of this technology is the ΦC31 integrase system, which is a phage-derived system mediating the integration of plasmids bearing an *attB* site into pseudo-*attP* sites of the genomes of humans and mice (Thyagarajan et al. 2001; Karow and Calos 2011). This system, however, possesses risks for DNA damage and chromosomal rearrangements in mammalian cells (Ehrhardt et al. 2006; Liu et al. 2009). Site-specific genomic integration was used by Howden et al. (2008) to co-transfect a plasmid containing the P5 integration efficiency element derived from adeno-associated viruses, and mRNAs coding for Rep68/78 proteins. Gersbach et al. (Gersbach et al. 2011) inserted recombinase target site throughout the genome using *piggyBac* transposase, and then integrated plasmids into those sites and multiple transposons with an engineered zinc-finger recombinase. The site-specific genomic integration technology, together with inducible gene expression systems (that precisely control the expression of transgenes by bioactive compounds (Centlivre et al. 2010; Weyler and Morschhauser 2012)), radiation (Ito et al. 2001) or heat (Tang et al. 2008), has taken the development of gene therapy to the next level.

1.3 Importance of Systemic Delivery to Anti-Aging Medicine

With the advances in genetic manipulation technologies, silencing or overexpressing specific genes is no longer a technical difficulty at the cellular level. A significant challenge, however, lies in the delivery of therapeutic agents to somatic cells body-wide. For instance, in order to perform genetic manipulation, delivery of therapeutic nucleic acids is often required. Naked nucleic acid molecules are unstable and can be degraded shortly after administration (Musacchio and Torchilin 2013; Gavrilov and Saltzman 2012; Seth et al. 2012). Also, nucleic acid molecules may increase the risk of triggering immunogenic response (Ma et al. 2005; Draz et al. 2014). Taking the aforementioned lysosomal storage diseases for example (Eto and Ohashi 2000; Sands and Davidson 2006). Although gene therapy can be applied in a localized

(ex vivo) or a systemic (in vivo) manner, the localized treatment is carried out using macrophages obtained from cultured cells and can only be applied to tissues in which cell transplantation is viable. To genuinely tackle the disease, therapeutic nucleic acids which generate a persistent endogenous reservoir for lysosomal enzymes are needed. Also, the nucleic acids need to be delivered body-wide so as to offer a one-off solution to the disease. With advances in genetic engineering, synthesis of plasmid encoding the enzyme is no longer technically challenging, and the development of systemic delivery approaches has become the key to success.

As genetic manipulation is designed to be a treatment across the whole body, systemic delivery systems have to be well developed before the technology can be moved on to clinical trials. In fact, delivery systems have been developed rapidly in recent years mainly by investigating their therapeutic potential in various in vivo models. For instance, Zamora-Avila et al. (2009) reported that with aerosol delivery of poly(ethylenimine) loaded with RNAi agents that silence Wilms' tumour gene 1 in mice with B16F10 lung metastasis, the number and size of the tumour foci were reduced and the mean survival time of the mice was extended. Dar et al. (2015) used lipid-based nanoparticles as a carrier to deliver siRNAs to silence *ErbB2* and *AURKB* genes in mice with tumours. An improved cellular uptake by the tumour tissues and the subsequent tumour suppression were observed upon the application of the carrier. A safe and effective delivery approach plays an important role in interventive biogerontology and treatments of pathologies (Odom et al. 2007; Yannaki et al. 2010). Using delivery systems in interventive biogerontology has a number of advantages, including (1) low immunogenicity due to inertness and a small particle size; (2) easy access to the blood stream and crossing other cellular barriers due to a small particle size; (3) an enhanced circulation time that allows them to penetrate and accumulate in cells more efficiently; (4) readiness for tracking and imaging in some cases; (5) stimulation of interferon production and enhancement of natural killer cells resulting in an activation of anti-tumour activity, in cases of anti-cancer therapies (Tatiparti et al. 2017). The most frequently studied drug delivery systems are listed in Table 1.1. Although further optimization and manipulation are still required, many of these systems have already shown great potential to enhance the efficiency and bioavailability of therapeutic agents in tackling diseases.

1.3.1 Viral Nanoparticles

Viral nanoparticles (VNPs) are composed primarily of proteins and are thereby known for their innate biocompatibility, biodegradability, the ability to cross cellular barriers and effective delivery of cargo in a systemic manner (Huang et al. 2011). Viruses have evolved naturally to deliver nucleic acids and can therefore be subverted for the delivery of other molecules (Koudelka et al. 2015). VNPs can function as prefabricated nano-scaffolds with unique properties and can easily be modified (Huang et al. 2011). The interiors of VNPs can encapsulate sensitive compounds while the exteriors can be chemically modified to covalently carry drug molecules in

Table 1.1 Summary of the strengths and potential limitations of current drug delivery technologies

Types		Strengths	Limitations	References
Viral nanoparticles		• Natural stability • Innate biocompatibility • Biodegradability • Easy crossing of biological barriers • Modifiability with atomic precision • Prodigious replication • Target selection after surface functionalization	• Pathogenicity • Immunogenicity • Dose concerns	(Huang et al. 2011; Koudelka et al. 2015)
Inorganic carriers		• Bio-consistency • Extreme small size (<10 nm) • Theragnosis	• Non-biodegradability • Non-biocompatibility • immunogenicity	(Tomalia 2009; Kim and Hyeon 2014)
Organic carriers	Polymeric	• Easy surface modifications • Target selection • Theragnosis • Delivery of imaging agent	• High production costs • Potential toxicity • Undesirable entrance of blood–brain barrier	(Mallapragada et al. 2015)
	Albumins	• Biocompatibility • Versatility • Outstanding half-life	• Low loading capacity • Inconsistency in loading capacity, drug release rete	(Cortes and Saura 2010; Lamichhane and Lee 2020)
	Exosomes	• High biocompatibility • Low immunogenicity • Low cytotoxicity • High targeting accuracy • Low required dosage • Minimum side effects • Effective penetration of cell membranes	• Ineffective isolation and purification methods • Difficulties in characterization • A lack of specific biomarker • A lack of high-resolution visualization techniqe	(Li et al. 2019; Yuan et al. 2017)

(continued)

Table 1.1 (continued)

Types		Strengths	Limitations	References
	Lipid-based	• Biocompatibility • Biodegradability • Easy modifiability • Penetration of cell membranes • Low immunogenicity	• Toxicity of cationic lipids • Instability in blood • Non-specific distribution • Low transfection efficiency • Unstable for long-term storage	(Xue et al. 2015; Tatiparti et al. 2017)
	Micro/nanobubbles with ultrasound	• Exclusive cellular penetration • Well controllable release rate • Ability to carry gaseous contents	• Challenging in preparation of smaller sizes	(Duan et al. 2020; Husseini et al. 2000)
Hybrid carriers		• Combined benefits of both components • Fabricability to overcome various limitations	• Time consuming and costly to produce	(Taylor-Pashow et al. 2010; Li et al. 2014)
Xenobot		• Reconfigurability for shape, behaviour and function • Biocompatibility • Nontoxicity • Self-limiting lifespan • Self-renewing	• Potentially costly to produce • Technically challenging for development for a broad range of applications	(Kriegman et al. 2020; Kriegman et al. 2019)

precisely defined arrays (Koudelka et al. 2015). This makes VNPs a natural versatile platform for the delivery of RNAi agents (Galaway and Stockley 2013; Choi et al. 2013), conventional small-molecule drugs (Pokorski et al. 2011), imaging reagents (Koudelka et al. 2015), photosensitizers (Rhee et al. 2012) and even heterologous viral genomes for gene therapies (Azizgolshani et al. 2013). Some suggested that VNPs are almost the only efficient means for systemic delivery of nucleic acid hitherto (Lai 2013). For example, studies showed that Adeno-associated virus can safely and effectively deliver therapeutic genes for the treatments of a number of genetic diseases such as haemophilia, lipoprotein lipase deficiency, inherited retinal disease and spinal muscular atrophy with positive results preclinically and clinically (Gaudet et al. 2013; Mingozzi and High 2011; Nathwani et al. 2011). In addition, viruses were naturally designed to deliver nucleic acid and hijack the intracellular machinery of

the host to prodigiously replicate the components of progeny viruses, which allows a massive production of mammalian tissue-derived VNPs for gene therapies and inexpensive manufacture at an industrial scale (Koudelka et al. 2015).

A particular advantage of VNPs over other synthetic nanomaterials is the modifiability with functional surface appendages attributed to their monodisperse structures, which allows surface tailoring for targeting specific cells including cancer cells and immune cells (Brown et al. 2002; Lewis et al. 2006; Ren et al. 2007). In addition, the encapsidation of nucleic acids serves as a protection mechanism making the particles extremely stable naturally, which therefore allows a range of chemical modifications to be applied and facilitates structural integrity in plasma and even gastric conditions (Rae et al. 2008). For this reason, VNPs modified for targeting specific cell types allow for the application of toxic payloads (loaded into the cavity of VNPs rather than the exterior) which selectively eliminate diseased cells without off-targeting healthy ones (Brown et al. 2002; Yildiz et al. 2013; Cao et al. 2014). For example, Hibiscus chlorotic ringspot virus was developed as a carrier for a chemotherapeutic drug doxorubicin which displays a high level of cytotoxicity (Ren et al. 2007). With the conjugation of folic acid onto the capsids, the VNPs were found to selectively target ovarian cancer cells. Huang et al. (Huang et al. 2011) engineered HK97 VNPs with transferrin for tumour cell-specific targeting, and demonstrated an effective targeting via transferrin receptor in vitro.

In addition to standard chemotherapy, VNPs are often used to carry photosensitizers for photodynamic therapies. For instance, a metalloporphyrin derivative was loaded in bacteriophage Qβ VNPs with a glycan surface ligand targeting cells bearing the CD22 receptor (Rhee et al. 2012). A multifunctional MRI contrast media and a photodynamic therapy agent (named chelated Gd^{3+} and Zn^{2+} phthalocyanine) were encapsulated in an engineered cowpea chlorotic mottle virus, which was the first demonstration of the use of VNPs in **theranostics** (Millan et al. 2014). Further, retargeted adenoviral vector carrying gold nanoparticles was used for photothermal therapy for tumour cells (Everts et al. 2006). Due to the unique properties of VNPs, viruses can be employed not only for the development of drug delivery systems and novel therapeutic approaches, but also to serve as a model tool for exploring the key mechanisms behind the interactions between nanoparticles and cells (Vanova et al. 2019).

Despite multiple superiorities of VNPs as a drug carrier, limitations such as pathogenicity and immunogenicity are not to be neglected. The pathogenicity of mammalian viruses often triggers natural virus–host interactions resulting in a compromised treatment effect or even lethal immunogenic responses (Guenther et al. 2014; Wirth et al. 2013; Yla Herttuala 2012). For this reason, bacteriophages and plant viruses are regarded to be safer candidates for the development of therapeutic VNPs due to their inability to infect humans (Koudelka et al. 2015). Otherwise non-viral **nanocarriers** would become an option for drug delivery. The pathogenicity problem, however, may not be highly significant if the VNPs are functionalized to target specific cells instead of the whole body or when the required dose is minimal. Nevertheless, biological aging is a systemic degenerative process where interventions require body-wide transfection (de Grey 2003). This essentially means that a

considerably high dose would be required, and consequently a higher risk is to be confronted. Koudelka et al. (Koudelka et al. 2015) suggested that the dosage issue could be mitigated by loading the drugs using encapsulation method instead of covalently attaching the cargo molecules to internally exposed side chains, in which case the loading capacity could be increased by up to three folds (Ren et al. 2007; Aljabali et al. 2013). However, this solution would still be far from adequate to confront the dosage issue.

1.3.2 Inorganic Carriers

Inorganic carriers are consisted of hard and insoluble nanoparticles that are biopersistent and non-biodegradable, such as metal, metal oxides, carbon nanotubes, carbon fibres, magnetic particles, etc. (Tomalia 2009; Kim and Hyeon 2014). Gold nanoparticles are versatile as they can be applied as a delivery system or as a therapeutic agent (Everts et al. 2006; Bhattacharyya et al. 2011) as they exhibit anti-angiogenic property and anti-tumour activity that interfere certain cellular processes (Bhattacharyya et al. 2011). Nano-diamonds are diamond particles in an extremely small size (from 4 to 5 nm). They are suitable for surface modification, stable in photoluminescence which allows analysis for intracellular localization (Alhaddad et al. 2011) and often used to deliver therapeutic siRNA (Tatiparti et al. 2017). Alhaddad et al. (2011) employed nano-diamonds as a vector to deliver siRNA to Ewing sarcoma cells. Quantum dots are colloidal semiconductor nanocrystals produced by quantum confinement effects. They possess outstanding optical and electronic characteristics (Young et al. 2016). They are self-tracking vehicles for siRNA for cancer treatments (Tatiparti et al. 2017). Tan et al. used Quantum dots to deliver siRNA molecules that silence human epidermal growth factor receptor 2 to breast cancer tissue (Tan et al. 2007). Carbon nanotubes are a theragnostic agent that simultaneously serve imaging and therapeutic purposes (Lee et al. 2013; Zhang et al. 2014). Due to the nanoneedle structure, carbon nanotubes are capable of independently translocating into cytoplasm without causing cell death (Bhattacharyya et al. 2011). Zhang et al. (2006) used functionalized carbon nanotubes to carry an siRNA that was released from the side wall of the nanotubes and silenced the expression of telomerase reverse transcriptase in cancer cells. This activity prevented cancer cells from acquiring replicative immortality and thereby suppressed tumour growth. Another theragnostic agent is super-paramagnetic iron oxide nanoparticles. Their large surface area allows for conjugation of targeting ligands and encapsulation of both drugs and imaging agents. This dual-action probe performs non-invasive imaging task and siRNA delivery and is often used for tumour treatments. A unique advantage of this nanocarrier is that the delivery is highly target-oriented with the aid of an external magnetic field (Bhattacharyya et al. 2011; Lee et al. 2013). Despite the various advantages that inorganic nanocarriers exert, their applications in clinics have been limited by their immunogenicity, non-biodegradability and, in some cases, non-biocompatibility.

1.3.3 Organic Carriers

Organic carriers are usually based on "soft" nanomaterials that can be natural or synthetic. Organic nanoparticles are typically made of polymeric organic substances or surfactant molecules that can be assembled into large aggregates. These aggregates could be biodegradable or non-degradable. Examples are proteins, liposomes, exosomes, polyesters, chitosan, etc. (Tomalia 2009; Mallapragada et al. 2015).

1.3.3.1 Polymeric Nanoparticles

Polymeric nanoparticles are a group of (in most cases) synthetic polymeric products that are often used for both diagnostic and therapeutic purposes. They have been used to combat degenerative, inflammatory and genetic diseases associated with aging, such as cancer and developmental, infectious and immune disorders (Mallapragada et al. 2015). As drug carriers, a considerable number of polymeric nanoparticles have been approved by the FDA for clinical applications (Weissig et al. 2014). With their inherent chemical properties which allow them to be modified for targeting functions and an excellent versatility to carry various drugs, they have often been used in site-targeted therapies and to deliver antioxidants, anti-inflammatory agents, immunomodulatory compounds, growth factors, genes, RNAi agents, bioactive compounds and antimicrobials (Mallapragada et al. 2015). They are also commonly used for cell imaging and tracking (Shao et al. 2013; Chen et al. 2013; Vande Velde et al. 2012; Ren et al. 2013; Miyoshi et al. 2005). Some examples are listed below.

Poly(alkyl cyanoacrylates) is the most well-established polymeric nano-delivery system and has been used to deliver compounds that include hexapeptide dalargin (Kreuter et al. 1995; Kreuter 2015), doxorubicin (Gulyaev et al. 1999; Steiniger et al. 2004), loperamide (Alyautdin et al. 1997) and tubocurarine (Alyautdin et al. 1998). For example, Kreuter et al. (1995) reported the first successful attempt of delivering dalargin adsorbed to the surface of poly(butyl cyanoacrylate) nanoparticles to the central nervous system via intravenous injection. Apart from poly(alkyl cyanoacrylates), polyesters have also been adopted for delivery purposes. Polyesters are commercially available and have been approved by the FDA for human use (Weissig et al. 2014). They are recognized as a promising biodegradable delivery system. One of their important properties is their low cytotoxicity attributed to their rapid degradation into metabolites (Gunatillake and Adhikari 2003). For the same reason, all polyesters undergo bulk erosion (Tamada and Langer 1993; Burkersroda et al. 2002) and often cause premature release of drugs. Polyesters are often used to carry loperamide (Gelperina et al. 2010; Tosi et al. 2007), active peptides (Li et al. 2013), ritonavir (Rao et al. 2008) and doxorubicin (Gelperina et al. 2010; Wohlfart et al. 2011). Polyanhydrides also exhibit good biocompatibility and drug delivery potential (Rosen et al. 1983). A notable example of their application is the implantable wafer systems for central nervous system-directed delivery, which is

often used as therapeutics for Alzheimer's disease (Wu et al. 1994; Howard et al. 1989) and brain cancer (Brem et al. 1989; Jampel et al. 1991; Lesniak et al. 2005). An important advantage of these polyanhydride implants is their degradability into biocompatible metabolites that are readily eliminated (Domb et al. 1994). In addition, polyethers have recently attracted extensive attention for their potential as a drug carrier. These polymers are naturally derived polymers. A typical example is chitosan, a cationic polysaccharide, which is generally deemed to be a promising drug delivery vehicle (Ta et al. 2008; Shamji et al. 2009). Polyethers are not particularly susceptible to hydrolytic degradation since their ether bonds are considerably stable in water. Elimination of polyethers often takes place through enzymatic degradation either by oxidation or by dissociation prior to excretion (Ohta et al. 2005; Kawai 2002). Pille et al. (2006) reported a 90% growth inhibition of xenografted aggressive breast tumours in mice induced by anti-RhoA siRNA loaded on chitosan-coated polyisohexylcyanoacrylate nanoparticles via intravenous injection.

In summary, polymeric nanoparticles are promising and generally recognized drug delivery systems that serve diagnostic, imaging and therapeutic purposes. In addition, with appropriate chemistries and functionalization, their performance can be further improved for safety, effectiveness and target/site-specificity (Voigt et al. 2014). However, it was suggested that some of the polymeric chemicals were found to be cytotoxic to cells in the central nervous system (Mallapragada et al. 2015). In addition, future application research should focus on reducing the high cost in fabrication of these polymers and scaling up the production (Mallapragada et al. 2015).

1.3.3.2 Albumin-Based Carriers

Albumin is a highly biocompatible, non-immunogenic and negatively charged protein that is abundant in blood plasma. It has been developed into a versatile drug delivery system often used for the transportation of hydrophobic molecules such as fatty acid, hormones, bilirubin, fat-soluble vitamins, exogenous drugs (e.g. warfarin and ibuprofen) (Cortes and Saura 2010) and positively charged compounds (Lamichhane and Lee 2020). An outstanding advantage of albumin as a drug carrier is its excellent half-life that draws particular research interest. This advantage makes albumin a preferable delivery system for anti-cancer agents (Choi and Han 2018). The first attempt of using albumin as a drug carrier was performed by Stehle et al. (1997) who chemically coupled methotrexate with albumin using carbodiimide as a cross-linking agent.

Albumin molecules can be modified in various ways to serve different purposes. Due to the nature that albumin molecules carry negative charges, which limits their applications in delivering co-charged compounds, cationization has been performed to modify albumin by adding ethylenediamine to a buffered albumin solution (Byeon et al. 2016). Cationized albumin exhibited favourable pharmacokinetic properties with a longer serum half-life and an enhanced selectivity to brain tissues as compared to other organs (e.g. liver, heart, lung, etc.) (Bickel et al. 2001). Vaidya et al. (2020) demonstrated that using bovine serum albumin cationic nanoparticles as an inhalable

delivery system significantly improved the therapeutic efficacy of quinacrine against lung cancer. Surfactants have also been used as a stabilizer to modify albumin. Due to the amphiphilicity of surfactants, a surfactant–protein complex is more hydrophilic than the protein alone, resulting in an increased retention time of the drug in systemic circulation (Mishra et al. 2006), and also in a reduction in the protein–protein surface interactions and the consequent aggregation (Ruizeña et al. 2010). Taneja and Singh (2018) reported that irinotecan loaded in human serum albumin (stabilized by polysorbate 80) showed an improved loading and entrapment efficacy and an enhanced stability of up to twelve months. Moreover, Elsadek et al. (2010) developed an albumin-binding prodrug by conjugating a hydrazone derivative of doxorubicin to human serum albumin modified with an enzymatically cleavable peptide linker. The prodrug was designed to target prostate cancer tissues as the peptide linker can be cleaved by prostate-specific antigen, which initiates drug release.

However, significant limitations of the albumin delivery system exist. Firstly, the loading capacity and efficiency of albumin are relatively low as compared to other types of drug carriers (Xu et al. 2011). Additionally, one of the drawbacks of using protein as a drug carrier is the inconsistency in loading capacity and drug release rate from batch to batch (Sleep 2015). To overcome the inconsistency problem, recombinant protein technology (involving placement of cross-linking groups and binding moieties) has been adopted and has provided protein molecules with precisely defined properties (Lamichhane and Lee 2020).

1.3.3.3 Exosomes as a Vehicle

Exosomes, a type of extracellular vehicle (with a size ranging from 30 to 140 nm), are secreted by most living cells and mediate transmission of information intercellularly (Niel et al. 2018; Sun et al. 2019). During the information exchange process, information of the parent cell in the form of lipids, proteins and nucleic acids is packed into and carried by exosomes to a neighbour cell (Waldenström and Ronquist 2014). Attributed to the function and properties of exosomes, they are developed as a natural drug delivery vehicle with high targeting accuracy, low required dosage, minimum side effects, versatility for delivery of a wide range of cargoes and excellent penetrability of cell membranes (Li et al. 2019). For example, exosomes as a drug carrier were found to be able to penetrate blood–brain barrier and enhance cellular uptake by the central nervous system (Yuan et al. 2017).

Many in vitro and in vivo attempts have been made using exosomes as a drug carrier for therapeutic purposes. Kim et al. (2016) demonstrated that paclitaxel encapsulated in macrophage-derived exosomes exhibited preferential accumulation in cancer cells with minimal metastasis, as compared to liposomes and polymeric carrier systems. Yang et al. (2015) loaded different anti-cancer drugs into brain endothelial cell-derived exosomes and successfully delivered them to human glioblastoma astrocytoma cells in vitro and to brain tissues of zebrafish in vivo. Tian et al. (2014) engineered exosomes with targeting proteins to deliver doxorubicin specifically to

breast cancer cells and found an inhibition of tumour growth with a reduced toxicity and immunogenicity of the drug.

Despite numerous advantages of using exosomes as a drug delivery system have been revealed, and their applicability for therapeutic purposes has repeatedly been demonstrated, clinical application of exosomes would be challenged by a number of limitations, including a lack of efficient isolation and purification methods, difficulties in characterization, and a lack of specific biomarkers and high-resolution visualization techniques, etc. (Li et al. 2019).

1.3.3.4 Lipid-Based Nanocarriers

Lipids and phospholipids are often used as drug carriers due to various advantageous properties, among which, the most favourable one is the similarity of their chemical composition to cell membranes. This property results in a natural tendency of lipid-based carriers to interact well with cell membranes and thereby an improved cellular uptake of the cargoes (Xue et al. 2015). There is a large number of highly biocompatible and biodegradable lipid-based materials that are commercially available without a need for chemical synthesis. In addition, lipids show a significantly lower risk of undesired immunogenic reactions in contrast to other polymeric nanomaterials (Xue et al. 2015). Therefore, a solid track record of clinical applications of lipid-based materials is noteworthy (Xue et al. 2015; Tatiparti et al. 2017; Chakraborty et al. 2017). Among non-viral drug delivery systems, lipid-based nanoparticles appear to be a promising candidate over other systems in terms of biocompatibility, biodegradability, immunogenicity, preparation costs, etc. Nevertheless, lipid-based systems are challenged by toxicity issues, instability in blood, non-specific distribution and a low transfection efficiency after intravenous administration (Vhora et al. 2015).

A typical example of lipid-based nanocarriers is liposome, which is a closed bilayer phospholipid that consisted of biocompatible lipid materials and is used to delivery therapeutic drugs (Allen and Cullis 2013). Liposome molecules possess an aqueous core which allows them, unlike other lipid-based systems that are exclusively used for loading lipophilic compounds, to carry hydrophobic, hydrophilic and ionic drug molecules (Pathak et al. 2011; Mallick and Choi 2014). For this reason, liposomes are probably the optimal candidate, thus far, for encapsulation of nucleic acids and have thereby become the most commonly used non-viral vehicles for gene therapies (Xue et al. 2015). An advantage of this encapsulation pattern is that the RNA molecules are wrapped in the core of liposome carriers resulting in a reduced risk of triggering immunogenic response (Xue et al. 2015). In addition, cationic liposomes have been the standard carrier for RNA molecules (that are poly-anionic by nature) due to a favourable electrostatic interaction (Mallick and Choi 2014). However, cautions should be taken for the spontaneous formation of siRNA-lipid complex during the encapsulation process, which results in exposed RNA molecule on the surface of the carrier and in turn inducing immunogenic responses (Tagami et al. 2011). Moreover, the structure of liposomes can be conveniently modified with functionalized lipids such as polyethylene glycol-conjugated lipids for extended

circulation time or with functional side chains for specific targeting (Xue et al. 2015). Yamada et al. (2014) demonstrated the potential of using liposomes as a drug delivery system for treatments of diabetes. In this study, 2′-OMe RNA was loaded into liposome nanoparticles and delivered to pancreatic cells. Results indicated a high affinity between the nanoparticles and the cells and a significant enhancement in insulin secretion. Apart from this, liposomes were also used for virus-free transfection to pluripotent cells (Park et al. 2012), gene delivery to mesenchymal stem cells (Madeira et al. 2010), targeting peripheral neurons and Schwann cells for an enhanced uptake (Lee et al. 2013) and targeting the central nervous system (Begley 2004; Leonor Pinzon Daza et al. 2013). Notably, several limitations of the liposome systems are of concern. Cationic liposomes are rather toxic in general as they disrupt the integrity of membrane structure causing cytoplasm vacuolization and in turn cell shrinkage (Lappalainen et al. 1994; Mishra et al. 2004), and even cell lysis and necrosis if the level is sufficiently high (Wu et al. 2001), while unfortunately negatively charged liposomes often have a short in vivo circulation time due to quick clearance (Xue et al. 2015).

Compared with liposomes, solid lipid nanoparticles with a solid and lipophilic core are inherently unfavourable for encapsulating hydrophilic poly-anionic RNA molecules (Xue et al. 2015). Consequently, solid lipid nanoparticles are not suitable candidates for RNA delivery, unless RNA molecules are linked to the outer surface of the nanocarriers while the core region is packed with lipophilic drugs (Bae et al. 2013). For example, Chen et al. (2010) developed a novel multifunctional anionic lipid carrying VEGF siRNA on the surface and doxorubicin in the interior. The complex was shown to inhibit the growth of ovarian tumour cells. With this loading method, immunogenicity may be of concern as the RNA molecules are exposed.

Finally, nano-emulsions as a lipid-based system have also been widely studied for drug delivery. Nano-emulsions are a thermodynamically stable isotropic system prepared by emulsifying two immiscible liquids into one phase (Souto et al. 2011). In this system, the lipid phase may serve as a protector for RNA from enzymatic degradation (Brito et al. 2014). For instance, a cationic nano-emulsion was reported to protect an RNA-based vaccine from RNase treatment for up to 16 h (Brito et al. 2014). Also, nano-emulsions are capable of mixing with multiple ingredients in a single formulation. For example, Oh et al. (2013) formulated a nano-emulsion system incorporated with paclitaxel and Bcl-2 siRNA and found that the system was able to simultaneously deliver the two drugs to tumour cells. Despite the advances of nano-emulsion systems, relatively few nano-emulsions have been developed for RNA delivery in clinics, mainly due to a number of limitations. As the fine oil droplets in the emulsion are unable to stably immobilize the hydrophilic RNA molecules, the RNA molecules have a tendency to migrate to the oil–water interfaces and eventually diffuse into the external medium with limited control (Xue et al. 2015). In addition, nano-emulsions are negatively charged by convention, resulting in an unstable entrapment of RNA molecules (Xue et al. 2015). Another concern is the shelf life of nano-emulsion products. After loading with large molecular compounds, the sizes of the oil droplets are unable to remain small for a long period of time (Souto et al. 2011).

1.3.3.5 Microbubbles/Nanobubbles with Ultrasound

Microbubbles/nanobubbles (MNBs) are spherical vehicles comprising a gaseous core that is encapsulated in a shell of biocompatible materials such as lipids, proteins etc., with a particle size ranging from 0.1 to 10 μm (Khan et al. 2018; Duan et al. 2020). MNBs have been used to deliver various cargoes including chemotherapy agents, RNAi agents and even gaseous contents such as oxygen (Owen et al. 2016; Li et al. 2019; Zhong et al. 2020). When exposed to ultrasound, MNBs, as ultrasound contrast agents, contract and expand, resulting in backscattering of the ultrasound, cavitation, or even bursting of the shell (Duan et al. 2020). For this reason, ultrasound plays a role in controlling drug release, permeability and retention especially in tumour tissues (Husseini et al. 2000; Ahmed et al. 2019).

Ultrasound can stimulate the release of the cargo from MNBs and improve its retention in targeted cells. The release rate is controlled by certain acoustic parameters such as frequency, power density and pulse duration (Ahmed et al. 2019). For example, Alexander et al. (2001) reported that doxorubicin uptake by HL-60 cells was increased with the pulse duration and reached a plateau at 2 s. For cell membrane permeability, ultrasonic at 1 MHz and 0.25 W/cm^2 was found to promote the penetration of platelet by gold nanoparticles. Liposome nanobubbles were used to deliver a polyethyleneimine conjugated shRNA targeting the surviving gene in tumour tissues (Li et al. 2019). With the assistant of ultrasound, the nanobubble complex significantly enhanced the transmission of the shRNA to the tumour cells and improved the gene silencing effect. Nittayacharn et al. (2019) reported that the use of a type of synthesized lipid nanobubbles enhanced the cellular uptake of Doxorubicin by LS-174 T human colorectal adenocarcinoma cells and in turn reduced the cell viability to a range from 30.6 to 23.8%. The application of ultrasound further reduced the viability to 15.9%. The enhanced anti-cancer effect was also observed in an in vivo mouse model. Despite the exclusive controllability of drug release and permeability by the MNBs-ultrasound system, the manipulation of particle size remains a major challenge limiting its application. It was suggested that preparation of nanobubbles with a size of less than 200 nm is extremely challenging (Duan et al. 2020).

1.3.4 Hybrid Nanocarriers

Hybrid nanoparticles are synthesized by combining two or more inorganic or organic materials for diagnostic, therapeutic and imaging purposes (Mallapragada et al. 2015). Hybrid carriers exhibit not only the advantageous properties of each individual ingredient but also synergistic advantages of the composite (Taylor-Pashow et al. 2010; Li et al. 2014). Hybrid nanoparticles are often fabricated for theranostic (i.e. both diagnostic and therapeutic) functions, an extended storage-life, controllable drug release and reduced side effects (Tatiparti et al. 2017; Ryu et al. 2014; Muthu et al. 2014). Muthu et al. (2012) loaded docetaxel into a complex of liposome and quantum dots, which served diagnostic (by quantum dots) and therapeutic (by

docetaxel) purposes for breast cancer. Apart from this, hybrid carriers are often fabricated with lipid-based materials and polymeric materials taking the advantages of their properties (Xue et al. 2015). Lipid-based materials exert outstanding biocompatibility, low immunogenicity and highly controllable drug release rates, whereas polymeric materials exhibit excellent affinity for drugs such as RNAi agents, and hybrids of them have been designed for an improved encapsulation and delivery efficacy (Narvekar et al. 2014; Xue and Wong 2011; Xue and Wong 2011). Also, a synergistic effect of lowered cytotoxicity is often shown. For example, hybrid particles of lipid and polyethylenimine were shown to reduce both acute (necrotic and apoptotic) and longer term (proliferation suppression) cellular toxicities to non-cancer breast cells and prostate cells as compared to non-lipidated polyethylenimine (Xue and Wong 2011; Xue et al. 2013). Other hybrid nanomaterials including Nanoscale Metal–Organic Frameworks (Della Rocca et al. 2011; He et al. 2014; Huxford et al. 2010) and functionalized nanotubes and nanogels (Lai et al. 2003) have also been extensively studied. However, challenges to the development and application of hybrid nanocarriers include reducing the high cost and time associated with fabrication and scaling up the production (Mallapragada et al. 2015).

1.3.5 Xenobot, a "Futuristic" Drug Delivery System

Very recently, Kriegman et al. (2020) reported a ground-breaking technology of engineering a living robot, named **xenobot**. They harvested stem cells from embryos of *xenopus laevis*, an African clawed frog, and differentiated them into heart cells which naturally contract, and skin cells that do not. With the natural inclination of the cells, heart cells allowing spontaneous movements and skin cells with bonding ability were combined and formed a "biological machine". The "machine" is computationally programmable for its shape, behaviour and function by evolutionary algorithms. The history-long dream of European alchemists in the Middle Age of creating life has now been fulfilled. For the reconfigurability of the xenobot, some have suggested to program it into a structure with a cavity that can pick up and carry cargoes, and thereby to create a soft machine that safely delivery drug and biological materials (such as RNAi agents) inside the human body (Patra et al. 2013). A safety feature of xenobot is that, in the absence of certain metabolic functions (which is the default setting of the robot, unless purposely engineered otherwise), it has a naturally limited lifespan (Kriegman et al. 2020). In addition, these reconfigurable organisms not only self-maintain their externally imposed configuration, but also self-repair for damages, such as automatically closing lacerations (Kriegman et al. 2019). Provided their biocompatibility, nontoxicity, self-renewing, self-limiting lifespan, they could serve as a smart vehicle for drug delivery (Patra et al. 2013) or a tool for internal surgery (Li et al. 2017).

Further, if equipped with express signalling circuits and/or proteins for enzymatic, sensory and mechanical deformation functions, they could locate and digest toxic waste or any undesired substances in vivo (such as removing plaque from artery

walls), or identify molecules of interest in the human body that are physically inaccessible to current surgery techniques (such as identifying cancer, differentiating or controlling biomarkers in local disease) (Kriegman et al. 2020). However, in addition to the unknown cost of manufacturing xenobot at an industrial scale, the "evolution" of this artificial life at this stage is still premature for a broad range of applications. Numerous technical challenges need to be conquered by multidisciplinary teams before this technology can be brought into clinics.

1.4 Clinical Trials

To convert a therapeutic technology into clinical application, there are five phases of clinical trials to go through. Phase 0 is the preliminary human trials where a subtherapeutic dosage of the tested drug is administered to a small population (10–15 participants) to gather initial data on pharmacodynamics and pharmacokinetics (Murgo et al. 2008); Phase I aims to determine a safe dosage range and potential sides effects on a population size of 20 to 80 individuals (Eisenhauer et al. 2000; Zuckerman et al. 2014); in Phase II trials, the efficacy and long-term safety of the drug are evaluated involving a larger group of participants (100–300) (Rubinstein et al. 2005); Phase III aims to confirm the effectiveness of the therapy by comparing with other existing treatment methods and to continue to monitor potential side effects. A large population of participants is involved (1000–3000 individuals) (Sonnad et al. 2013); the final stage Phase IV aims to further identify treatment risks, benefits and optimal usage by post-marketing studies (Hennessy 1998).

RNAi therapies, for example, has been taken to clinical trials at an unprecedentedly fast pace (Xue et al. 2015; Young et al. 2016; Chakraborty et al. 2017; Oh and Park 2009). Some examples of clinical trials are shown in Table 1.2. Despite many clinical trials having been conducted for future therapeutic applications, most of the technologies have not reached routine clinical use. For instance, anti-Bcl-2 antisense, as a promising therapeutic agent against chronic lymphocytic leukemia, has been rejected twice by the FDA because no difference was found in the five-year survival rate of patients in phase III clinical trials (Resnier et al. 2013). CALAA-01, a cyclodextrin polymer-based systemic delivery system has been in Phase I-II trials for delivering siRNA (Oh and Park 2009). In Phase I trials, siRNA targeting and silencing the expression of M2 subunit of ribonucleotide reductase was intravenously administered to patients with solid tumours (Guzman Villanueva et al. 2012). The delivery system exploits the endogenous RNAi machinery to reduce mRNA levels to a desired level for up to one month. Transferrin was used to modify the cyclodextrin based nanoparticles for targeting tumour cells, which prevented nuclease mediated degradation of CALAA-01 siRNA in the system. Phase I trials have attained exciting success in three patients whose tumour tissues were effectively suppressed by the drug (Zuckerman et al. 2014). Hence, the trials have moved forward to Phase II to study the safety and a suitable dosage range (Zuckerman et al. 2014). Another example lies in SNALP-formulated delivery system for carrying siRNA targeting

Table 1.2 Clinical trials of RNAi therapeutics 49, 102, 163, 217

Drug	Delivery system	Target disease	Phase of trial
CALAA-01	Cyclodextrin nanoparticle, Transferrin, PEG	Solid tumour	I
Atu027	Liposomes (Lipoplexes, Cationic lipid)	Solid tumour	I
ALN-VSP02	SNALP	Solid tumours with liver involvement	I
ALN-VSP03	SNALP	Solid tumour	I
TKM 80,301	SNALP	Solid tumour	I
siRNA-EphA2-DOPC	Neutral liposomes	Solid tumour	I
siRNA versus ApoB	Lipid nanoparticles	Hyper-cholesterolemia	I
siRNA versus VEGF and KSP	Lipid nanoparticles	Solid tumour	I
siRNA versus PLK1	Lipid nanoparticles	Solid tumour	I
siRNA versus PCSK9	Lipid nanoparticles	Hyper-cholesterolemia	I
siRNA versus ZEBOV L polymerase, VP24 and VP35	Lipid nanoparticles	Ebola virus infection	I
siRNA versus HSP47	Vitamin A- coupled lipid nanoparticle	Fibrosis siRNA	I
siRNA versus TTR	Lipid nanoparticles	Transthyretin mediated amyloidosis	II
siRNA versus PKN3	Liposome (cationic lipid, AtuFECT01)	Pancreatic cancer	II
siG12D LODER	Polymer matrix (LODER polymer)	Pancreatic ductal adenocarcinoma	II

vascular endothelial growth factor and kinesin spindle protein, which was the first dual targeting siRNA drug (Young et al. 2016). Phase I trials confirmed its efficacy in treating solid liver tumours. However, the study has not reached the maximum tolerated dosage, and the trials continued to enrol more patients for dose evaluation (Lee et al. 2013; Tabernero et al. 2013). In most of the cases, current clinical trials have shown satisfying outcomes with limited side effects, yet very few have been taken to clinical application (Young et al. 2016). Moreover, concomitant treatments have generated promising results, which suggested the possibility of personalized cancer therapies in the future (Resnier et al. 2013).

1.5 Summary and Outlooks

Prolongation of lifespan has been in progress of becoming scientifically achievable. Its achievability has been supported by various evidence, while, at this stage, it will not happen immediately but in a distanced but foreseeable future. To shorten the timeframe of this achievement, an enormous amount of dedications is required in developing novel anti-aging technologies and improving the existing ones.

Based on the current understanding of the association between genetics/genomics and longevity and the advances in transgenic technologies, we are one step closer to the intervention of the aging process. However, before theories can become practical, development of safe and effective drug delivery technologies will be an essential step to be accomplished. By far, nanoparticle-based delivery systems have been deemed to be the most promising approach to conquer the technical barrier. However, as mentioned above, limitations such as cytotoxicity, biocompatibilities, immunogenicity, biodegradability, effectiveness in delivery, target selection, costs of manufacturing, etc. are major problems to be addressed. In addition, as nanoparticles are often administered in a systemic fashion, prospective research should focus on studying the interactions between nanoparticles and blood components to further identify potential safety risks (Shajari et al. 2017). Moreover, co-delivery of gene-based therapeutics and conventional chemotherapy agents might offer a new approach to treat aging-associated diseases in a more effective manner although development of synchronized delivery systems is still technically challenging at this stage (Chakraborty et al. 2017). In essence, the development of safe, effective and reliable drug delivery systems is an imperative goal to take anti-aging medicine to the next level.

Despite gerontological intervention approaches such as RNAi technology have yet to be generally accepted as a therapeutic modality, innumerable in vitro and in vivo evidence supporting its feasibility and practicality have led basic research towards clinical trials. However, limited success has been seen primarily due to product failure during clinical trials, which includes the safety and effectiveness concerns, the design of clinical trials and commercial considerations (Chakraborty et al. 2017). All these concerns have to be thoroughly addressed before a successful drug can emerge. Last but not least, a question researchers must ask themselves prior to reporting a success is that: is the effect of an interventional technology significant enough to make a difference? Scientists are not trying to extend mouse lifespan by ten or more years, as this is absolutely unrealistic at this stage or in a near future. Instead, studies should focus on a more practical goal, such as an extension by 50% (around one year), which is already an enormous challenge at the moment especially if interventions are not performed at an early stage of life (de Grey 2003). If the answer to the question is no, researchers should not be overly optimistic and ambitious in taking the research output to the application level. It is unwise to overclaim a laboratory success by exaggerating its clinical potential. Studies at this stage should focus on the optimization of current technologies, which will attract more

funding opportunities, lead to a more promising success in future clinical trials and eventually fulfil the history-long dream of mankind for longevity prolongation.

Important Notes

- Anti-aging studies have shifted from geriatrics to biogerontology, which is a significant breakthrough.
- Biological aging has been found to be closely associated with genetics/genomics.
- Drug delivery in a systemic fashion plays an important role as gene therapies are designed to be a body-wide treatment.
- Despite various drug delivery systems having been successfully developed, safety, biocompatibility and effectiveness are still of great concern.
- Numerous clinical trials for anti-aging interventions have been conducted with limited success.

Questions for Future Research

- **How are biogerontological interventions compared to geriatrics in terms of practicality when biogerontology is still at the laboratory stage?** In contract to geriatrics, biogerontology is more proactive and less-palliative, while less cost-effective. Therefore, dedication should be taken to further develop and maturate biogerontological technologies to improve effectiveness and reduce costs.
- **Do laboratory successes in anti-aging research have substantial clinical potential to be taken to clinical trials?** Despite numerous successes have been seen at a laboratory level, researchers should focus on optimization of current anti-aging technologies before bringing them to the clinical level, in order to avoid failure in clinical trials that causes unnecessary costs and procrastination in product development.
- **When applying systemic drug delivery, what is the best solution to the dose loss problem?** Common causes of the dose loss issue include undesired immune interactions, undesired excretion, interactions between delivery systems and blood components, etc. Non-immunogenic materials with a well-controlled particle size would be a sensible option.
- **How to simultaneously improve safety, biocompatibility, target selection, effectiveness and reduce the costs of a drug delivery system to a clinical standard without compromising the effect of the drug?** Nanoparticles are often employed in drug delivery systems for their nano-scale sizes that minimize immunogenicity, prolong circulation time and enhance

delivery efficacy. Biocompatible and biodegradable materials such as liposomes are preferred materials for synthesizing nanoparticles. Target selection can be achieved by using materials that are easily modifiable. However, it is not easy to find a material that matches all criteria. Hybrid nanocarriers are therefore a good option if the production costs can be brought down to a certain threshold.

- **How to design an effective clinical trial to avoid product failure that is not due to the product itself?** It is essential to properly design the population size and entry criteria of participants, control groups (e.g. historical controls, placebo, active controls, etc.), endpoints, blinding strategies, in order to minimize variation and randomness.

Glossary

Biogerontology A discipline concerning the biological aging process, with regard to its evolutionary origins and potential interventions

Genome-wide association studies An approach involving rapid scanning of specific markers across the genomes of numerous individuals in order to find genetic variations associated with a particular disease

Mammalian target of rapamycin A serine/threonine protein kinase that monitors the availability of intracellular amino acids and cellular energy status and thereby affects cell growth and proliferation

Microbubbles/nanobubbles Spherical drug carriers comprising a gaseous core that is coated by a shell of biocompatible materials

Nanocarriers Nanosized materials used as a transport module for drugs

RNA-interference technology A biological process in which exogenous RNA molecules are administered systemically to inhibit undesired gene expressions

Theranostics A field of medicine combining specifically targeted therapeutics and diagnostic tests

Viral nanoparticles Drug carriers composed of natural or engineered virus particles in nanoscale sizes

Xenobot An engineered "bio-robot" derived from frog stem cells, which is computationally programmable for its shape, behaviours and functions

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Chapter 2
Current Status of Systemic Drug Delivery Research: A Bibliometric Study

Saba Ale Ebrahim, Maysam Zamani Pedram, and Nader Ale Ebrahim

Abstract The previous chapter has presented an overview of the roles and limitations of systemic delivery in anti-ageing medicine. This chapter will examine the publication trend of research in systemic drug delivery by using a bibliometric approach. The data will be collected from the Scopus database to study the research trend from 1974 to 2019, and will be analyzed using network analysis of research outputs. Journals that have published related articles will be analyzed based on a diverse range of factors (including document citations, countries, topics and keywords). According to the publication trend identified, research in systemic drug delivery is a hotspot. With the bibliometric study presented, this chapter will offer an overview of the current status and future directions of research in systemic drug delivery.

Keywords Publication trend · Bibliometrics · Drug delivery · Literature research · Research direction

2.1 Introduction

Systemic drug delivery has been widely adopted in biomedical applications. Generally, systemic drug delivery refers to a method that uses a medium or a specific carrier (also known as "vehicle") to deliver a therapeutic agent body-wide or towards

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a specific region of the body. By optimizing the properties of the carrier, the performance of the drug can be enhanced. Moreover, performance optimization studies with various delivery mechanisms and delivery models have attracted extensive research interest (Bhagwat and Vaidhya 2013). Current administration methods include subcutaneous injections and non-invasive routes such as buccal, oral, transdermal and nasal administrations. Drug delivery via different routes requires different technical strategies and often encounters different challenges (Anselmo et al. 2019). Similarly, targeted drug delivery is also performed by systemically delivering a drug in a predefined way and towards the specific region of interest. Optimization of targeted delivery technologies focuses extensively on the improvement of delivery efficiency and the reduction of side effects. There are generally two types of targeting: passive targeting which relies solely on the molecular properties of the target tissues (e.g., permeability), and active targeting in which the design of the vehicle plays an important role. Nanoparticle- and carbon nanotube (CNT)-based drug delivery systems are examples in this field (Svenson 2004).

Bibliometrics is a widely adopted approach for measuring academic and organizational performance based on various indices such as the number of publications, the number of total citations and the average number of citations per year (Davidson et al. 2014; Ghanbari Baghestan et al. 2019). Bibliometric studies analyse the connection between the derivatives of publication output, the citation impacts, countries' scholarly outputs, assessment of scientific activity and compare relevant contributions to specific research areas, groups or institutions (Nordin et al. 2019; Jamali et al. 2015). Web-based citation **databases** such as **Scopus** and **Web of Science** are frequently used for deriving bibliometric data (Das 2015). Over the last six decades, bibliometric studies in the field of drug delivery analysed publication trends (Ale Ebrahim et al. 2019), the growth in the amount of literature published (Robert et al. 2017), collaboration networks (Huang et al. 2015), the evolution of nano-enabled drug delivery (NEDD) systems (for brain cancer and Alzheimer's disease) (Ma et al. 2015) and controlled drug delivery technology (Park 2014).

The first article related to systemic drug delivery was published in 1974. It determined the administration dose of intra-arterial drug delivery (Eckman et al. 1974). The latest related articles published in 2018 have focused on topics such as bioinspired nanocarriers for bioimaging and drug delivery (Liu et al. 2018), biomimetic liposomes for tumour therapy (Pitchaimani et al. 2018), plasma membrane-responsive nanoparticles for intracellular drug delivery (Jia et al. 2018), colloidal nanoparticles for drug delivery (Gagliardi et al. 2018), bile salt enhancers for inhalation delivery (Sørli et al. 2018), silk sericin-based nanoparticles for biological media (Hu et al. 2018), polymer-coated liposomes for oral administration (Klemetsrud et al. 2018), ketoprofen loaded particles for sustained drug delivery (Floriano et al. 2017), liquid crystal nanocarriers for photodynamic therapy (Nag et al. 2018), systemic delivery of simvastatin and lovastatin (Shahrezaei et al. 2018), local drug delivery systems for thyroid cancer treatment (Yoo et al. 2018), delivery of aerosols for pulmonary proteins (Wilson et al. 2018), etc. However, as far as we are concerned, none of

the authors has paid adequate attention to bibliometric research in "systemic drug delivery". This research aims to explore the research status in the field of "systemic drug delivery" from 1974 to 2018 using bibliometric approaches.

2.2 Methodology

Two databases, Scopus and Web of Science Core Collection (WoS) (along with PubMed database), were used in this bibliometric analysis. Specific inclusions and exclusions were applied to determine relevant articles for our bibliographic dataset. The term "systemic drug delivery" was used as the search item in the former databases on 7 September 2019. The first search strategy consisted of all the documents published in journals, indexed by Scopus, WoS or **PubMed** databases, using "systemic drug delivery" as the keyword in their titles. There were very limited (under 50) publications met the criteria, which may not cover all publications in the field.

In order to extend the data set, a search was conducted with individual words (i.e. "systemic", "drug" and "delivery") included in the titles of articles. The results included a relatively small number of 80–150 documents obtained from different databases. Moreover, some documents were found to be irrelevant to the field by an initial inspection. Scopus, WoS and PubMed databases allow a topic search from articles' titles, abstracts and keywords (especially Scopus database). A topic search for the words "systemic" and "drug" and "delivery" was accomplished to cover all publications in the field. The search results contained from 7000 to over 20,000 publications (as illustrated in Fig. 2.1). After investigating the most cited and the latest documents in the result, most documents were found unrelated to the research area. Therefore, changing the search strategy was required to retrieve relevant documents.

A second topic search "systemic drug delivery" was designed to compile a bibliography of all publications related to systemic drug delivery. The search retrieved documents containing "systemic drug delivery" in the title, abstract or keywords. These searches returned 372 documents from Scopus, 264 documents from WoS and 250 documents from PubMed. After a comparison of different databases, Scopus database was chosen for the final bibliographic dataset because it was more comprehensive among others and covered more documents in the field of "systemic drug delivery" (Aghaei Chadegani et al. 2013). Hence, a limitation of this study is that the data were collected solely from the Scopus database and those obtained from WoS or PubMed were not considered (except for the common ones). However, the Scopus database listed "in-press" articles while Web of Science did not (Harzing 2019). Therefore, this limitation did not significantly affect the results.

The final literature search was performed using Scopus search engines with the term "systemic drug delivery" on 7 September 2019. The search returned 372 documents from the Scopus database. **PRISMA** (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) consisted of a checklist and a flow diagram was employed. PRISMA flow diagram showing the data selection procedure has been generated (Fig. 2.1) (Moher et al. 2019). The data (372 documents)

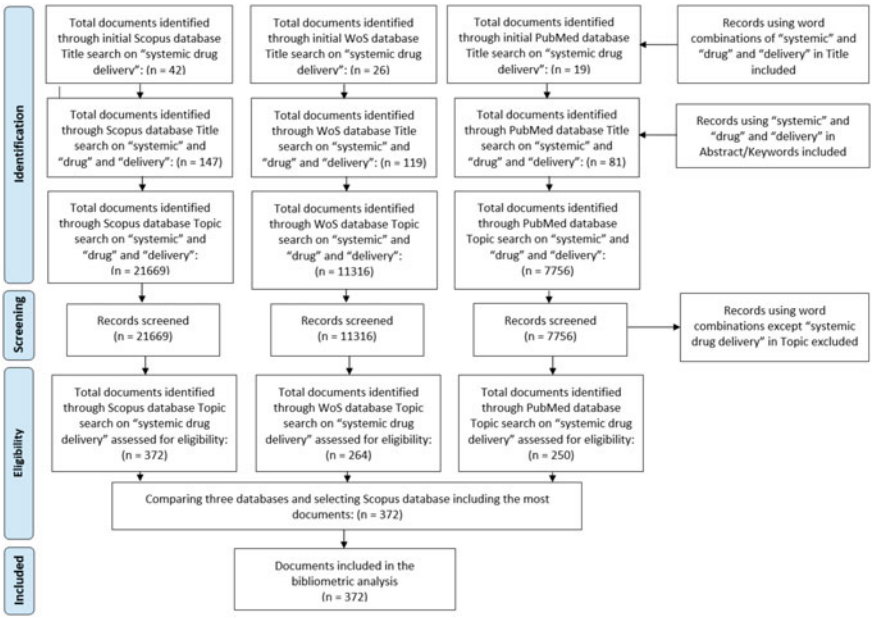

Fig. 2.1 The PRISMA flow diagram for the bibliometric analysis in the field of systemic drug delivery

were analysed by Bibliometrix-package (https://www.bibliometrix.org/) which is an **R tool** for comprehensive science mapping analysis (Aria and Cuccurullo 2017). The bibliometrix-package is designed for quantitative research in **scientometrics** and bibliometrics. The tool provides various routines for importing bibliographic data from well-known databases such as Scopus (https://scopus.com) and Clarivate Analytics Web of Science (https://www.webofknowledge.com/). Table 2.1 shows a summary of the primary information on bibliometric data. The details of the quantitative analysis included publication years, document citations, sources, authors, countries, topics and keywords.

2.3 Analysis Results

2.3.1 Analysis of Publication Years

In Fig. 2.2, the publications trend on systemic drug delivery from 1974 to 2018 is presented. There are a total number of 372 documents published within this period of time, including 206 articles, 133 reviews, 17 book chapters, 12 conference papers, 2 notes, 1 book and 1 short survey. The number of relevant publications per year increased from one article in 1974 to over 30 articles in 2018. As shown in Fig. 2.2,

Table 2.1 Summary of the main information of the collected bibliometric data

Description	Results
Documents	372
Sources (Journals, Books, etc.)	226
Index keywords	4922
Author's keywords	1153
Period	1974–2019
Average citations per documents	36.87
Authors	1532
Authors of single-authored documents	37
Authors of multi-authored documents	1495
Documents per author	0.243
Authors per document	4.12
Co-Authors per documents	4.42
Collaboration index	4.52

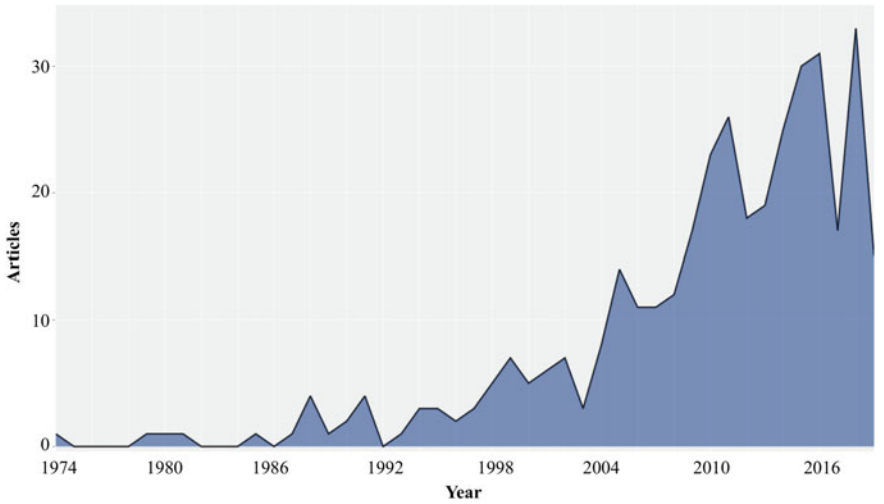

Fig. 2.2 Annual scientific productions in the field of systemic drug delivery

the number of publications per year has fluctuated within the time span. There were four main turning points in 2004, 2009, 2014 and 2018, where the publication rate dropped immediately after each time point. As there was a twofold increase in the number of publications from 2017 to 2018, it seems that research interest drawn by this area has been growing rapidly.

2.3.2 *Analysis of Citations*

The annual average numbers of article citations in the area of systemic drug delivery from 1974 to 2018 are shown in Fig. 2.3. An average number of 36.87 citations was found for each publication in this field. Notably, publications in 2008 had the highest number of average total citations per year. According to Fig. 2.3, the mean number of citations has been oscillating, while the mid-value has been increasing steadily from 1974 to 2018.

The publications found were published in 226 different sources including journals, books, conference proceedings series, etc. Figure 2.4 shows the top 20 sources that published articles in this field most frequently. The Journal of Controlled Release, International Journal of Pharmaceutics and Advanced Drug Delivery Reviews were ranked as the top three most productive journals with 19, 15 and 13 related publications, respectively.

Figure 2.5 ranked the **Hirsch index** (h index) of the sources and listed the top 20. The Journal of Controlled Release had the highest h index of 16 and a citation count of 1375. Advanced Drug Delivery Reviews and the International Journal of Pharmaceutics (shown in dark blue colour) had more than ten papers with at least ten citations and were therefore ranked second and third. As shown in the figures, these three journals had high rankings in all essential features (such as frequency, citation and h index).

Since 1974, journals have increased the number of publications in the area of systemic drug delivery. Figure 2.6 illustrates the growth in the number of related publications in five major sources. The Journal of Controlled Release has had a high growth rate since 1998 and reached more than 1.5 in the rate of annual occurrence in

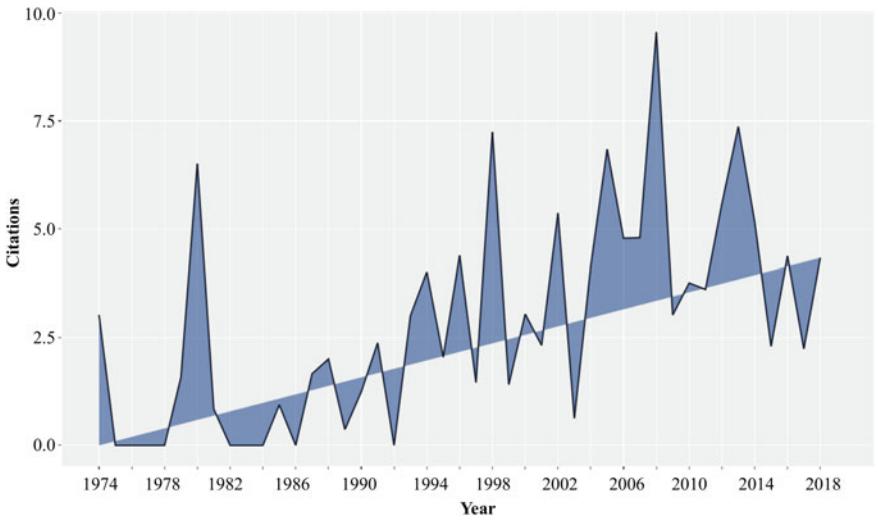

Fig. 2.3 Average article citation per year in the field of systemic drug delivery

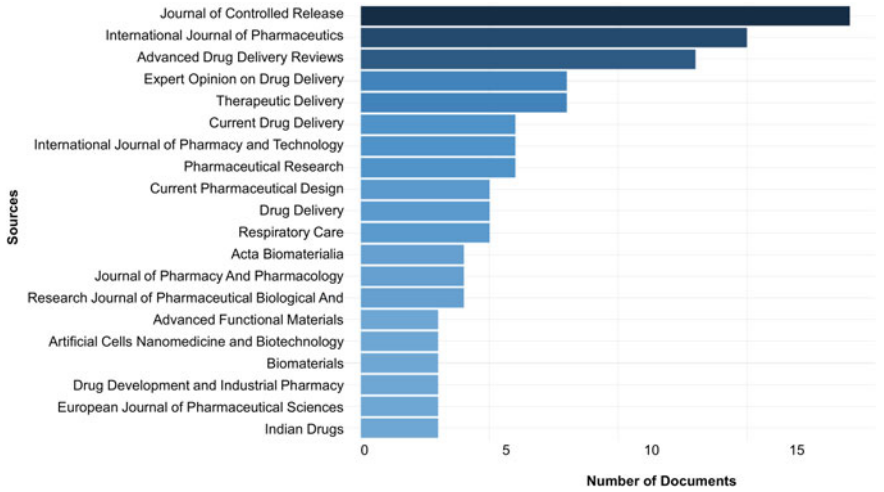

Fig. 2.4 Most relevant sources in the field of systemic drug delivery

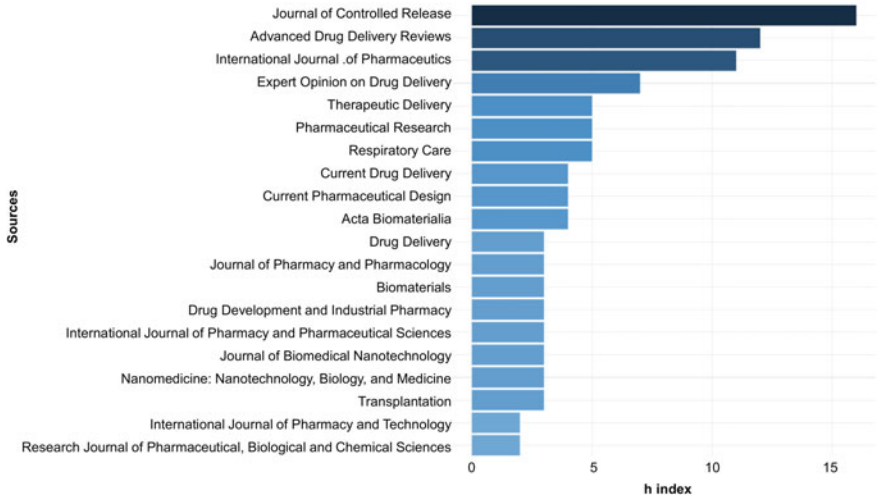

Fig. 2.5 Source impacts in the field of systemic drug delivery

2019. The International Journal of Pharmaceutics and the Journal of Expert Opinion on Drug Delivery, on the other hand, have had a decrease in the rate since 2010. Interestingly, the Journal of Therapeutic Delivery has been increasing the number of printed articles dramatically since 2004, and the rate had doubled (from 0.5 to 1.0) in 7 years. The rate of Advanced Drug Delivery Reviews has risen rapidly since 1980 but followed by a slight fall in 1998. Its production seems to be remaining steady after 2018.

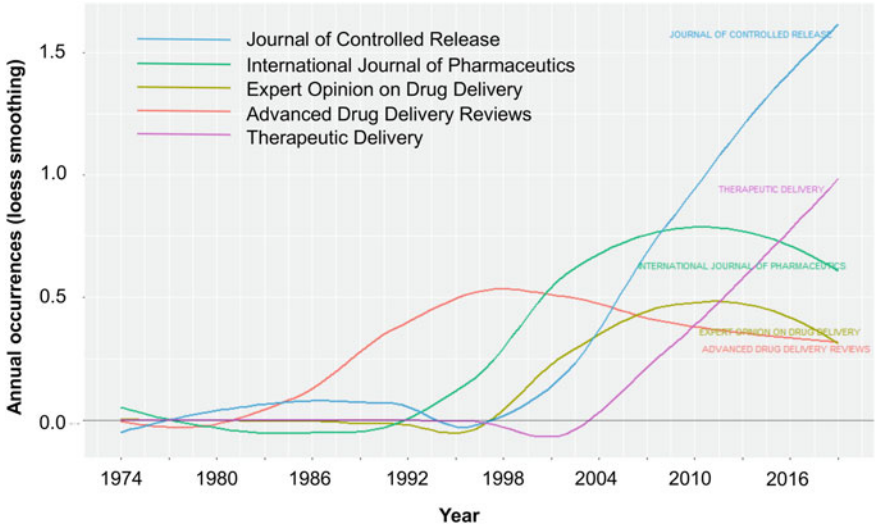

Fig. 2.6 Annual source growth in the field of systemic drug delivery

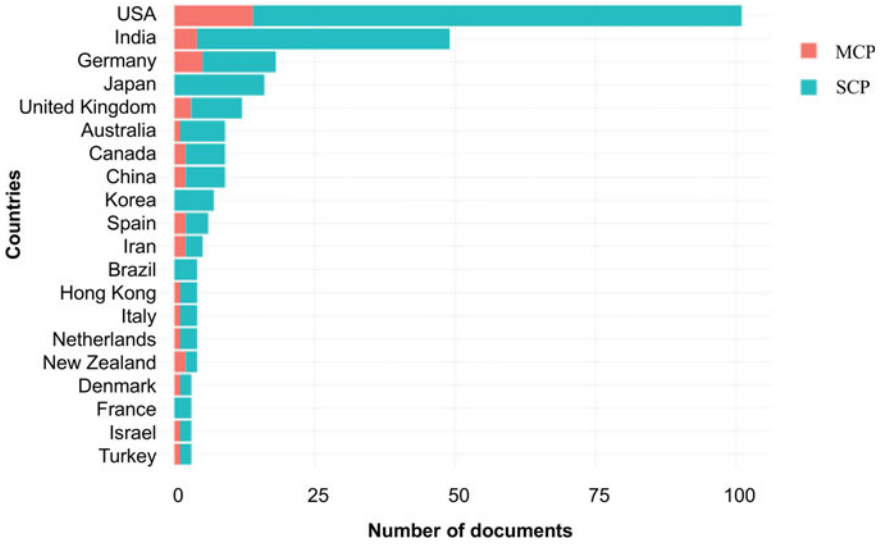

Fig. 2.7 Corresponding author's country. Abbreviations: MCP: Multiple countries publication; SCP: single country publication

2.3.3 Analysis of Countries

Figure 2.7 ranked the country of origin of corresponding authors. Red bars and blue bars indicate multiple countries' publication (MCP) and single country publication

(SCP), respectively. The MCP of each country specifies the number of published articles with a corresponding author from the country and at least one foreign co-author. As shown in Fig. 2.7, the USA had the highest total publication count (over 100) from 1974 to 2019, and India took second place in the list. Notably, Germany had only one-third of the total publication count but a higher MCP count as compared to India. Japan, Korea, Brazil and France were found to have no MCP.

Figure 2.8 shows the top 20 countries whose publications have had the highest citation counts. The USA, India and Germany were the top three on the list. Remarkably, the USA had over 6000 counts, which was six times higher than the second (India).

Figure 2.9 shows a map illustrating the productivity of each country from 1974 to 2018 (demonstrated by the intensity of the colour). It can be seen that the USA and India had the darkest colour and therefore the highest publication counts. Figure 2.10 shows a collaboration map of the countries. The USA had been the most frequently collaborated country and often collaborated with the United Kingdom, India, Germany and China. According to our bibliographic dataset, America and Australia were the most prolific continents, while Antarctica and Africa were the least prolific ones.

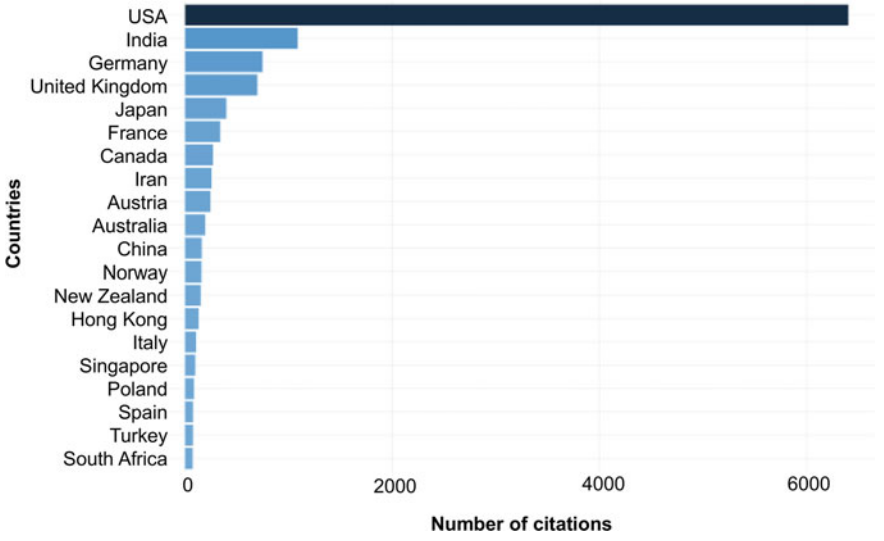

Fig. 2.8 Most cited countries in the field of systemic drug delivery

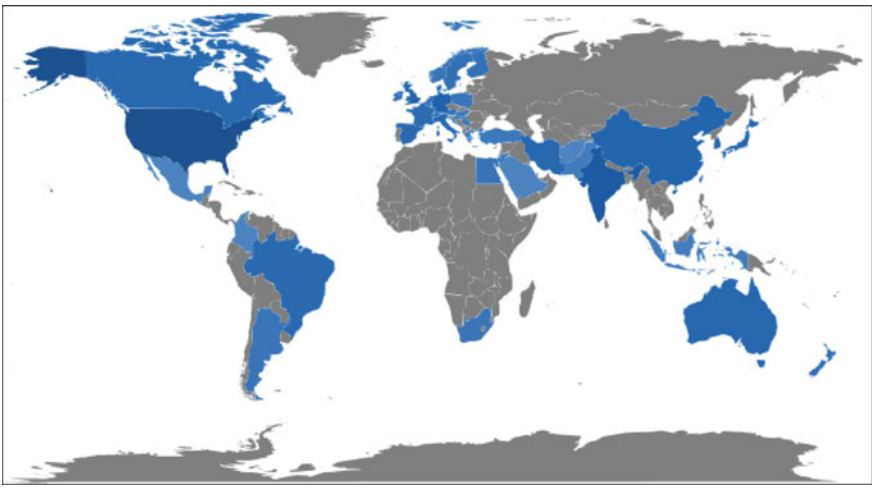

Fig. 2.9 Scientific production map

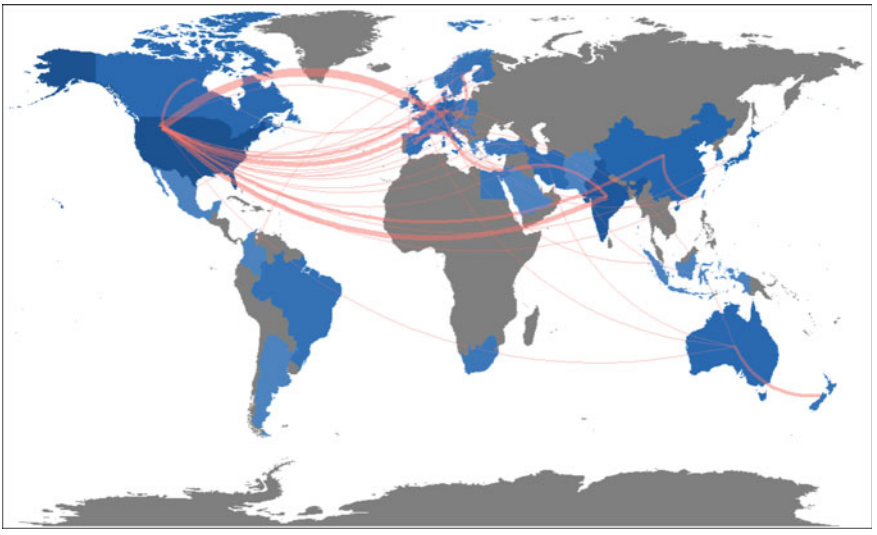

Fig. 2.10 Country collaboration map

2.3.4 Analysis of Topics

Topics in systemic drug delivery are divided into four sections as shown in Fig. 2.11 where each bubble indicates a network cluster. The four sections include highly developed and isolated themes in the upper left, motor themes in the upper right, emerging or declining themes in the lower left and basic and transversal themes in

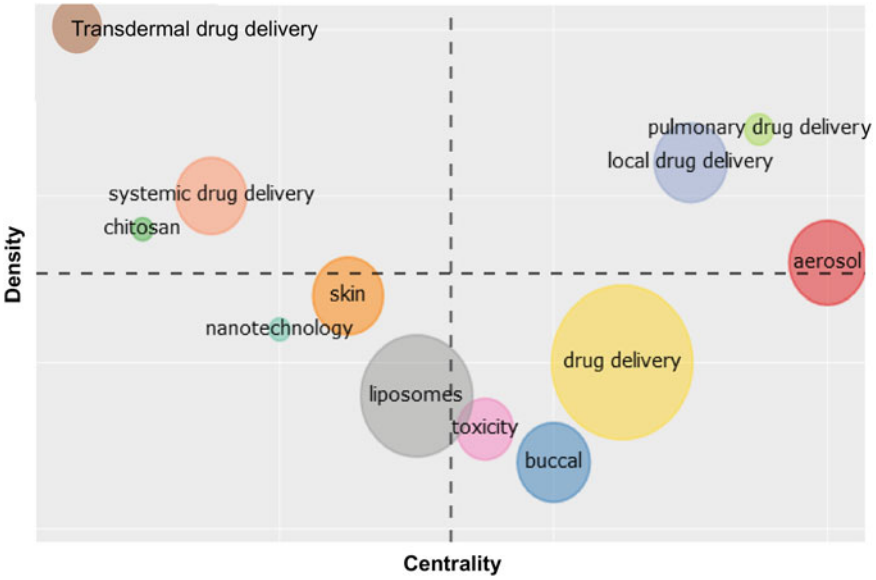

Fig. 2.11 Thematic map of keywords clustering in the field of systemic drug delivery

the lower right. The y-axis and x-axis represent density and centrality of the word clusters, respectively. The most frequently occurred word in each cluster was selected as the bubble name, and the total number of occurrences was indicated by the bubble size. The word cluster represented by Drug Delivery, as a basic and transversal theme, was the most relevant one in systemic drug delivery research. The term "Liposomes" was classified as an emerging or declining theme that has been frequently used. Pulmonary Drug Delivery, Local Drug Delivery and Aerosol clusters had the highest density and centrality and have been widely used as motor themes. Although Systemic Drug Delivery was the main keyword for our bibliographic collection, it was located in the upper left section with a medium bubble size, indicating that it is an extensively studied and isolated theme.

2.3.5 Analysis of Keywords

Figure 2.12 shows the top 20 most frequently used keywords plus (an automatic set of words or phrases often appeared in titles, keywords and references). There were 1153 author's keywords and 4922 keywords plus identified in the area of systemic drug delivery from 1974 to 2019. Drug Delivery System was the most relevant keyword plus in the area with almost 300 (out of 4922) occurrences. In the bibliometric analysis, keywords plus are as crucial as the author's keywords because they represent the current knowledge structure in the research field rather than the article's content.

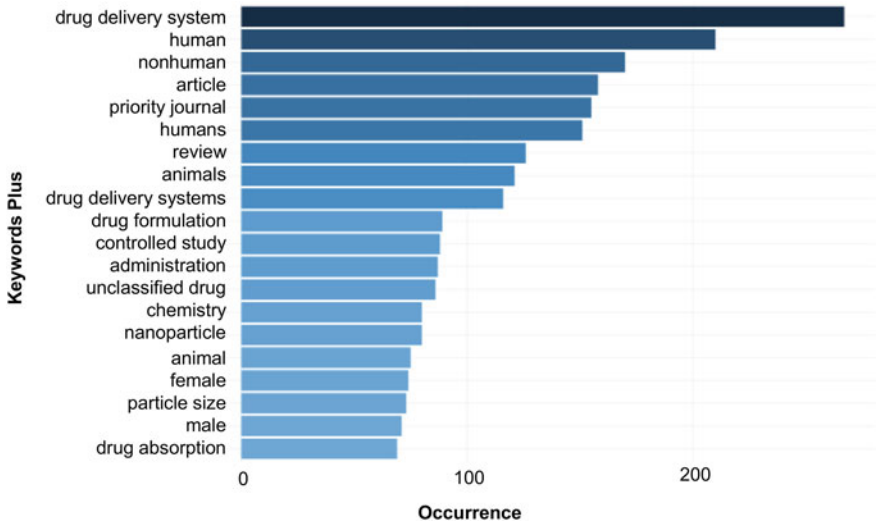

Fig. 2.12 Most relevant keywords plus in the field of systemic drug delivery

The conceptual structure **dendrogram** of keywords plus used in the field of systemic drug delivery research from 1974 to 2019 is shown in Fig. 2.13. The data dimensions were reduced by several factorial approaches, including Correspondence

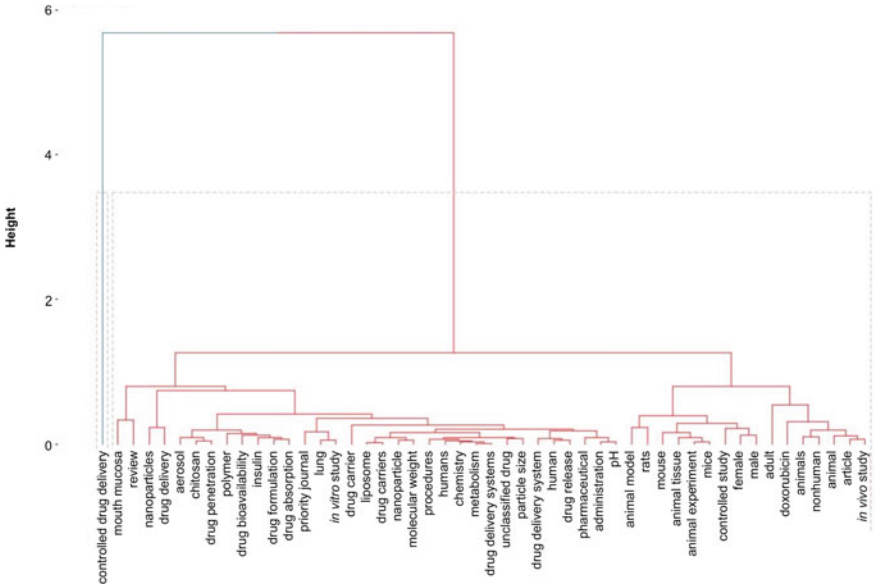

Fig. 2.13 Conceptual structure dendrogram of keywords plus in the field of systemic drug delivery

Analysis (CA), Multiple Correspondence Analysis (MCA) and Multidimensional Scaling (MDS). The y-axis shows the distance measurements of the words. Clusters were labelled with colours (where red refers to tech and blue refers to policy), productivity and innovation cluster headers.

Chitosan and Drug Penetration, Drug formulation and Drug Absorption, Lung and in vitro Study, Liposome and Drug Carries, Nanoparticle and Molecular Weight, Drug Delivery Systems and Metabolism, Unclassified Drug and Particle Size, Human and Drug Release, Administration and pH, Animal Experiment and Mice, Female and Male, Animals and Nonhuman, and Article and in vivo study were paired up in the dendrogram because they were the relevant keywords plus that belonged to the same concept/topic and are often appeared together in systemic drug delivery-related publications. Lastly, the keywords plus of Controlled Drug Delivery was labelled as policy, productivity and innovation and differed from other keywords.

Figure 2.14 shows tag clouds that represent the metadata of the author's keywords, where the font size of each tag cloud was directly correlated to its prominence. As shown in the figure, Drug Delivery and Systemic Drug Delivery were the top two most frequently used keywords because both of them consist of the term used to set up the bibliographic query. Nanoparticles, Transdermal Drug Delivery, Local Drug Delivery, Liposomes, Mucoadhesion, Pharmacokinetics and Aerosol were the top ones in the list as authors considered them to be suitable terms to describe the content of the publications. Figure 2.15 illustrates the increase in occurrence of the keywords plus from 1974 to 2018. All of the keywords plus showed an increase in the frequency of occurrence. Drug Delivery System exhibited the highest increase rate among others, while Animals, Review and Drug Formulation reached a plateau after 2016.

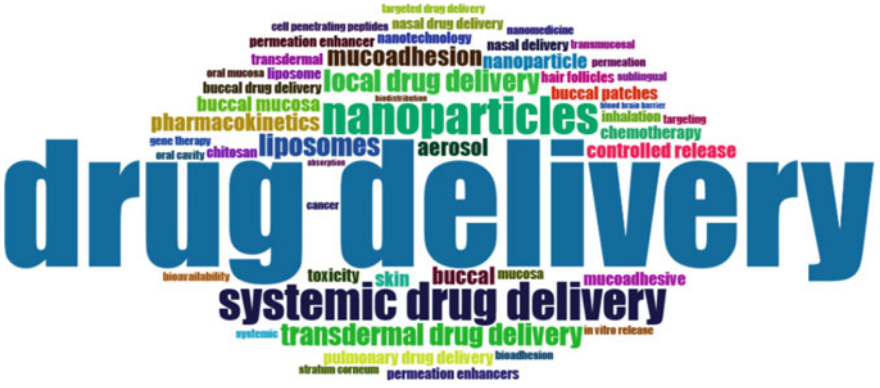

Fig. 2.14 Wordcloud of author's keywords in the field of systemic drug delivery

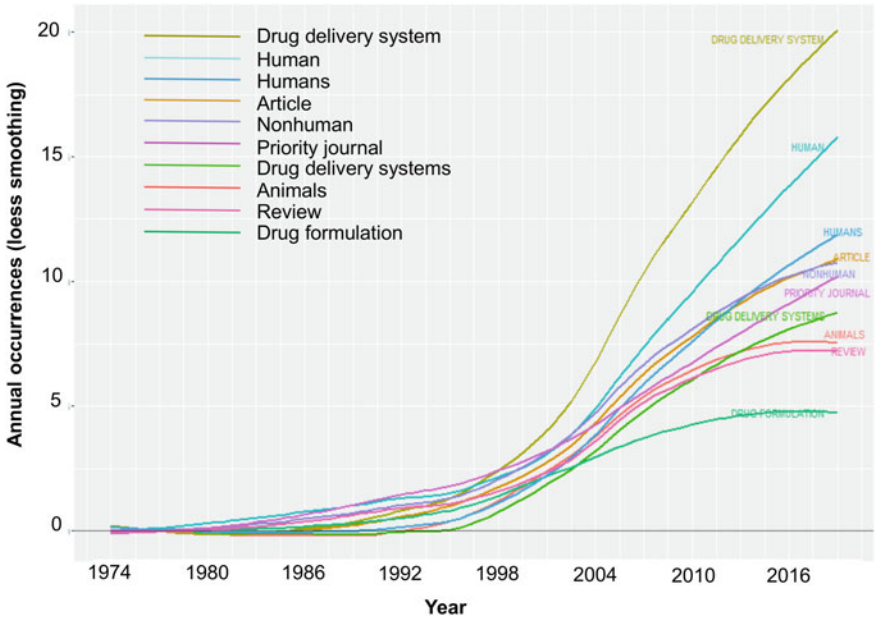

Fig. 2.15 Growth in occurrences of keywords plus in the field of systemic drug delivery

2.4 Summary and Outlooks

A global scientific production trend in the area of systemic drug delivery from 1974 to 2018 was quantitatively analysed with the Scopus database in terms of citations, most relevant sources, authors, countries and topics. The results showed that the annual scientific production in the field of systemic drug delivery has been growing in general. The United States, India and Germany produced the highest amounts of publications that have been cited most frequently. The keyword "liposomes" has been an emerging or declining theme that occurs frequently. Many studies in the categories of "pulmonary drug delivery", "local drug delivery" and "aerosol delivery" had the highest density and centrality among others. The occurrence of "Drug delivery system" has been growing rapidly according to the keywords plus analysis, while "Drug formulation" has been the least frequently used keyword plus growing in a steady manner. This bibliometric study serves as a guide to researchers for the selection of future research directions in the area of systemic drug delivery.

Important Notes

- Systemic drug delivery has been widely studied in biomedical applications.

- Bibliometrics is a useful tool for measuring academic and organizational performance based on various indices such as the number of publications, number of citations, average citation per year, etc.
- The final search strategy covered all documents published in journals indexed by Scopus using "systematic drug delivery" as a keyword.
- The average number of authors listed in each publication was 4.12 from 1974 to 2019 (1646 authors were listed).
- The research interest in the area of systemic drug delivery has been growing in general, which is indicated by a doubling in the number of publications from 2017 to 2018.

Questions for Future Research

- **What are the most popular research topics in the area of systemic drug delivery, and how are they related to the treatments of age-associated diseases such as cancer?** The bibliometric analysis of topics and keywords provides an insight into different hotspots.
- **What are the most appropriate model systems and the most effective methods of drug administration for systemic drug delivery?** The selection of model systems as well as the route of drug administration are two important factors to be considered when research on systemic drug delivery is carried out. Performing a systematic literature review is one of the feasible methods of choosing and tuning appropriate factors.

Glossary

Bibliometrics Adiscipline in which the progress of a given discipline is studied, evaluated and monitored by applying mathematical and statistical methods to analyse publications.

Databases Structured collections of data.

Dendrogram A type of diagrams displaying the results of hierarchical clustering.

Hirsch index Serves as an alternative to traditional journal impact factor metrics for the evaluation of the impact of the work done by a particular researcher.

PRISMA A set of guidelines governing the items to be reported in systematic reviews and meta-analyses.

Pubmed A database developed by the National Center for Biotechnology Information at the National Library of Medicine.

R tool A software for statistical computing and graphic plotting.

Scientometrics A discipline to quantitative study science, communication in science, and science policy.

Scopus An indexing database held by Elsevier, containing abstracts and citations for journal articles, book chapters and conference papers.

Web of science An indexing database within Web of Knowledge. It is held by Thomson Reuters.

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Chapter 3
Characterization Techniques for Studying the Properties of Nanocarriers for Systemic Delivery

Aditi Mehta and Olivia M. Merkel

Abstract As presented in the last two chapters, effective systemic delivery is critical to the execution of biogerontological interventions, and is a hot research topic. In systemic delivery, carriers are generally in the nanoscale. The physicochemical properties of these nanocarriers determine their in vivo pharmacokinetics, biodistribution, and tolerability. The most analyzed among these physicochemical properties are shape, size, surface charge, and porosity. Several techniques (including SEM and dynamic light scattering) have been adopted in the literature to characterize these specific properties. These different techniques assess the particles under varying conditions (such as physical state, and solvents) and as such probe, in addition to the particles themselves, artifacts due to sample preparation or environmental conditions during measurement. In this chapter, we will discuss the use of different methods, including their advantages or disadvantages, to precisely evaluate the properties of nanocarriers for systemic delivery. Here it is worth mentioning that, in several cases, there are physical properties that can be evaluated by more than one technique. Different strengths and limitations of each technique complicate the choice of the most suitable method, while often a combinatorial characterization approach is needed.

Keywords Nanoparticle characterization · Nanoparticles · Porosity · Shape · Size · Surface charge

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3.1 Introduction

Nanomaterials are generally defined as materials wherein "50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm" (European Commission definition of nanomaterial 2011), and are important components in novel drug formulation. Due to their small size and large surface area, nanoparticles can help increase solubility and thus enhance bioavailability, can mediate additional ability to cross the blood–brain barrier (BBB), enter the pulmonary system, and be absorbed through the tight junctions of endothelial cells of the skin (Kohane 2007). Carriers used in drug delivery systems are made of polymers (polymeric carriers, matrices, **micelles** or dendrimers), lipids (liposomes or solid lipid carriers), gold carriers, viruses, nanotubes, and magnetic carriers (Fig. 3.1) (Cho et al. 2008). They have been shown to efficiently transport therapeutic agents to cells influencing the **pharmacokinetics** of transport, drug release, and distribution of the active drug. The advantages of using nanocarriers include increased bioavailability of the drug, protection against degradation and stabilization of more sensitive agents (e.g., proteins, antibodies), resulting in their higher concentrations in the target tissue and reduced side effects (Allard et al. 2009). Moreover, nanocarriers can be attached to targeting ligands (Xie et al. 2016; Peer et al. 2007; Allen 2002) such that their specificity to target cells/tissues can be increased. In addition, nanocarriers can also be used

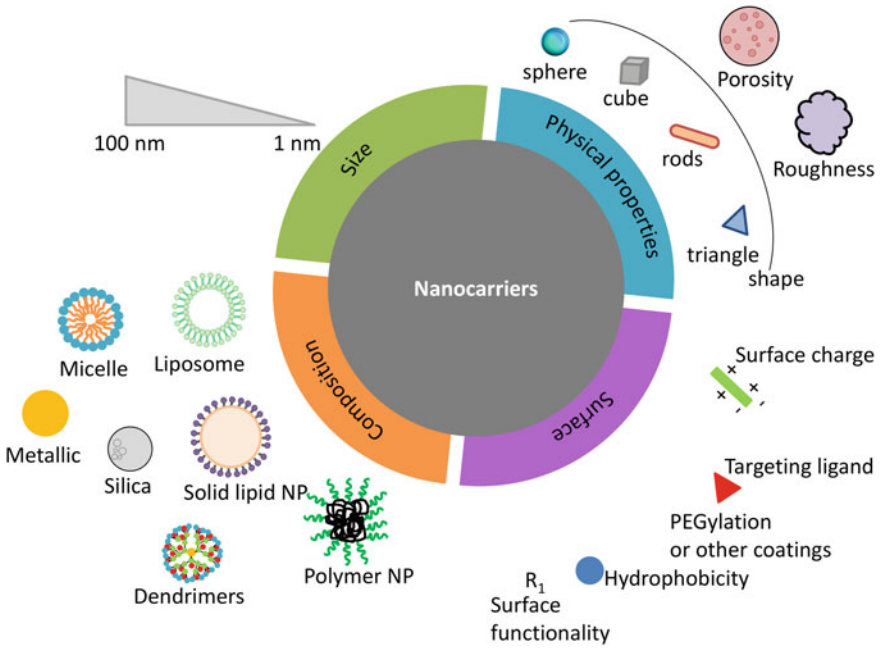

Fig. 3.1 Nanocarriers used in drug delivery. A summary of nanocarriers explored as carriers for drug delivery, together with illustrations of their biophysicochemical properties

to deliver hydrophobic or poorly water-soluble drugs, for instance, by using micelles which assemble into a hydrophobic core and hydrophilic shell. A remarkable example is Doxil®. The first FDA approved nano-drug composes the drug doxorubicin loaded within PEGylated nano-liposomes, which demonstrates prolonged drug circulation time and avoids clearance by the **reticuloendothelial system** (RES). Nanocarriers also allow for synergistic therapy options via the codelivery of multiple drugs at the same time to the same location with the same pharmacokinetics.

Over the last several decades, nanocarriers have become an attractive option to deliver therapeutic molecules to target tissues after systemic delivery. However, the physical and chemical properties of nanocarriers affect their **biodistribution** and tissue retention within the body (Hirn et al. 2011; Huang et al. 2011). Nanocarriers can be administered either by direct injection, inhalation, or via oral intake. Once they are part of the systemic circulation, they interact with serum proteins (Mu et al. 2014), adsorb small molecules, such as amino acids, folate, biotin, and many others (Guo et al. 2008). Specifically, their shape and size strongly influence cellular uptake. It has been shown that 100 nm particles exhibited a 2.5-fold greater uptake compared to 1 μm diameter particles in vitro (Desai et al. 1997). Another challenge to these nanoparticles is the immune system. While small particles (<30 nm) are rapidly cleared by the kidney, while those > 30 nm in size are cleared by the reticuloendothelial system (RES), including macrophages in the liver and spleen. Whether nanocarriers are taken up by macrophages or not, depends on **opsonization** by the innate immune system (Gaumet et al. 2008). On the other hand, the size and surface properties of the nanoparticles also influence their in vivo stability (Alexis et al. 2008). For instance, PEGylation, i.e., conjugation of a polymer polyethylene glycol (PEG, $(CH_2CH_2O)_n$) on the nanoparticle surface can at least partially protect them from opsonization (Hoffman 2008).

In fact, nanoparticle size in particular affects immune cell sequestration and subsequent clearance from the blood stream. It was observed that particles greater than 200 nm activate the lymphatic system and are removed from circulation quicker (Prokop and Davidson 2008). Moreoever, nanoparticle size considerably influences its **cytotoxicity**. Xiong et al. demonstrated size-dependent cytotoxicity, with smaller (60–100 nm) particles triggering more damage, as measured by the release of TNF-α as compared to those above 200 nm (Xiong et al. 2013). For systemically delivered nanocarriers, the nanocarriers-drug complexes should also remain soluble and stable, escape aggregation in the blood, or prevent exposure of their 'cargo' to degrading enzymes within the blood or inter-tissue fluid. Determining the physicochemical properties of nanoparticles and exploring their structure–function-interaction relationships is, therefore, a critical part of nanomedicine. Characterization parameters, *shape, size, surface charge, porosity,* and *viscosity,* and the different characterization techniques, each based on different physical properties, are described in this chapter.

3.2 Shape and Size Distribution

As discussed above, the shape and size of nanocarriers are the most important and most studied parameters in their characterization, influencing their size distribution, degree of aggregation, surface charge, and area as well as in vivo biodistribution, tolerability as well as pharmacokinetics. Moreover, due to the synthesis process of nanocarriers, the product is often prone to be a polydispersion of nanoparticles which sometimes might be of a broad distribution, making size distribution analysis crucial to understanding nanoparticle behavior in vivo. The morphological characterization of nanocarriers can be performed by direct or indirect methods. Direct methods, including for example microscopy, X-ray diffraction and scattering, radioimmunoassay, rely on directly observing the nanocarriers together with the therapeutic material and distinguishing this assembly from its individual components, the carrier and the therapeutic material. On the other hand, indirect techniques focus on the parameters of a suspension or solution containing all three entities, the carrier-therapeutic assembly, the therapeutic material, and the carrier alone, thereby remaining unable to identify the differences between the pure components. Indirect methods include, for example, static and dynamic light scattering, absorbance, linear and circular dichroism, zeta potential.

3.2.1 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is one of the most efficient tools for nanomaterials characterization. TEM is based on the interaction between a uniform current density electron beam and a thin sample. The extent of this interaction is dependent on the size, sample density, and elemental composition of the sample. As the electron beam reaches the sample, the electrons interacting with specimen are transformed to unscattered electrons, elastically scattered electrons or inelastically scattered electrons (Williams et al. 1998). The scattered or unscattered electrons are then focused by a series of electromagnetic lenses and projected on a screen to generate an electron diffraction, amplitude-contrast image, a phase-contrast image or a shadow image of varying darkness according to the density of unscattered electrons (Fig. 3.2a) (Williams et al. 1998).

Both TEM and light microscopes operate on the same basic principle, however, due to the much shorter wavelength of electrons, TEM achieves a much higher level of resolution up to the level of atomic dimensions (<1 nm). Moreover, TEM provides the most accurate estimation of nanoparticle homogeneity. TEM is undoubtedly the most important and frequently used nanoparticle characterization technique.

The abovementioned advantages of TEM are also accompanied by serious limitations. Due to the high resolution provided by TEM, sampling is often challenging, allowing the user to only view a small section of the sample. Another problem of sampling is that for TEM imaging, very thin (electron transparent) specimen are

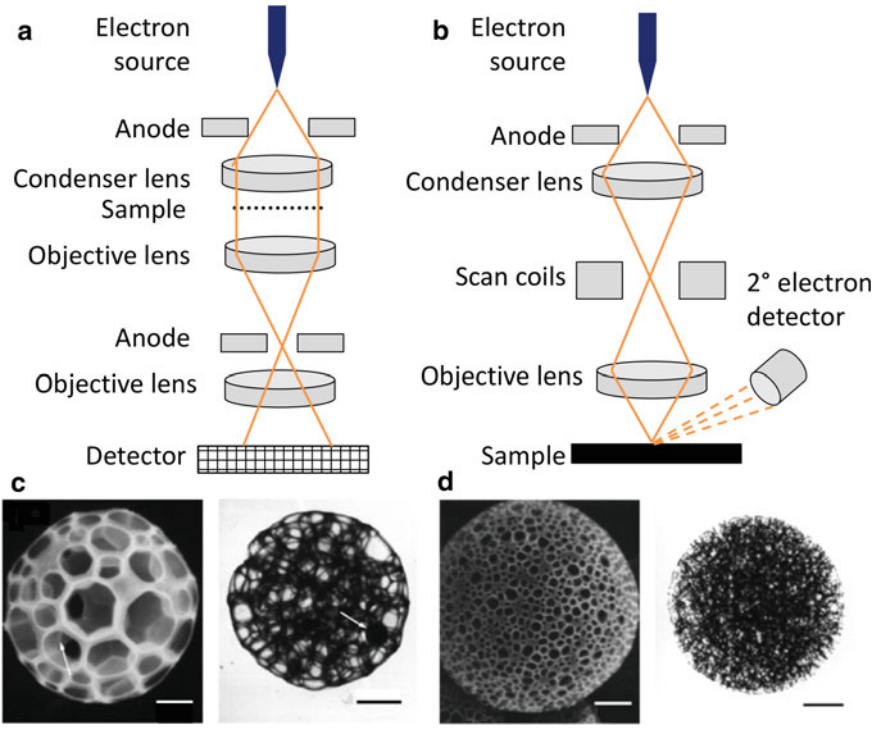

Fig. 3.2 Scanning versus Transmission electron microscopy. Simplied schematic diagram of **a** SEM and **b** TEM. **c**, **d** SEM and TEM micrographs of **c** poly(methyl methacrylate) (PMMA) microspheres and **d** poly(vinyl acetate) (PVA) microspheres prepared under the same conditions. The scale bar = 2 μm. Reproduced from He and Liu (2005) with permission from American Chemical Society

required, and specimens <100 nm in thickness should be used wherever possible. However, the thinning processes used affect the specimens, changing both their structure and chemistry. In addition, due to the high energy, the electron beam can damage organic, polymer, and hybrid nanoparticles. This problem can be addressed by either reducing the acceleration of the electron beam (however simultaneously reducing the attained resolution) or using Cryo-TEM (discussed in Sect. 1.4). Another problem is that TEM presents 2D images of 3D specimens, averaged through the thickness of the sample, thereby lacking depth sensitivity, also known as 'projection-limitation' (Williams et al. 1998).

3.2.2 Scanning Electron Microscopy

In scanning electron microscopy (SEM) a focused beam of high-energy electrons is incident across a sample in a raster pattern. The emitted electrons are detected

by a detector for each position in the scanned area, generating an image by the interaction signals obtained at the surface of solid samples (Fig. 3.2a). The intensity of the emitted electron signal is displayed as brightness on a display monitor and is stored in a digital image file that represents the morphology of the sample surface. Since SEM uses electron beams that are less powerful than for TEM, it limits their penetration depth and therefore, the results are sensitive to the surface morphology, with the advantage of minimal to no damage to the sample. However, due to the low energy electron beams, the resolution limit of SEM is typically around 3 nm. While SEM only yields information on the sample surface structure, TEM interacts with the whole sample volume, thereby providing information on the sample's internal structures. A direct comparison of SEM and TEM for the same nanoparticles was described by He and colleagues confirming these observations (Fig. 3.2c–d) (He and Liu 2005).

Although sample preparation for SEM is straightforward and simple, the samples are dried and imaged under vacuum, which may alter the topography of the sample. In addition, for high resolution imaging, the samples are required to be conductive. Non-conductive samples need to be coated by a thin layer (<10 nm) of a metallic film before being analyzed. An alternative method, **environmental or wet SEM**, is performed at low pressure instead of high vacuum and allows the analysis of hydrated materials without fixing, drying, or coating of the specimen (Bogner et al. 2007). However, environmental SEM has a lower spatial resolution than standard SEM imaging and has so far been limited to the characterization of microspheres and microcapsules (Xiong et al. 2013).

SEM can be operated in the transmission mode, i.e., through the technique called "scanning-transmission electron microscopy" (STEM), which combines both operational modes, SEM and TEM. In STEM mode, a convergent electron beam is focused to a small area of the sample. To register an image, the electron probe is raster scanned and subsequently propagated through the sample. Due to the electron–matter interaction, the trajectory of the electrons is scattered away and different kinds of signals are registered in sync with the electron probe scanning. Using STEM, advanced nanoparticle analysis can be carried out in the transmission mode by gaining in-depth information, and analysis of ensembles of particles is possible (Hartl et al. 2019). One of the main advantages of STEM over TEM is that the electrons scattered out to high angles on a high-angle annular dark field detector (HAADF) are chemically sensitive, and a sample with a definite crystalline arrangement is not a requirement.

3.2.3 Cryo-TEM

Cryo-TEM was developed in the 1980s to visualize biological samples in their vitrified, frozen-hydrated state, i.e., in a near-native state at the resolution of TEM (0.1–2 nm). Although originally developed to image biological samples in water, Cryo-TEM currently plays an important role in visualizing viruses (Adrian et al. 1984),

lipids- and polymer-based nanocarriers (Belliveau et al. 2012; Bonnaud et al. 2014) validating the structural integrity of nanoparticles. Cryo-TEM can be applied to samples in organic solvents or in aqueous surroundings, thereby allowing the visualization of nanoparticles under different solvent conditions as well as evaluate the changes during the development or self-assembly of the particles.

Typically, in Cryo-TEM, samples are suspended in a thin layer of frozen buffer stretched across a carbon grid in a specialized holder, which also contains a small dewar for liquid nitrogen as cooling agent at its end. Adequate sample cooling is essential to avoid sample damage by freeze-drying. In Cryo-TEM, the frozen sample grid is kept at liquid nitrogen temperature during imaging in a TEM, thereby taking images of the sample in its frozen but hydrated state. Since the sample is flash-frozen, Cryo-TEM avoids artefacts that result from sample drying.

Currently, Cryo-TEM is considered the gold standard for liposome imaging (Baxa 2018). An interesting recent example is the imaging and characterization of a widely used anticancer agents, namely, doxorubicin encapsulated in liposomes. In 2016, Wibroe et al. assessed liposome morphology of four liposomal doxorubicin formulations, Doxil®, Caelyx®, DOXOrubicin, and SinaDoxosome (Fig. 3.3) (Wibroe et al. 2016). They observed that while Doxil, Caelyx, and DOXOrubicin show intact spherical and prolate ellipsoidal unilamellar vesicles, SinaDoxosome, revealed co-existence of flat circular disks along with unilamellar vesicles.

Despite its many advantages over traditional TEM, Cryo-TEM also suffers some drawbacks. A significant concern of Cryo-TEM is that since the samples are frozen, the density difference between the sample and the frozen water is minimal resulting in

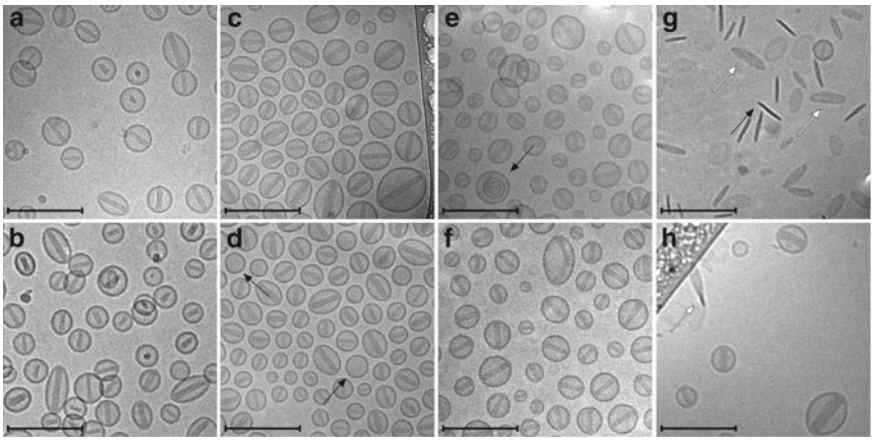

Fig. 3.3 Cryo-TEM analysis of four liposomal doxorubicin formulations. Cryo-TEM images of Doxil® (**a**, **b**), Caelyx® (**c**, **d**), DOXOrubicin (**e**, **f**) and SinaDoxosome (**g**, **h**). Scale bars: 200 nm. Black arrows indicate empty liposomes (**d**), an oligolamellar vesicle (**e**) and disks (**g**). White arrows represent face on view of disks (**g**, **h**). Reproduced from Wibroe et al. (2016) with permission from Elsevier B.V

a reduced contrast obtained in micrographs. To circumvent this problem, often Cryo-TEM micrographs are taken a few micrometers out of focus to generate phase contrast in the image (Baker et al. 1999). In addition, frozen samples are more sensitive to electron damage and can only tolerate lower electron doses, approximately, 10^3 fold lower than in TEM, before significant damage occurs to the samples.

Cryo-TEM images provide useful information about the shape, size, and importantly, the integrity of the nanoparticles. Furthermore, Cryo-TEM can also be used to determine 3D structures of nanocarriers at atomic resolution. For structurally and chemically homogenous particles, such as icosahedral viral vectors, protein-based nanoparticles (Kler et al. 2013), or gold nanoparticles (Tian et al. 2015), single-particle reconstruction may be performed by averaging multiple Cryo-TEM micrographs taken in various orientations. On the other hand, for irregularly shaped or heterogenous nanocarriers, such as liposomes or multipolymer micelles (Lobling et al. 2014), Cryo-electron tomography (Cryo-ET) may be performed, wherein the sample is tilted through a large angular range (for instance, $-80°$ to $+80°$) collecting a tilt series of images of a single specimen area. The electron dose is a critical factor, especially for Cryo-ET images, and should be maintained at approximately 20 electrons/Å^2. Irrespective of the method used, 3D reconstructions provide a complete representation of the sample as well as spatially accurate and quantitative measurements of each sample.

3.2.4 Atomic Force Microscopy

Atomic force microscopy (AFM) belongs to the family of scanning probe microscopy (SPM) techniques and was developed in 1985 combining the principles of scanning tunneling microscopy and the stylus profilometer (Binnig et al. 1986). AFM uses a sharp tip probe at the end of a cantilever of a probe to scan the surface properties of the specimen. AFM can be used to assess surface properties, such as morphology and mechanical properties of materials at an exceptionally high (nanometer) resolution (Binnig et al. 1986). This high resolution of AFM is achieved due to a combination of its probe (normally a sharp tip), carefully controlled tip-specimen forces, the optical level, and high-precision movement of the scanner. The probe is generally less than 5 μm in length and 10 nm in diameter at the apex and is located at the end of a microscale cantilever which is 100–500 μm long (Fig. 3.4a, b) (Kim et al. 2007). This tip moves over the sample surfaces and due to the tip-surface attractive or repulsive forces, the cantilever moves vertically, and a laser beam focussed on the back of the cantilever is deflected and detected. Therefore, the movement of the tip can be monitored by alterations in the laser which is then ultimately translated into a 3D image. A piezoelectric scanner is used to precisely control the probe—sample position and the accurate movement of the probe tip over the sample surface (Binnig et al. 1986; Lovley and Malvankar 2015). AFM permits quantitative, high-resolution, non-destructive imaging of surfaces, including biological ones.

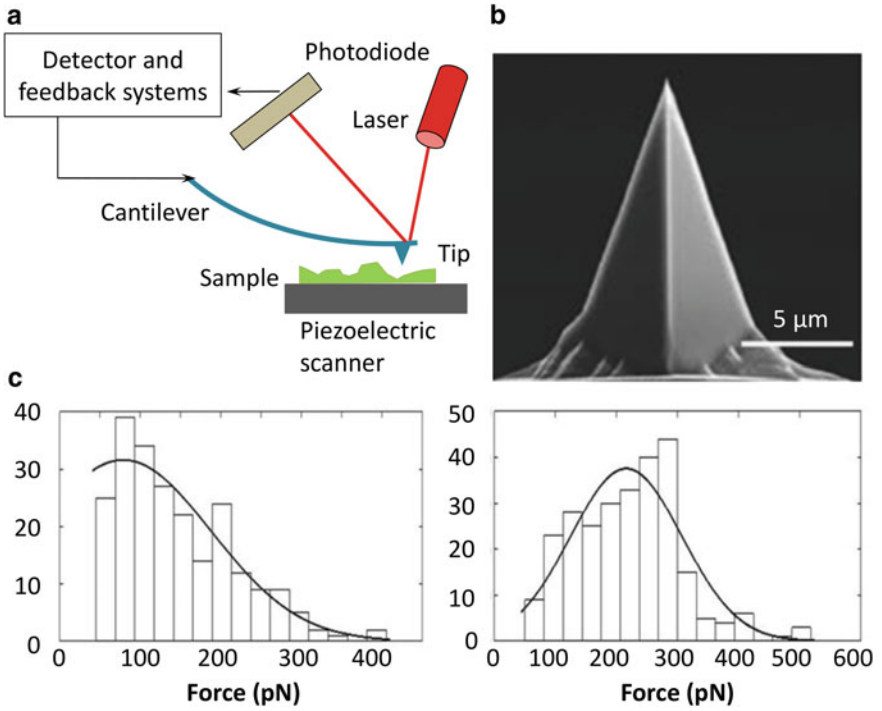

Fig. 3.4 Atomic force microscopy. **a** Simplified schematic of AFM. **b** SEM micrograph of a AFM probe tip. Reproduced from (Kim et al. 2007) with permission from AIP publishing. **c** Rupture force histogram plotted for substrate functionalized with free folic acid (left) and functionalized with folate decorated nanoparticles (right). Reproduced from Jones et al. (2017) with permission from Elsevier B.V

AFM allows shape and size measurements of nanocarriers under different conditions, such as various charge ratios, pH ranges, and salt concentrations, without any special treatment or vacuum conditions (Lovley and Malvankar 2015). AFM can be operated in different modes, of which the two most popular are the contact and non-contact mode. In the contact mode, as implied by the name, the AFM tip is in contact with the sample surface. As the scanner moves over the sample surface, the cantilever deflection is sensitive to changes in surface topography (Lovley and Malvankar 2015). In this case, the interaction between the tip and the sample is repulsive and coupled to the frictional force; it can damage softer samples and is therefore ideal for imaging relatively hard samples (Xiao et al. 2014).

In the non-contact mode, the cantilever is oscillated above the sample surface (5–15 nm above, amplitude <10 nm) near its resonant frequency (100–400 kHz). The attractive forces between the tip and surface change according to the distance between them, which induce alterations in the resonant behavior of the oscillating cantilever. These changes in frequency or phase and amplitude are used to generate images. The main advantage of the non-contact mode is that the tip never comes

in contact with the sample and therefore the sample remains undisturbed making it suitable for soft or vulnerable samples, such as biological samples.

An intermediate and the most commonly used mode is the tapping mode, wherein the cantilever is oscillated over the sample and to achieve the highest resolution, comes very close to the sample, often making intermittent contact with the sample. The short contact further dampens the oscillation amplitude which can be further translated to an image. This mode circumvents the lateral forces in the sample while minimizing frictional forces (Gadegaard 2006; Stylianou et al. 2019). In the tapping mode, topography and phase images are simultaneously acquired so as to obtain information on different properties of the sample (Etzler et al. 2012). The tapping mode is appropriate for samples weakly bound to the surface or soft samples, such as polymers, lipid bilayers, DNA, or proteins. (Zhou et al. 2015; Zhong et al. 2007; Shamitko-Klingensmith and Legleiter 2015).

Common AFM probes include silicon or silicon nitride probes and carbon nanotube tips (Hafner et al. 2001). The tip, however, can be modified according to different applications. For instance, for micrometer-scale imaging and mechanical testing, spherical tips can be constructed by gluing colloid or glass spheres to the AFM tips (Zhou et al. 2015). Interestingly, AFM probes can also be functionalized by coating polymers or proteins onto the tips thereby allowing measurements of the force required for the interaction between the substrate and protein/polymer. Using folic acid receptor (FRα) coated cantilevers, Jones et al. demonstrated the interactions between free folic acid or folic acid decorated micelleplex nanoparticles (Jones et al. 2017). The nanoparticles investigated in the latter study consisted of micelles formed with a FA conjugated triblock copolymer (PEI-g-PCL-b-PEG-FA) which condensed siRNA to form micelleplexes. Using this modified cantilever, over 1000 force measurements were made for each substrate and the binding probability as well as rupture force was determined (Fig. 3.4c). They demonstrated that the folate decorated micelleplexes had a significantly higher binding force as compared to free folic acid.

3.2.5 X-ray Diffraction

X-ray techniques are generally non-destructive and provide information about the ensemble average of many particles, in contrast to direct imaging techniques such as electron microscopy where only a very small sample of particles is analyzed which may not be truly representative of the material, for example, in case of polydisperse particles. X-ray techniques provide direct measures of particle size and lattice dimensions, in contrast to other indirect methods such as UV–visible spectroscopy, where the particle size is inferred from the systematic shift in the position of the absorption peak.

The importance of X-ray diffraction (XRD) was evident soon after its discovery. X-ray diffraction was proposed in 1912 by Max von Laue, who was awarded the Nobel Prize in 1914 for the same. In the next year, father and son William Henry

Bragg and William Lawrence Bragg received the Nobel Prize for determining the first crystal structures using X-rays. They characterized the atomic order of sodium chloride and other similar compounds and since then, crystal structures of more and more complex compounds have been elucidated. XRD allows for the determination of the atomic or molecular structures of all types of materials, which is a prerequisite for understanding their properties.

XRD is based on the constructive interference of a crystalline sample and monochromatic X-rays directed toward the sample, generated by a cathode ray tube, filtered to produce monochromatic radiation. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda = 2d \sin\theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. The larger the collection angle (or smaller the wavelength), the higher the achieved data resolution. These diffracted X-rays are then detected, processed, and counted. By scanning a sample through a range of angles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. XRD provides information about the crystal components, their average shape and size, nature of the phase, lattice parameters and crystalline gain size. For the latter, the Scherrer equation is used by broadening of the most intense peak of an XRD measurement.

XRD is performed in dry, powdered samples, commonly after evaporating their colloidal suspensions. In the area of nanoparticles, X-ray scattering and diffraction allow the non-destructive, direct evaluation of the crystal and particle size and their crystallographic phase. Interestingly, Upadhyay et al. determined the average crystal size of magnetite nanoparticles in the range of 9–53 nm. However, the TEM deduced size of the same was found to be higher than that calculated from XRD. They further showed that when the particle size was bigger than 50 nm, there was more than one crystal boundary on their surface, which could not be distinguished by XRD (Upadhyay et al. 2016). Similarly, another study with copper telluride nanostructures of different shapes showed that the relative intensities between the different XRD peaks depend on the particle shape (Liao et al. 2006).

3.2.6 Small Angle X-ray Scattering

X-ray scattering (XRS) techniques are used to characterize the crystal and particle size and the crystallographic phase, which all together determine the physical properties of the nanoparticle. Due to their small volume and limited coherence, XRS of nanoparticles is much weaker than that of bulk materials. The signal can be increased by either measuring the sample over a longer time or with high-flux sources (increased photons/sec). While high-flux sources provide superior signal-to-noise, they can have a detrimental effect on the sample due to radiation damage, which is particularly relevant for polymers or organic molecules such as surfactants that may be present as stabilizers on the nanoparticle surface (Ingham 2015).

Small angle X-ray scattering (SAXS) is widely used to determine the shape and size of materials in the range of 1–100 nm. SAXS is based on the elastic scattering of the electron cloud of each atom present in the sample and the difference in the electronic density of the scattering object and the medium. Typically, a SAXS sample is highly concentrated and can be a solid, powder, composite, or a nanoparticle dispersion in liquid medium. Samples are then irradiated by a monochromatic X-ray beam and the X-ray detector records its scattering pattern, which can be expressed as a function of momentum transfer as $q = 4\pi \sin \theta/\lambda$, where λ is the wavelength of the incident beam and 2θ is the scattering angle. Being an ensemble method, SAXS probes a very large number of nanoparticles simultaneously and gives a statistically relevant average over a large proportion of the sample. Wang et al. compared SAXS and XRD to monitor the structural changes of platinum nanoparticles with temperature (Wang et al. 2008). They observed that for some conditions, the sizes from XRD and SAXS did not correlate since SAXS is more sensitive to the size of the fluctuation region of electron density during thermal treatment.

3.2.7 Dynamic Light Scattering

Dynamic light scattering (DLS) is one of the most commonly used methods to analyze nanoparticle size. When particles are suspended in a liquid, they undergo constant random motion, known as Brownian motion, wherein each particle is constantly moving, and its motion is not correlated with that of other particles. The diffusion of spherical particles can be described by the Stokes–Einstein equation (Eq. 3.1):

$$D = \frac{\kappa_B T}{3\pi \eta d}, \tag{3.1}$$

where κ_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, D is the diffusion coefficient of the particles, and d is the diameter of the particles. Accordingly, smaller particles move more rapidly in solution as compared to larger particles.

When a particle is irradiated by a visible light, a part of the light will be transmitted through the sample and a part can be absorbed by the sample (Fig. 3.5a, b). For particles considerably smaller (at least 20-fold) than the wavelength (λ) of the incident radiation, the radiation will be scattered in different directions, without altering its wavelength or energy. This elastic scattering of light is known as Rayleigh scattering. As light scatters from the moving particles, the distance between particles varies with time which creates constructive and destructive interferences in the intensity of scattered light, resulting in time-dependent fluctuations in the intensity of the scattered light, which in DLS are measured by a fast photon counter. This fluctuation of scattered light intensity as a function of time reveals information on the velocity of the particles, known as the translation diffusion coefficient. As expected, larger particles

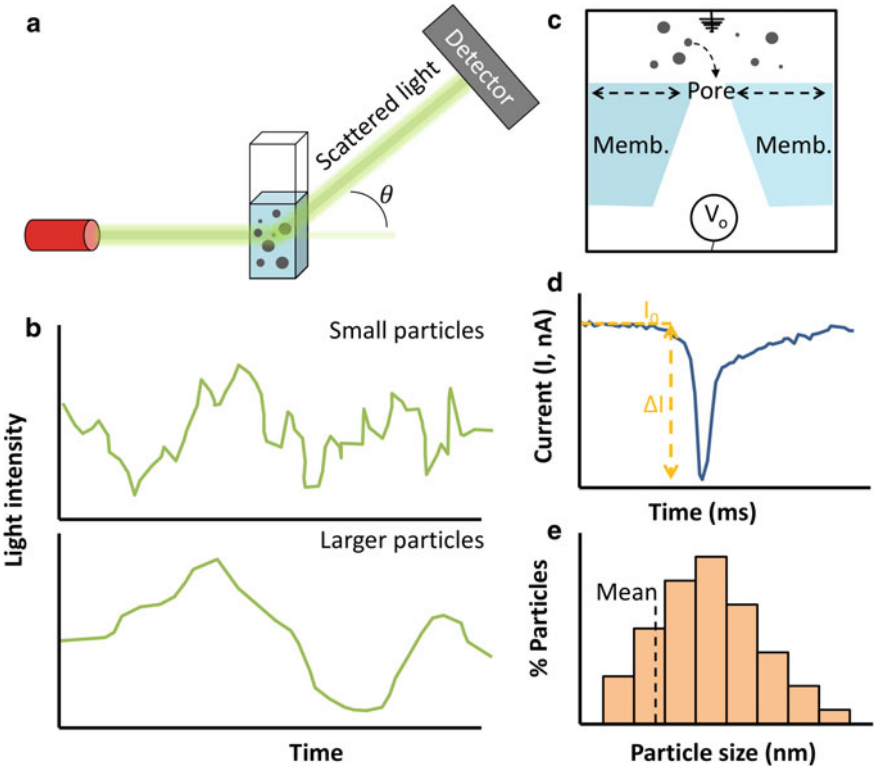

Fig. 3.5 DLS versus TRPS. **a** Schematic illustration of DLS. **b** Hypothetical DLS scattering plots of smaller particles (top) and larger particles (bottom). **c** Schematic illustration of TRPS. Tunable pores are located in the central septum of a polyurethane membrane (Memb.), placed within a fluid cell. **d** Representative data of one typical pulse in detail. **e** Number-based size distribution obtained from TRPS analysis

will cause smaller fluctuation rates in the scattered light, whereas smaller, faster particles will result in higher fluctuation rates. From the translation diffusion coefficient, the hydrodynamic diameter of particles can be calculated using the Stokes–Einstein equation. The practical upper limit of the particle size determined using the DLS method is around 1–3 μm.

DLS is one of the most frequently used methods for size estimation of nanoparticles. Sample preparation and the measurement method for DLS are simple and straightforward. Since DLS depicts the intensity of scattered light as a function of particle size distribution, which can be converted to their contribution per volume or relative number, DLS can be used to observe subtle changes in particle sizes. For instance, diameter changes after silica coatings on gold nanoparticles have been described (Sharma et al. 2018). Due to the low contrast of silica, measurements by TEM correspond only to the metallic cores, whereas the results obtained by DLS correspond to the total size of the metallic core and the coating layer, enabling the

assessment of the thickness of the coating layer. Another application is evaluating particle stability over different conditions. For example, Guidelli et al. determined the minimum concentration of a stabilizing agent required to prevent particle aggregation (Guidelli et al. 2012). In 2013, Borissevitch et al. (2013) used DLS to study the interaction and complex formation between CdSe/ZnS-PEGOH 570 Quantum Dots with negatively charged meso-tetrakis(p-sulfonato-phenyl)porphyrin (TPPS4). DLS has also been utilized to study the changes in particle diameters after encapsulation of small molecule drugs into polymeric micelles (Yokoyama et al. 1998) or liposomes (Zaru et al. 2009).

On the other hand, DLS measurements are also very sensitive to the salt concentration, pH, or the buffer in which the nanoparticles are suspended. In a comprehensive study, Huang and Zhang (2018) demonstrated impact of polymer concentration, type of organic solvent, temperature, aqueous phase ionic strength, organic phase injection rate, aqueous phase agitation rate, gauge of the needles, and final polymer concentration on the size of the poly(D,L-lactic-co-glycolic acid) nanoparticles measured by DLS.

An important parameter for DLS measurements is the **dispersity** of the particles. This is usually manifested in the form of the polydispersity index (PDI). The intensity size distribution of particles is highly sensitive to small numbers of aggregates. If particle size distribution can be fitted to a Gaussian distribution, the PDI can be calculated as (Eq. 3.2)

$$\mathrm{PDI} = \frac{\sigma}{R_h}, \tag{3.2}$$

where σ represents the standard deviation and R_h the average hydrodynamic radius. A higher PDI indicates that the mixture contains particles of different sizes and the hydrodynamic diameter is the average of this mixture, irrespective of the relative contribution of each different particle (i.e., if 90% of the mixture has a diameter of 100 nm and only 10% has a diameter of 1000 nm, DLS measurements will result in an average diameter of 200–400 nm). In addition, size analyses of nonspherical particles by DLS must be performed with caution. Since the calculated diameter for DLS is calculated by the Stokes–Einstein equation, the diameter of a non-spherical particle will be approximated to that of a sphere diffusing in the same medium at the same velocity regardless of the particle shape. To circumvent this problem, Badaire et al. (2004) used depolarized light for DLS measurements of the size of carbon nanotubes in suspension.

Taken together, considering that the limitations of DLS are mainly associated with particle geometry, it provides one of the most practical and fastest ways to study particle size distributions in monodispersed and polydispersed systems and the kinetics of size evolution under different conditions.

3.2.8 *Tuneable Resistive Pulse Sensing (TRPS)*

Due to its ease of use, high throughput nature, and broad applicability DLS is currently the preferred method for nanoparticle size characterization. However, when analyzing polydisperse systems, the Z-average value obtained after DLS measurements is not indicative of the neither population's actual hydrodynamic diameter. Recently, tuneable resistive pulse sensing (TRPS) has shown to be a highly sensitive method to determine individual particle sizes as well as the real size distribution, with similar accuracy to TEM, for a nanoparticle suspension. TRPS can be used for size estimations (Kozak et al. 2012), with a lower detection limit of 40 nm, for concentration analysis, to analyze nanoparticle shape (Golibersuch 1973; Platt et al. 2012), conductivity (Holden et al. 2011), and also surface charge (Ito et al. 2004, 2003).

TRPS is based on the **Coulter principle**, wherein whenever a particle passes through a single pore in a thin membrane, separating cells filled with electrolytic solutions, the ionic current passing through that pore is blocked for a short period of time resulting in a "resistive pulse". This electric signal, proportional to the particle volume, is recorded and analyzed for each particle, one after the other, thereby resulting in a particle-by-particle size estimation, providing in the end number-weighted population statistics. TRPS uses a polyurethane membrane wherein the size of the nanopore can be 'tuned' (Sowerby et al. 2007) (Fig. 3.5c–e). Since TRPS relies on changes in electric current, it requires conductive solutions for the analysis, making it incompatible to characterize nanoparticles under physiological buffer conditions.

Our group recently demonstrated that for siRNA-polymer polyplexes, which were largely monodisperse, particle sizes depending on used N/P ratios (ratio between excess polymer to siRNA) followed a similar trend (Hartl et al. 2019). Interestingly, DLS measurements performed in HEPES showed the smallest particle sizes and most efficient siRNA packaging at a polymer per siRNA excess of N/P 5.5. On the other hand, in the high ionic strength TRPS electrolyte solutions, the smallest particles were observed at N/P 4. Although the TRPS data displayed slightly higher mean diameters, the average sizes as well as the number-weighted distribution profiles were in acceptable agreement with DLS data.

Pal and colleagues directly compared TRPS and DLS to characterize polydisperse dispersions of engineered nanomaterials in complex cell culture medium, containing serum, mimicking in vitro testing conditions (Pal et al. 2014). They performed serial dilutions of the engineered nanomaterial dispersions over the 0.5–50 μg/mL concentration range in RPMI + 10% FBS. In nanotoxicology studies, lower nanoparticle concentrations have shown to be better tolerated (<1 mg/ml), thus the characterization of nanomaterials at low doses is critical. Their results, summarized in Fig. 3.6 and Table 3.1, show that DLS produced very broad unimodal size distributions across all concentrations. The measured average hydrodynamic diameter decreased from 311 nm (at 50 μg/mL) to 43 nm (at 0.5 μg/mL), this later peak corresponding to serum proteins (confirmed with blanks). In addition, the PDI increased from 0.3 to 0.4

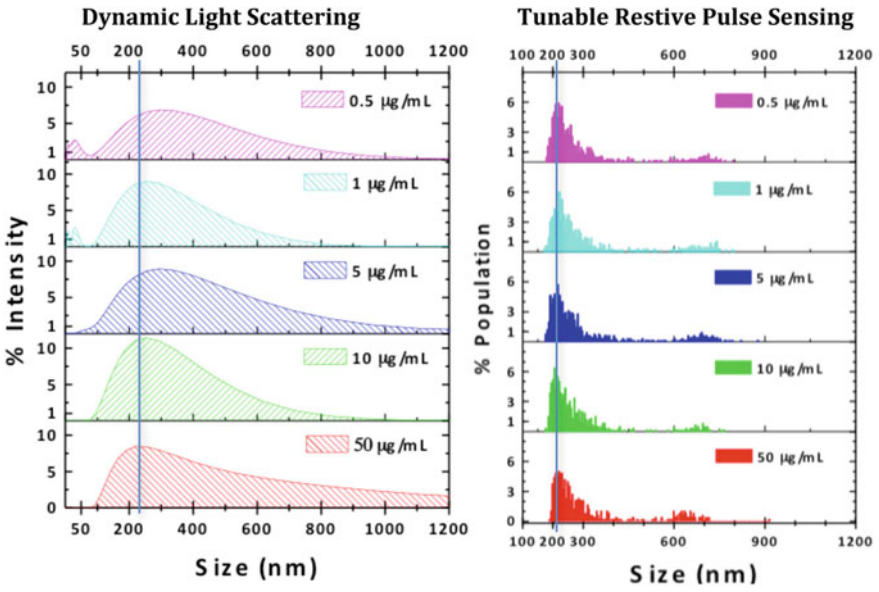

Fig. 3.6 Comparative evaluation of TRPS and DLS in characterizing sensitivity and stability of size distribution measurements of a series of sequential dilutions of nanoparticles from 0.5–50 µg/mL, prepared from a stock solution of 500 µg/mL in RPMI +10% FBS. The graphs represent averages of triplicate measurements. Note changes in the DLS size distributions below 5 µg/mL, especially left-side broadening of the peak and appearance of a smaller peak <50 nm, related to proteins in serum. At higher concentrations (50 µg/mL) the peak broadened to the right. In contrast to DLS, the TRPS size distribution remained fairly constant over the whole concentration range. Reproduced from Pal et al. (2014) with permission from Americal Chemical Society

Table 3.1 Effect of dilution on hydrodynamic size by DLS and TRPS

	DLS		TRPS	
NP dilution in medium	$d_{h,\,z\text{-ave}}$ (nm)	PdI	Size mean (nm)	Size mode (nm)
1:10	311±11	0.37	317	228
1:50	223±1	0.28	291	204
1:100	240±4	0.48	315	210
1:500	70±2	1	313	223
1:1000	43±2	1	297	208

Table modified from Pal et al. (2014)

to 1 below 1 µg/mL. On the other hand, TRPS size distributions were bimodal (peaks at 220 and 660 nm), which did not change notably as a function of their concentration. In addition, since TRPS has a lower cut-point at 40 nm, serum proteins were not measured. As expected, the frequency at which the particles went through the

pore also dropped from 1000 particles/min (at 50 μg/ml) to 134 particles/min (at 0.5 μg/ml).

Further, TRPS also provides indirect information on particle shape. For particles of similar dimensions, for instance, it was shown that the resistive pulse signal of a rod is significantly different from that of a sphere (Platt et al. 2012). The resistive pulse of a particle with different shapes can be distinguished by the blockage event magnitude, revealing particle size and the full width at half maximum duration, related to the time taken for the particle to traverse the pore, dependent on its speed and length.

3.2.9 Nanoparticle Tracking Analysis

One of the more recent techniques, nanoparticle tracking analysis (NTA), is an innovation system to characterize the size of nanoparticles. In NTA, the particles, moving under Brownian motion, are illuminated by a laser beam and the light scattered by them is recorded by a microscope camera (Fig. 3.7). Thus, each individual particle can be tracked and its hydrodynamic diameter can be calculated based on a modified Stokes–Einstein equation. For NTA, the measurable size range is between about 50–1000 nm, depending on the refractive index of the analysed particles. With real-time monitoring, subtle changes in the characteristics of particle populations, such as aggregation or disassembly under different conditions (e.g., thermal stress) can be observed and confirmed by visual validation. In addition, NTA can also provide approximate particle concentrations. Filipe et al. (2010) compared NTA and DLS measurements of polystyrene particles and protein aggregates. Both techniques showed good sizing accuracy and narrow distributions for all monodisperse samples (polystyrene beads). However, when beads of two different sizes were mixed together to result in a polydisperse system, NTA was able to resolve and distinguish the two

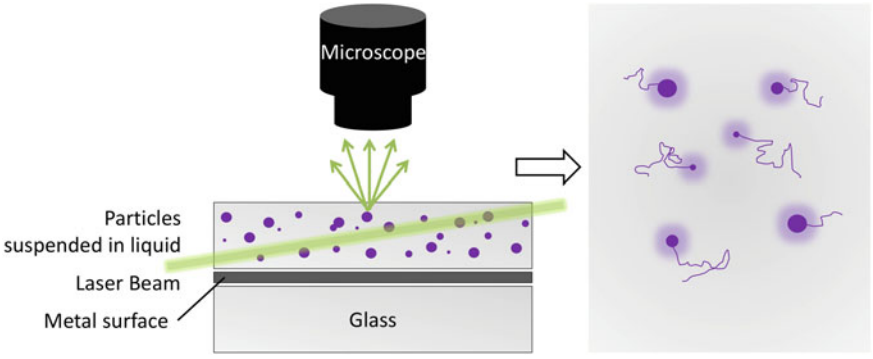

Fig. 3.7 Schematic representation of NTA

size populations in all mixtures, resulting in accurate size estimations of the beads in the mixtures (Fig. 3.8). On the other hand, DLS resulted in a broad single peak, shifted toward the larger sizes present.

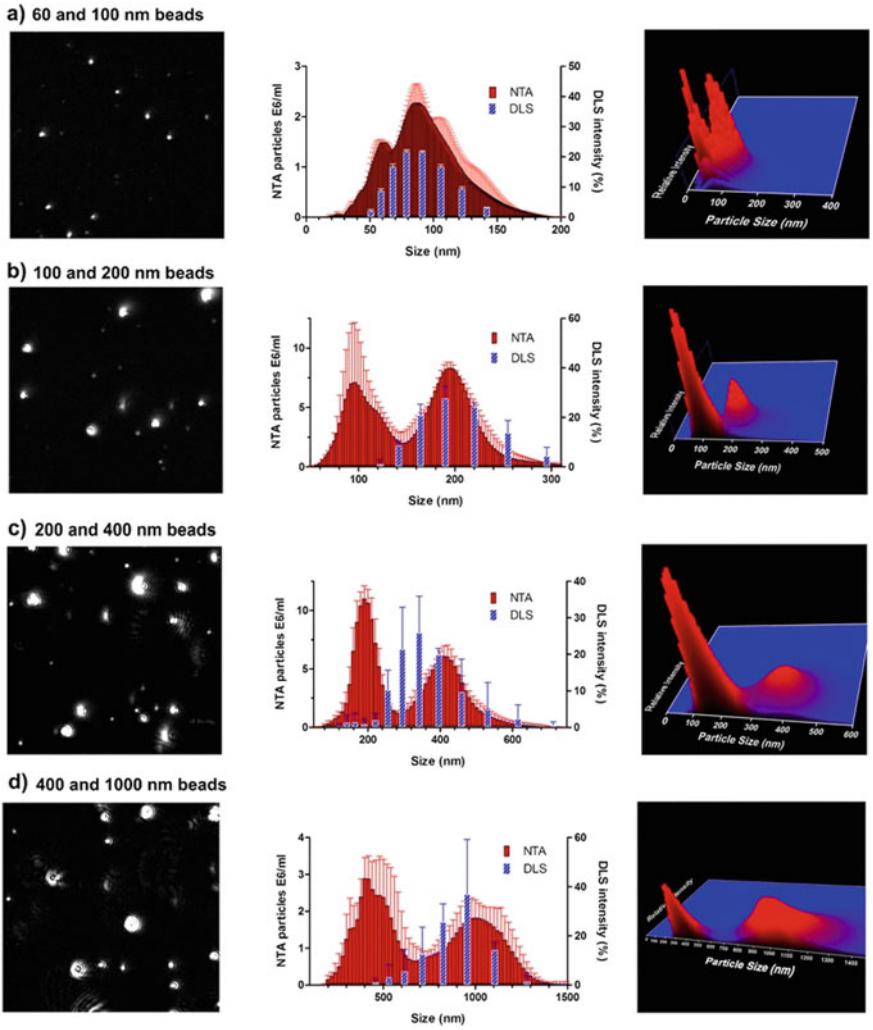

Fig. 3.8 Size distribution from NTA and DLS measurements of mixtures of monodisperse polystyrene beads (middle panels) with the corresponding NTAvideo frame (left panels) and 3D graph (size versus intensity versus concentration; right panels). **a** 60/100-nm beads at a 4:1 number ratio; **b** 100/200-nm beads at a 1:1 number ratio; **c** 200/400-nm beads at a 2:1 number ratio; **d** 400/1000-nm beads at a 1:1 number ratio. Reproduced from Filipe et al. (2010) with permission from American Association of Pharmaceutical Scientists

3.3 Surface Charge

Surface chemistry and charge play critical roles in nanoparticle stability and aggregation, cellular uptake (Cho et al. 2009; Xiao et al. 2011; Saha et al. 2013; Jo et al. 2015), in vivo biodistribution (Elci et al. 2016), cytotoxicity, activation of the immune system (Moyano et al. 2012) and the development and composition of the protein 'corona' that develops around the nanoparticles in vivo (Fleischer and Payne 2014).

Generally, positively charged nanoparticles have been shown to be taken up more efficiently via phagocytosis than neutral or negatively charged particles, irrespective of their composition (Jo et al. 2015). On the other hand, slightly negatively charged nanoparticles were shown to be taken up by tumor cells more efficiently with low liver uptake (Xiao et al. 2011). In addition, negatively charged samples also did not significantly adsorb proteins thereby reducing their clearance by the reticuloendothelial system (RES) and improving in vivo compatibility. By varying the surface charges, one can thus vary the electrostatic interaction between the nanoparticles and serum proteins thereby affecting the fate of nanoparticles administered in biological systems.

The surface charge of nanocarriers can be inferred by measuring their zeta potential (ζ-potential), which describes the electrokinetic potential in colloidal dispersion. The ζ-potential represents the electrostatic potential at the plane of shear and typically samples with ζ-potential values higher (or equal to) ± 20 to 30 mV form stable colloidal suspensions that do not tend to agglomerate (Mourdikoudis et al. 2018). Current characterization methodologies are based on ensemble measurements (e.g., phase analysis light scattering, laser doppler anemometry, streaming potentiometry) that measure the average **electrophoretic mobility** of particles in suspension. However, while dealing with polydisperse systems (such as polyelectrolyte complexes) that contain a heterogeneous mixture of a range of ζ-potentials, an ensemble approach is problematic. Using resistive pulse sensing, Deblois et al. (1977) first performed single particle electrokinetic measurements, which are discussed at the end.

3.3.1 Laser Doppler Anemometry

One method for the measurement of the ζ-potential https://www.sciencedirect.com/topics/chemistry/zeta-potential is based on the relative electrophoretic motion of particles and electrolytes within an applied electric field. In this technique, voltage is applied across a pair of electrodes at the ends of a cell containing the particle suspension and is irradiated with laser light. The particles are attracted to the oppositely charged electrode, and the velocity of the particles can be measured by observing the Doppler shift in the scattered light. The direction and velocity of motion of the nanoparticles is a function of their charge, the suspension medium, and the strength of the applied electric field. Their mobility can then be calculated as the ratio of the

velocity to the applied electric field strength (Eq. 3.3).

$$U = \frac{\lambda . Vd}{2E.n.\sin\left(\frac{\theta}{2}\right)}, \tag{3.3}$$

where λ is the wavelength of the laser light; Vd, the particle velocity determined by the Doppler shift; E, the applied electric field strength; n, the refractive index of the solvent used; and the scattered light angle, θ.

Subsequently, the ζ-potential can be calculated according to the electric potential of the particle at the shear plane using the following relationship (Eq. 3.4).

$$\zeta = \frac{U\eta}{\varepsilon f(ka)}, \tag{3.4}$$

where U is the electrical mobility; ε, the dielectric constant of the solvent; η, the solvent viscosity; and $f(ka)$, the Henry coefficient.

Interestingly, Liao and colleagues demonstrated the pH dependence of ζ-potential of Titanium oxide (TiO_2) particles irrespective of their size and shape (Liao et al. 2006). Similarly, Sharma et al. observed pH-dependency of the ζ-potential of magnetic iron oxide nanoparticles coated with citrate, PEG-PEI, CM-dextran, dextran, and methoxy-PEG-phosphate + rutin over a pH range from 2 to 10 (Sharma et al. 2018). They observed that the citrate or PEG-PEI coated precursor magnetic iron oxide particles had a strongly positive ζ-potential at pH < 3, i.e., around 40 mV while other polymers displayed a mildly positive ζ-potential (<10 mV) at low pH values (Fig. 3.9). Interestingly, all polymer coatings except PEG-PEI demonstrated negative ζ-potentials at higher pH (pH > 5) while PEG-PEI had a positive ζ-potential across the entire pH range tested. The advantages of laser Doppler anemometry are that the method requires minimal sample preparation, can analyze multiple

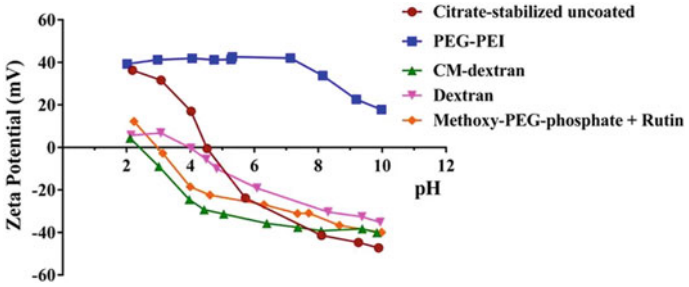

Fig. 3.9 The measured pH-dependent zeta potential of magnetic iron oxide nanoparticles (MNPs) coated with citrate (red), PEG-PEI (blue), CM-dextran (green), dextran (magenta), and methoxy-PEG-phosphate + rutin (amber) in water. All MNP constructs displayed a negative surface charge at pH 7, except PEG-PEI MNPs. Reproduced from Sharma et al. (2018) with permission from Springer Nature

samples, provides results with good statistics, and, by using disposable cuvettes, avoids cross-contamination between samples.

3.3.2 Single Particle Electrokinetic Measurements

TRPS can be used to measure the surface charge of particles in suspension, enabling single particle surface-charge measurements leading to robust and reproducible ζ-potential measurements. This property is based on the resistive signal duration as a function of the applied pressure or voltage across the pore. The average electrophoretic mobility shift is then calculated with respect to the calibration standards (carboxylated polystyrene particles, for example) with known average ζ-potentials. The step by step calibration process and the consecutive zeta potential calculation of the sample on a particle-by-particle basis have been explained in detail by Blundell et al. (2016) and Vogel et al. (2016,2017). Briefly, there is a linear relationship of electrokinetic (electroosmotic and electrophoretic) particle velocities of sample and calibration and their respective ζ-potentials, based on the Smoluchowski approximation (Sikora et al. 2015).

3.4 Porosity

Over the last decade, mesoporous nanoparticles have been actively investigated in the areas of drug delivery and imaging. Mesoporous nanoparticles can be made of inorganic materials, often silicon or silica. The most remarkable advantage of mesoporous nanoparticles as drug carriers is their extremely high surface to volume ratio, large surface area (700–1000 m^2/g), and large pore volume (>0.9 cm^3/g) (Salonen et al. 2008; Vallet-Regi 2010) while still maintaining a thermally, mechanically and chemically stable and rigid framework (Santos et al. 2011). The small size of the pores confines the space of a drug and engages the effects of surface interactions of the drug molecules and the pore wall. Pore diameters of mesoporous materials lie between $2 - 50$ nm, allowing high payloads of therapeutic molecules while protecting them from premature release and degradation (Salonen et al. 2008). Thus, they can be used to deliver large doses of hydrophobic drugs to target organs, at a controllable release rate (Hudson et al. 2008).

Porosimetry is a useful technique for the characterization of porous materials, providing information about the pore size, pore volume, and surface area of a sample (Giesche 2006). The technique is based on the intrusion of a non-wetting liquid (such as Mercury) into the voids in a porous sample. As pressure is applied, mercury fills the larger pores and further proceeds to fill the smaller pores as the applied pressure increases. Using mercury porosimetry, pores between about 250 μm and 3.5 nm can be investigated (Giesche 2006). Using the Washburn Equation the pore diameter (D_P) can be calculated as (Eq. 3.5)

$$D_P = -\frac{4\sigma \cos\theta}{(P_L - P_G)}, \tag{3.5}$$

where σ is the surface tension of mercury; θ, the contact angle of mercury (between 135° and 142° for most solids); P_L the pressure applied to mercury; and P_G, the gas pressure (since the assay is usually performed in a vacuum, this value is 0) (Giesche 2006).

3.5 Viscosity

The viscosity of a nanoparticle suspension significantly influences its injectability, since high viscosities require high injection forces. In addition, highly viscous fluids should not be injected intravenously due to the risk of pulmonary embolism (Weir et al. 1986) and should be administered subcutaneously. Interestingly, a very low viscosity of a subcutaneously injected solution has also been associated with an increased sensation of pain. Berteau and colleagues compared the pain perceived after subcutaneous injections of three different fluid viscosities (1, 8–10, and 15–20 cP) and observed that high viscosity injections (up to 15–20 cP) were less painful and, consequently, the most easily tolerated ones (Berteau et al. 2015). Since the application route of nanoparticle suspensions depends on their viscosity, knowledge of their rheological properties becomes crucial. Particle size, shape, concentration, and temperature affect the nanosuspension viscosity (Mahbubul et al. 2012), and Rudyak and colleagues reported that nanoparticle size had the strongest impact on viscosity as measured by rheology (Rudyak and Krasnolutskii 2014).

Rheology studies flow behavior and are normally applied to fluid or 'soft solid' materials, such as hydrogels. Flow is typically measured using shear stress and its parameters, stress (τ) and strain rate (γ) are calculated from measurements of torque and flow rate. Viscosity (η) is defined as Eq. 3.6

$$\eta = \frac{\tau}{\gamma}. \tag{3.6}$$

Experimentally, a rheometer can measure the viscoelasticity, yield stress, thixotropy, extensional viscosity, and stress relaxation behavior of the suspension. There are three main types of rheometers: capillary, torque, and dynamic rotational. For a capillary rheometer, the sample is forced to flow through a capillary of well-defined dimensions under high pressure, and the pressure drop across the capillary is measured resulting in pressure-flow rate data for the fluid, from which viscosity is calculated. Temperature and shear rate can be closely controlled to simulate the processing environment of interest and smaller sample volumes can be evaluated, which may be beneficial for more expensive formulations, such as nucleic acids or monoclonal antibodies (Hudson et al. 2015). A torque rheometer resembles an extruder and measures the torque on the mixing screws or rotors, which reflects how

hard it is to mix the material and can be correlated to viscosity (Ogah et al. 2014). While both capillary and torque rheometers typically provide data on viscosity and melt flow as material passes through the instrument, dynamic rotational or oscillatory rheometers probe into a polymer's molecular structure and viscoelastic properties. These instruments place the plastic sample between two components, one stationary and one that turns back and forth at adjustable speed and operate at relatively low shear stress.

An ideal fluid flows in Newtonian behavior, with a linear relationship between stress and strain rate and zero stress at zero strain rate. However, only a small number of fluids exhibit such constant viscosity. Most fluids show non-Newtonian behavior, of which most commonly demonstrate plastic or pseudoplastic behaviors. For plastic fluids flow only initiates after a certain level of stress is applied (yield stress), however, once attained, subsequently the relationship between stress and strain rate is linear. On the other hand, for pseudoplastic fluids viscosity decreases as strain rate increases (Barnes et al. 1989).

3.6 Summary and Outlooks

Nanomaterials have great potential for use in drug delivery, improving drug stability and release in vivo while minimizing toxic side effects. Over the last decade, a rapid growth in the development of nanocarrier systems has been described, which exist in various chemical compositions ranging from micelles to metals or metal oxides, synthetic polymers or biomolecules. Each of these materials features a completely different chemistry, surface properties, and interaction potential, particularly with proteins in vivo. In this chapter, different characterization methods have been discussed. They have been summarized in Table 3.2. The choice of the nanoparticle characterization techniques depends at first, on their physical form, i.e., solid samples and powders or suspensions. Solid or dry samples provide considerable freedom in the choice of technique and can be analyzed by electron microscopy, AFM, or X-ray scattering. Nanoparticle suspensions, on the other hand are more challenging, especially in case of high polydispersity, and can be evaluated by light scattering or NTA techniques. Characterization of the surface charge is almost always performed by electrophoretic methods, irrespective of particle state. Subsequently, it is imperative to characterize nanoparticles under the envisioned biological operating conditions of the nanomaterial. Components of biological fluids, such as proteins, often interact with and assemble with the nanoparticles, resulting in the formation of a protein corona, thereby altering their initial surface properties (Box 1). In summary, to get a full picture of the physico-chemical characteristics of nanoparticles, typically a combination of the techniques described here is essential. In fact, even to analyze a single parameter, such as size, a combination of techniques may need to be employed.

Table 3.2 Summary of characterization methods for nanoparticles

Method	Nanoparticle state	Parameter	Advantages	Disadvantages
Transmission electron microscopy (TEM)	High vacuum, dried sample	Size, size distribution Shape	Direct imaging of nanoparticles at very high resolution (<1 nm)	Tedious sample preparation High energy electron beam may damage sample
Scanning electron microscopy (SEM)	High vacuum, low pressure	Size, size distribution, surface structure	Single particle resolution, Lower energy electron beams as TEM	Limited resolution and penetration depth
Atomic force microscopy	Dry or liquid	Size, shape, binding force to modified cantilever	Allows high-resolution measurements in different conditions	Particles must adhere to a fixed surface
X-ray diffraction (XRD)	Dry, powdered	Crystallite size	Determines crystalline/amorphous phases and information about crystal structure	Low sensitivity No information about particle size, shape
Small angle X-ray Scattering (SAXS)	Dry, in suspension	Size, size distribution, and shape	High sensitivity	Information about particle morphology is required
Dynamic light scattering	In suspension	Hydrodynamic radius and intensity based size distribution	Large measurement range (0.6 nm to 1 μm) Rapid and high throughput	Biased toward larger particles in suspension, difficult data interpretation for polydisperse samples
Tuneable resistive pulse sensing (TRPS)	In suspension (in conductive liquid)	Size, shape, concentration, ζ potential	Tunable detection range, single particle resolution	Requires specific liquid (conductive) and careful initial calibration
Nanoparticle tracking analysis (NTA)	In suspension	Hydrodynamic radius, size distribution, concentration	Single particle resolution, suitable for highly polydisperse samples	Requires highly scattering particles
Laser doppler anemometry (electrophoretic mobility)	In suspension	Surface Charge (ζ- potential)	Rapid and high throughput, minimal sample preparation	Depends on the model applied to convert mobility to ζ potential

(continued)

Table 3.2 (continued)

Method	Nanoparticle state	Parameter	Advantages	Disadvantages
Porosimetry	Dry	Pore size, pore volume, and surface area of a sample	Compatible with polydisperse samples	Sample cannot be used subsequently

Important Notes

- Nanoparticle characterization is a major obstacle in nanoscience and is unfortunately cannot be addressed in a straightforward manner.
- Nanoparticles possess unique physicochemical properties due to their high surface area and nanoscale size, depending on their shape, size, and structure.
- Different physicochemical properties define the structure–function relationship (in vivo*)* of a nanoparticle and precise evaluation of each (especially shape, size, charge, and porosity) is critical.
- Measuring these properties is important for translating the potential benefits of nanoparticles into specific applications in drug delivery or as diagnostic tools.

Questions for Future Research

- **How does one decide what is the best method to use?** In most conditions, a combination of methods is required to fully characterize a sample. The choice of methods depends on prior the nanoparticles' physical form, whether dry, powders, or colloidal suspensions. In addition, if the nanocarriers are sensitive to high energy electron beams or whether they have crystalline structures. Polydispersity of the samples also adds an additional parameter to consider, especially when light scattering methods are used. Moreover, nanoparticles should be characterized in buffers that mimic the pH, temperature, and ionic strength that the nanoparticles would encounter in vivo as closely as possible.
- **Does the nanoparticle result in the formation of a protein corona in contact with plasma?** This depends on the particle size, surface topology, and composition. The formation of a protein corona around the nanoparticles results in decreased activity by masking the surface of the particles as well as resulting in immune recognition. Therefore, depending on the presence or absence of a protein corona, the same NPs can induce different biological outcomes.

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Glossary

Biodistribution A method of tracking where an administered agent localizes in a living organism. This can be an experimental animal or patient, and distribution is assessed after labeling the compound with either a fluorescent tag or a radioactive isotope. Expression vectors can be used as well in gene delivery experiments, and reporter gene expression, e.g., of luciferase or fluorescent proteins, can be assessed by whole-body imaging.

Coulter principle A principle developed by Wallace H. Coulter in 1940s. It can be applied to count the number of particles and to determine the particle size using impedance measurements.

Cytotoxicity Toxic effects on cells which can be caused by membrane toxicity, impact on mitochondrial activity or other intracellular processes, or induction of apoptosis.

Dispersity A measure of the heterogeneity of sizes of molecules or particles in a mixture.

Electrophoretic mobility The movement of ions in solution due to the electric potential applied across the media in which the ions exist.

Micelles Agregates of surfactant molecules dispersed as a liquid colloid. Typically, micelles form aggregates with the hydrophilic head in contact with the surrounding aqueous solvent, sequestering the hydrophobic tail regions within the center. The hydrophobic core can be used for encapsulating hydrophobic compounds.

Opsonization The coating of a particle with proteins that facilitate phagocytosis of the particle by tissue macrophages and activated follicular dendritic cells as well as binding by receptors on peripheral blood cells.

Pharmacokinetics A term describing the absorption, distribution, metabolism, and excretion of a compound within the body.

Reticuloendothelial system A diffuse system of cells that function in host defence by the phagocytic removal of abnormal or dead cells or foreign material from the circulation. It includes monocytes and macrophages which are primarily found in the liver and spleen.

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Chapter 4
In Vivo Assessment of the Efficiency of Systemic Delivery

V. K. Ameena Shirin, Renu Sankar, Sabna Kotta, and Kannissery Pramod

Abstract While the characterization of the physicochemical properties of a nanocarrier as presented in Chap. 3 is crucial to the evaluation and subsequent optimization of the carrier performance, as the ultimate goal of the development of a carrier is to enhance the efficiency of systemic delivery in a biological body, in vivo assessment of the carrier is unavoidable. In vivo assessment gives information about the mechanism of action, pharmacokinetics, and pharmacodynamics of the agent administered. Over the years, many animal models have been used for studying the in vivo performance of a drug, which in this case is either a free drug or a drug that has been pre-loaded into a carrier. Rodents like rats, mice, guinea pigs, and rabbits are the commonly used animals. Recently, zebrafish invertebrate models have also been established and used in in vivo studies. They are now used widely as a screening model to assess the in vivo performance of many drugs. Regarding the importance of in vivo assessment in the design and use of systemic delivery technologies in anti-aging medicine, in this chapter, we will describe the various in vivo techniques for the assessment of drug delivery.

Keywords Bioavailability · Drug delivery · In vivo · Pharmacokinetics · Systemic · Zebrafish

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4.1 Introduction

In vivo assessment includes experiments that are done in living organisms to determine the therapeutic, pharmacological, and toxicological effects of the drug. Usually, these studies are performed in animals including humans. Before a drug is made available in the market, the pharmacological and **therapeutic efficacy** of the drug should be monitored. Both clinical and preclinical studies are performed during drug discovery. The preclinical studies include both in vitro and **in vivo studies**. **In vitro studies** give limited information about the in vivo performance of the drug delivery and the interaction of the drug with organs and others. Hence, it is essential to perform the in vivo assessment of drugs. These studies give information about the mechanism of action of the drug, **pharmacokinetics**, and **pharmacodynamics** of the drug delivery systems.

As far as the development of bodywide biogerontological intervention is concerned, in vivo assessment of **systemic delivery** is an important step. **Bioavailability** studies in animal models are a pre-requisite for evaluation of the performance of a drug delivery system. The absorption, metabolism, distribution, and excretion characteristics of a drug could be explained only by in vivo experiments. So, the in vivo experiments are performed in laboratory animals and after assessment of the safety and efficacy of the drug in them, these are tested in humans. Different animals are used for the study which includes, rats, mice, rabbits, guinea pigs, etc. Both vertebrates and invertebrates are used for the study. The animal model is selected depending upon the objective of the study (Brake et al. 2017).

An exact model should respond to the treatment similar to the responses in humans. In vivo experiments are usually performed in rodents, but this model is found to be time-consuming and costly. Zebrafish model is a novel vertebrate model used for in vivo assessment of a drug. This particular model is having many advantages compared to a rodent in vivo model. The advantages of it include high reproducibility, low cost, availability of transgenic lines, etc. This model reduces the need for higher vertebrates for the studies. This model investigates the circulation behavior and toxic effects of the drug during the early stages of the discovery of drugs (Sieber et al. 2017). This chapter describes the in vivo assessment of drugs based on the route, molecule, and dosage form. Various in vivo assessment models are also discussed with a special focus on the emerging zebrafish model.

4.2 In Vivo Assessment Models

In vitro studies are essential to establish the fundamental characteristics of the drug release and the behavior of drugs inside the body, even though they cannot mimic a perfect live biological system. Moreover, in vitro results are unable to predict the interaction of a drug with other drugs. There is no guarantee for the performance of the drug in vivo even after a successful in vitro study. The in vivo experiments

in laboratory animals are, therefore, essential to ensure the safety and efficacy of the drug/formulation under question before human testing. Still, clinical trials are needed since animal experiments cannot predict exactly what will happen in the human subject.

4.2.1 Selection of Animal Models

The selection of the animal model solely depends on the aim of the research. There are two different classes of animal models: based on analogy and based on homology. The four major categories of animal models are experimental model, natural model, non-reactive model, and orphan model (Fig. 4.1). The first model, also called an induced model, tries to reproduce the conditions of original species, while natural or spontaneous models are the same in some conditions of the original species. Non-reactive or negative models are the typical equivalent of a disease model and, finally,

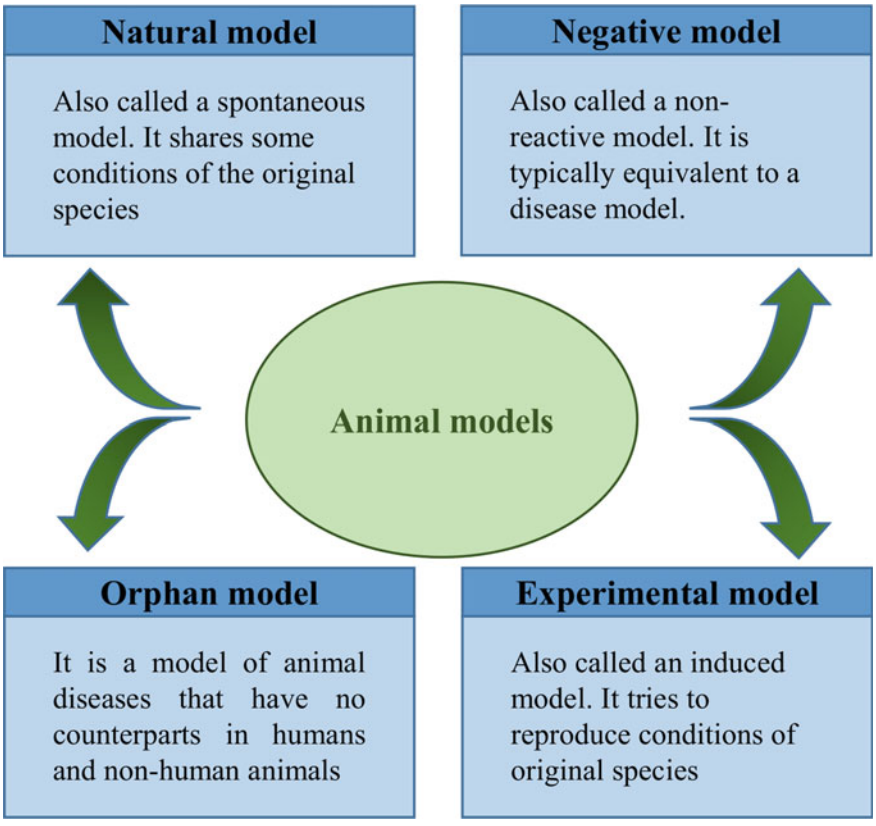

Fig. 4.1 Different types of animal model

the orphan models mean animal diseases for which no human or animal counterpart yet exists.

There are some typical criteria for the selection of animal models in research (Davidson et al. 1987). They are appropriateness as a model, genetic uniformity of the organism, transferability of the information and easiness for experimental manipulation, background information of biological properties, and generalizability of the findings. Apart from the costs and accessibility, ecological consideration and ethical involvement should also be considered (Davidson et al. 1987).

A perfect animal disease model will summarize the disease phenotype with similar pathophysiology like humans and react in a similar manner to the therapies as in patients. These kinds of models are very useful to predict the mechanism of action drugs, pharmacokinetics as well as dynamics. They are also invaluable to predict safety and toxicity issues associated with long-term use, so these models are helpful in predicting the dosage regimen for clinical studies. Thus, a model similar in pathology, physiology as well as response to treatment is said to be a homologous model. The model which resembles human disease but the disease should be induced by some method is called isomorphic models. While the predictive models have no similarity to human disorder, still they are somewhat helpful in making predictions of human disease, therapy, and its response (Sim and Kauser 2016). Invertebrate models like zebrafish and vertebrate models like baboons, cows, macaques, dogs, rabbits, guinea pigs, rats, and mice are used research. Invertebrate models help predict neurological, developmental, and genetic diseases that are genetically tractable. Vertebrate models are vital in translational research (Chow et al. 2008; Zon and Peterson 2005; Lieschke and Currie 2007).

4.2.2 Zebrafish as an Emerging Model for In Vivo Assessment

Assessment of the circulation behavior of a drug in the body at an early stage of drug discovery is a complicated task. To overcome this problem, many alternative approaches have been developed. The zebrafish model is a new model used for the in vivo tests. Zebrafish are small and they are easy to maintain. Earlier zebrafish was used to study the development of organs. Recently, they have been used for investigation of toxicity in pharmaceuticals.

Zebrafish vertebrate model can be used for the in vivo assessments that are having many advantages compared to a rodent in vivo model (Fig. 4.2) which includes high reproducibility, low experimental cost, availability of transgenic lines, and high level of genetic homology to humans. In this approach, transgenic zebrafish lines expressing green fluorescent protein injected with monodisperse preparations of fluorescently labeled liposome with similar size and zeta potential and further circulation behavior and vascular distribution pattern were analyzed both qualitatively and semi-quantitatively, and this circulatory pattern and published and experimental

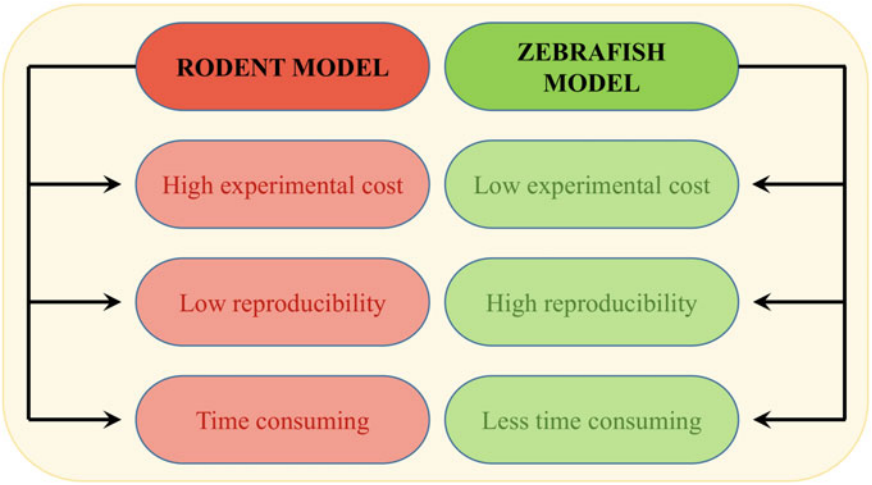

Fig. 4.2 A comparison of rodent and zebrafish models

pharmacokinetic data from mice and rat are correlated. Hence, this model can be used for the assessment of circulation and toxicological effects during the early stage of drug discovery, which reduces the need for higher vertebrates for this purpose (Sieber et al. 2017).

Graphene is one of the most important nanomaterials. Graphene oxide is used in biotechnology and nanomedicine. Graphene nanomaterials (GN) can be applied in drug delivery systems, bioimaging, and as anticancer therapy because of its special mechanical, electrical, optical, and thermal properties. Before the use of GN, we must check the **biocompatibility** and toxicity of graphene oxide. Graphene oxide is a toxic material; it can produce oxidative stress and cause injury to the pulmonary system. Also, it is genotoxic and produces reproductive damages.

Zebrafish is an important animal model because of its ease of maintenance and sensitivity to many pollutants. The evaluation of graphene oxide in zebrafish will provide knowledge about the graphene oxide toxicity and this in vivo assay is an important tool for getting data useful for the evaluation of toxic effects of graphene oxide. In this assay, embryos of zebrafish are collected. Both the embryo and larvae are kept at a temperature of 280 °C. Only the fertilized embryos are collected by observation using an inverted microscope. This fertilized embryo is placed in six-well culture plates and each well contains ten embryos. To this well, add a graphene oxide solution at different concentrations. At regular time intervals, heart rate, mortality, and length of the body were noted. These are the endpoints in this study. These endpoints are measured by using an inverted microscope. Increased mortality, heartbeats, and toxicity to the cardiovascular system occur. This reveals the developmental abnormalities produced by graphene oxide. Thus, the zebrafish model is very useful in vivo model for detecting the mechanism involved in the toxicity of nanomaterials (Bangeppagari et al. 2019).

Drug delivery by the use of nanomedicine is an efficient method to increase the particular drug concentration at the target site to avoid the unwanted side effects. In vivo studies in rodents are very expensive and time-consuming. So, the evaluation of nanomedicine is done biological models such as cell cultures. Zebrafish is recently used as an in vivo model. This model can be used to assess nanomedicines and thereby we can eliminate the problems associated with in vitro and in vivo rodent studies. Larvae of zebrafish have several advantages over rodents that make them suitable for screening of nanomedicines, cost of zebrafish is very low compared to rodents like mice and guinea pigs. Zebrafish larvae have optical transparency. Zebrafish and human plasma have similarities in conserving apolipoproteins.

Nanomedicines can be given to the zebrafish by using various administration routes. The selection of the appropriate route of administration is important and is based on the developmental stages. The formulation is given orally to adult zebrafish and its larvae. The injection is only given to zebrafish only during the late developmental stage. Direct injection into the blood only used in the infection model. The temperature of the experiment is another important factor. At an early stage of development, the incubation temperature affects the innate immune response. The processes depending on temperature can affect the test results. So, the temperature of the experiment must be carefully selected and standardized, especially for experiments involving studying the immune response because it is a temperature-dependent process.

Zebrafish in vivo models can be used to assess the delivery of enzymes and proteins. Nanomedicines for enzyme delivery require an assessment of the activity and immobilization of enzymes. Zebrafish larvae can be used for the in vivo assessment of enzyme delivery. Cancer models of zebrafish are also possible. Many tumor models can be developed using zebrafish. These zebrafish tumor models are comparable with tumors in humans. These models are developed by exposing zebrafish larvae to carcinogens by dissolving these chemicals directly into the zebrafish larvae solution (Sieber et al. 2019).

The rapid development of zebrafish embryos results in the use of this model for many in vivo assessment methods (Fig. 4.3). There are four important steps involved in the screening using zebrafish. They are mating of adults and collection of embryos, sorting of the embryo, chemical library administration, and, finally, analysis of data. Zebrafish can be used for studying absorption, distribution, metabolism, and excretion, and also in toxicological studies and the identification of prodrugs (Delvecchio et al. 2011).

In vivo assessments of formulations were usually performed in rodents but the assessment of formulations in nanomedicine form is limited within these animals. Hence, screening of nanomedicine was usually performed in cultures of human-derived cells. But, this method is found to be costly, time-consuming, and didn't exactly mimic the biological conditions. A zebrafish model is a new approach used for this purpose. This model is used for toxicity evaluation, systemic circulation, and bioavailability of nanoparticles, as a cancer model, etc. (Sieber et al. 2019).

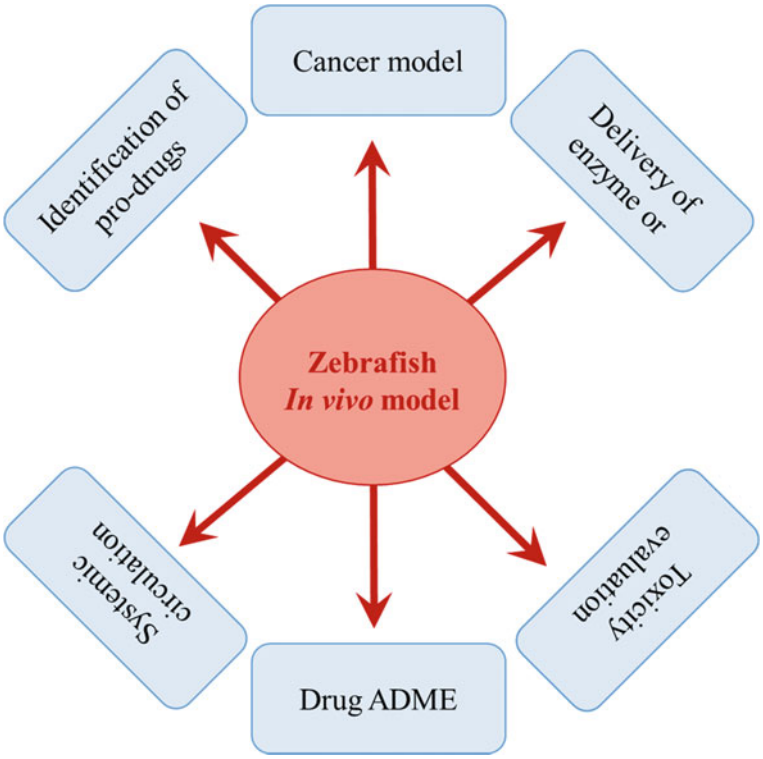

Fig. 4.3 Applications of zebrafish model

4.2.3 Animal Models and In Vivo Research Techniques

Dogs are most commonly used for pH-dependent absorption studies of drugs. Since dogs have high initial pH, it is a good model for drugs which is to be studied in fat and fed state. Rats and some rodent models are also used for studying pH-sensitive drugs even though the GI pH is not comparable with a man, unlike dogs. Since the rabbit has a non-keratinized buccal mucosa, it is only rodents that can be used for absorption and permeability studies (Sim and Kauser 2016; Shojaei 1998).

For studying the distribution pattern of drugs, mice and rat models are commonly used. Since rodents allow fast drug excretion, rodent models are very helpful in pharmacokinetic studies. Higher animals like rhesus monkeys, cynomolgus monkeys, and beagle dogs are generally used for metabolism studies due to the similarity in the enzyme kinetics. Rodents are not much used for distribution studies since the similarities with human cytochrome enzyme kinetics are not well established yet (Tang and Prueksaritanont 2010; Guengerich 1997). For in vivo excretion studies, animals in which glomerular filtration and passive reabsorption are the main mechanisms of excretion can be selected.

There is no single study available so far to determine all the in vivo characteristics of the drug under investigation. Different routes and different sampling techniques are also needed to investigate different parameters. The different routes include oral, i.v., i.m., s.c., ocular, transdermal, etc., tissue distribution can be measured from the concentration of drug in blood or tissue homogenate will be given information regarding protein binding, membrane permeability, partition coefficient, etc. But, this technique will not give a correct interpretation in accessing drug concentration in the tumor or brain. For this reason, techniques that are able to assess histological drug distribution are essential. So, this kind of measurement can be achieved by imaging techniques, equilibrium dialysis, microdialysis, PET (positron emission tomography), magnetic resonance spectroscopy, etc. These modern techniques have several advantages like semi- or noninvasiveness, continuous monitoring, direct concentration measurement even in a different site, etc., as compared to the conventional sampling techniques like tissue biopsy or saliva or skin blister sampling (Cheng et al. 2008; Cobice et al. 2015; Brunner and Langer 2006).

Equilibrium dialysis studies are useful for distribution studies and determining the protein binding behavior of the drug. Maintaining any organ like kidney, lung, brain in the living state with perfusion is called as isolated organ perfusion. The viability of the organ under study is important for the accuracy of the distribution studies. Microdialysis allows the determination of pharmacokinetics of a drug since it can consider the protein binding in vivo. This is also useful in predicting receptor phase pharmacokinetics and drug distribution. In this semi-invasive method, a probe that is attached with a dialysis membrane collects a minimal sample and circulation of perfusate. This method allows for generating a chronological data of distribution studies. Different pharmacodynamic studies are also possible at the same time, so it can be considered as the best technique for pharmacokinetic–pharmacodynamic studies (Brunner and Langer 2006; Rudin and Weissleder 2003).

Imaging techniques are widely used to study the mechanism of action of drugs in vivo. Various imaging techniques are useful for longitudinal studies with statistical relevance and they are especially useful for the sites which cannot be accessed by surgical methods. Autoradiography can be used in subcellular levels apart from tissue or cellular levels using radiolabeled isotopes. It allows tracing of drug distribution, but a greater number of animals are required for data collection (Lanao and Fraile 2005; Caro et al. 1962).

MRI uses magnetic fields and radiofrequency pulses for determining drug distribution. These radiations have excellent penetration and negligible interaction with tissues. Magnetic resonance spectroscopy has also used the same principle, which is more specific for the detection of drug and its metabolites. But both the techniques require a little more amount of drug concentration. PET can generate 3D images with high resolution and can also be used to get information about drug distribution. It has a very good translational applicability from animals to humans for studying neuropharmacokinetics by the measurement of in vivo drug concentrations. All these neuroimaging methods can give detailed metabolic, structural as well as functional

information inside the body and hence allows an easy understanding of the drug behavior in the brain (Wise and Tracey 2006; McGuire et al. 2008; Zhao et al. 2017; Gustafsson et al. 2017).

4.3 In Vivo Assessment of Systemic Delivery

A new delivery system must be characterized pharmacologically, therapeutically to make it available in the market. Drug discovery includes preclinical and clinical stages. The preclinical stage of drug discovery consists of in vitro and in vivo studies. This preclinical stage is necessary to ensure the safety, the efficacy of the new drug. In vivo studies of a new delivery system are important, because this study will provide understanding about all characteristics of the drug including its therapeutic effect, pharmacological effect, and also about its toxic effects. Many problems associated with in vivo studies can be avoided by selecting a suitable animal for the study, which means the selection of an animal having similarities with human beings.

In vitro studies have many disadvantages like they cannot predict the organ systems and also fail to predict the interaction of drugs with organs and with other drugs. They are important in drug development, but they fail to provide information about the absorption, distribution, metabolism, and excretion of drugs. Animal models are available for testing the characteristics of the drug in vivo. It can be used to assess the absorption, distribution, metabolism, and excretion of the drugs. There are some dissimilarities between animals and humans, so the data from the animal model is slightly different from the humans in the clinical trial. When a drug is tested by using animal models, we can predict its interactions, adverse drug reactions, pharmacokinetic characteristics, etc., before it reaches the clinical phase of drug development.

In drug testing, vertebrate and invertebrate models are used; here, invertebrate animal models are used to study neurological, genetic, and developmental diseases. One of the most important invertebrates is zebrafish. Vertebrates are large animals including rats, mice, rabbits, guinea pigs, dogs, etc. The animal is selected on the basis of similarities between the chosen animal and the human in terms of physiological and biochemical properties (Brake et al. 2017).

4.3.1 Based on the Route of Administration

Every drug delivery route has its own advantages and disadvantages (Verma et al. 2010). The pros and cons of some common routes of drug administration are given in Table 4.1.

Table 4.1 Pros and cons of some common routes of drug administration

Route	Pros	Cons
Oral	• Easy route of administration • Drug absorption occurs along the entire length of gastrointestinal tract	• First-pass effect • The drug destruction occurs by gastric acid and digestive juices • Unpleasant taste of drugs • Not possible to use for unconscious patients
Intravenous	• Sudden onset of action • Can be given to unconscious patients • Large amount of drug can be given	• Pain occurs at the site of action • Embolism may occur
Vaginal	• Can be given to unconscious patients • Can be given to patients with vomiting	• May show irritations • Absorption may be variable
Transdermal	• Avoid first-pass effect • Patient compliance • Easy to administer	• Low systemic availability • Skin irritation or sensitization

4.3.1.1 Nasal Drug Delivery System

Oral drug administration is one of the most common routes of drug delivery, but it has several disadvantages such as low bioavailability, inability to cross the blood–brain barrier (BBB), and undesired side effects. When the drug is given through the intranasal route, the drug can easily penetrate BBB, reduces the side effects, and also reduces the doses to be administered. Intranasal delivery can be easily evaluated by in vivo animal nasal absorption studies. The rat was the first animal used for this in vivo assessment and mouse, dog, and monkey were later used for this study. Rat and mouse models are effective for brain targeting, while other animals are used mainly for assessing pharmacokinetic properties. After animal studies, human studies must be conducted because of the difference in nasal cavities. Here, the drug is administered using a pipette by inserting it into the right nostril and the left nostril is the control. Animals are kept in a supine position to reach the drug easily to the olfactory region. There exist two possibilities for this drug to reach brain tissue. The drug may be directly absorbed either by the trigeminal nerve or by the olfactory nerve. Another possibility is that the drug which is in direct contact with the mucosal membrane gets absorbed into lamina propria reaches subarachnoid space through perineural space and finally reaches brain tissue (Erdő et al. 2018).

Subcutaneous insulin injection for insulin-dependent diabetes is having low patient compliance due to pain and discomfort and is not exactly showing the pulsative pattern of endogenous insulin secretin in non-diabetics. To solve this problem, an alternative system like nasal insulin delivery system based on thiolated chitosan (chitosan TBA—chitosan 4 thiobutylamidine) has been investigated. Different microparticulate delivery systems prepared by precipitation micronization techniques were nasally administered to non-diabetic rat and the insulin concentration in blood and decrease of blood glucose level as a pharmacologic response were

determined. Investigation of bioavailability of chitosan TBA insulin microparticulate system shows that this particular system has more than 1.5-fold bioavailability and more than 7-fold higher pharmacological efficiency than insulin loaded unmodified chitosan microparticles (Krauland et al. 2006).

4.3.1.2 Colon-Specific Drug Delivery System

Colon-specific delivery of a drug is one of the most effective treatment strategies for irritable bowel syndrome, ulcerative colitis, Crohn's disease. In vivo assessment of colon-specific delivery includes the determination of the rate of drug release in the colon for exerting a particular pharmacological effect. In vivo studies will provide a detailed information about the pharmacokinetics of colon-specific delivery. Animal models and human models can be used for this. Animal studies are mostly done by using guinea pig, rat, and dog. And, the human studies are done by using scintigraphy imaging (Yang et al. 2002).

Certain drugs used for the treatment of inflammatory bowel syndrome are having adverse reactions and lower therapeutic effect. Colon-targeted chitosan beads have been developed for the delivery of these drugs. The rabbit model is used for their assessments. The rabbits were divided into four groups, namely normal, colitis untreated, drug-treated, drug-loaded bead-treated group. Colitis was induced in them. The first two groups did not receive any treatment. The drug-treated rabbits were given acid-resistant capsule filled with drug for 3 days and the drug-loaded bead-treated rabbits were given acid-resistant capsule filled with drug-loaded beads. Then animals are sacrificed and colon-separated and analyzed. Results show that this formulation is having higher therapeutic efficiency (Helmy et al. 2017).

4.3.1.3 Ophthalmic Drug Delivery System

Ophthalmic delivery of drugs includes the administration of drugs into the eye. There are many formulations given through the ophthalmic route. The most commonly used formulation used is the eye drops which are applied topically. This route has better patient compliance also. But the delivery of drugs to the target site has to face ocular barriers thereby the therapeutic effect of the drug decreases. Researchers are focusing on the development of a novel system that is safe, effective, and higher therapeutic efficacy (Patel et al. 2013).

Topical ophthalmic formulations are most commonly used to treat diseases associated with eyes. Because of the complex nature of the generic formulations like ointment, suspension, gels, etc., and difficulties to determine the bioequivalence with the innovator formulation, these generic formulations face a problem in their approval. Rabbit model is widely used because of its anatomical similarity with the human eye. By using this model, we can determine the concentration of drugs in different tissues of the eyes. For the ophthalmic biodistribution study, adult male rabbits were used. They are divided into seven groups with each group containing

six rabbits. The formulation is only applied in the right eye and the tear sample is collected at a regular interval of time. Then, the collected sample is transferred to amber colored tubes and it is covered by using aluminum foil to protect from light. Thus, the rabbit model study is useful for determining the concentration of drugs in different tissues of the eye (Chockalingam et al. 2019).

In situ gelling system is one of the most promising strategies to improve the treatment of diseases of the eye. This system provides a safe, simple, and reproducible administration of drugs. One of the major drawbacks of ocular delivery is to obtain a concentration of drugs at the site of action, but it is difficult because of the rapid elimination so that only a small fraction of the drug is get absorbed. This problem can overcome by using solid and semi-solid formulations which will improve the drug release rate and bioavailability. In situ, gelling systems can overcome rapid elimination. In vivo assessment is done by using albino rabbits. Here, gamma scintigraphy is the method used for determining the retention time of in situ gelling systems. For gamma scintigraphy, the formulation must be radiolabeled with a radioactive isotope. In vivo toxicity study can be done by using the Draize test. In this, the formulation is instilled into the conjunctival sac of the rabbit eye and the eye is kept closed after installation. One eye is kept as a control for this study. Then any irritation on the conjunctiva is observed and scored after 1, 24, 48, and 72 h and 14 days (Destruel et al. 2017).

Fungal infections of the eyes are mainly treated by natamycin (NT) because it provides more ocular safety than other antifungal agents do. But, this drug when applied topically is having poor bioavailability thereby frequent administration of the drug is required. To solve all the problems associated with different formulations of NT, they are loaded to pegylated nanolipid carriers (NLC). The in vivo assessments were performed in albino rabbits. This formulation, NT-NLC, Natamycin alone were given to rabbits in the conscious stage for every 2 h for a time period of 6 h. Then they are anesthetized and then euthanized. Their intraocular tissues were separated and analyzed. The result shows that this particular formulation has more efficiency than conventional (Ahmed and Badr-Eldin 2019).

4.3.1.4 Transdermal Drug Delivery System

Dermatological studies involve the assessment of the absorption of drugs from transdermal delivery systems to the layers of skin. Table 4.2 shows various in vivo techniques that are used for this purpose. Assessment of drug bioavailability following topical application is a complicated process since the relationship between drug concentration at the site of action and in the systemic compartment is not clear. Hence, for this purpose, several approaches are under investigation. In a study, a comparison of bioavailability from three marketed products of diclofenac was done by a subcutaneous tape stripping approach. This method involves the collection of subcutaneous skin layers using adhesive tapes post-application of a drug containing formulation and further extraction and quantification of the drug in the subcutaneous tissue. Due to the presence of dimethyl sulfoxide, which is a skin penetration

Table 4.2 Common techniques in assessing drug absorption upon transdermal delivery

Technique	Description
Vasoconstrictor assay	Here balancing between skin and corticosteroids applied topically is considered as the endpoint to find bioavailability. The balancing between them occurs due to vasoconstriction, measurement of which is done by visual inspection, chromametry, or by digital imaging. Chromametry is the most preferred one because of its reliable and reproducible nature. Here, quantification of white light reflection is done. The digital imaging method involves capturing of skin site and further analysis. Visual inspection involves the use of the naked eye and it requires well-experienced and trained persons. Researchers have found that visual inspection and chromametry are equally comparable
Tape stripping	The amount of topically applied drugs in the human stratum corneum (SC) can be assessed by this method. Here tape strip is applied at different sites and SC is collected by removing the tape strip at different times after application. The amount of drug in the SC is determined by weighing the tapes, HPLC, radiolabeling method, etc. Then the loss of water from epidermal layers can be determined by using Fick's first law. By using Fick's first and second laws, k and D\L2 can be determined which can be used to find Cmax, Tmax, and AUC
Microdialysis	The amount of drug in the dermis and hypodermis can be monitored by this technique. By using a needle, a probe with the semi-permeable membrane is implanted into the dermis or hypodermis. The probe consists of an inlet and outlet for perfusing physiological solutions. perfusate used here is usually a Ringer lactate solution at a slow rate. The solubility of a lipophilic drug can be increased by the addition of solvents. Free drug concentration versus time graph is plotted and the Cmax, Tmax, and AUC were determined (Patel et al. 2016)
Skin biopsy	This is an invasive technique usually performed only after giving local anesthesia. This procedure is mainly used for the removal of warts and tumors from the skin. There are two types of biopsy shave biopsy and punch biopsy. Here skin samples are collected and separated into individual layers. Then drug content determined by extraction. A study was performed for the determination of the cutaneous bioavailability of radiolabelled acitrecin. The investigations were performed in the buttocks of human volunteers. Both punch biopsy and shave biopsy were performed and levels of acitrecin were found to be comparable. Here, the levels in the skin after topical and oral administration were found to be comparable
Follicle removal	This method can be used for the bioavailability determination after the topical application of the drug in disorders like acne. The method involves the application of an adhesive to the skin and a glass slide is applied over it. When the glue gets polymerized the glass slide is removed and then stratum corneum together with hair follicles is collected. Analyses of removed hair follicles were performed
Suction blister	A device in bell shape having several holes in the base portion is placed into the skin and then pressure is applied. The skin rises through the holes and forms a blister that gets filled with serum and interstitial fluid. The blister fluid usually carries the drug which is collected using insulin syringe and analyzed (Ruela et al. 2016)

enhancer, in a product, the uptake, and permeation of diclofenac from the formulation was more. It was much greater than that of the other product. The study concluded that subcutaneous tape stripping can be used for bioavailability assessment of topical drugs.

Transdermal delivery systems are novel alternative strategies that reduce systemic toxicity while increasing therapeutic efficacy. Transdermal delivery of low molecular weight heparin (LMWH) can be enhanced by using a cationic carrier, which is a permeation enhancer. Assessment of systemic toxicity is done by comparing the drug release from hydrogel form and aqueous solution. In vivo permeation study where performed by tape stripping method which involves the collection of subcutaneous skin layer using adhesive tapes after 24 h of its application and then it is extracted and quantified. Results show that LMWH released from hydrogel formulation has lesser systemic toxicity as compared to its aqueous solution (Taktak et al. 2019).

Transdermal delivery of drugs can be carried out by using proniosomes as a carrier by encapsulating the drug in proniosomal gels prepared by the process of coacervation phase separation. In vivo assessment of systemic delivery is conducted in rabbits. Proniosomal gel is applied to the rabbit and blood samples and blood samples are collected at regular intervals of time and then analyzed. In vivo assessment study results show that this preparation has increased relative bioavailability than conventional dosage forms (Ramkanth et al. 2018).

Nanoliposomal sludge can be used as an effective transdermal delivery system for bioadhesive, viscoelastic properties, and also pH or redox responsive properties. Due to this property, they can be used in inflammatory conditions, and it can provide response directly at the inflammation site. The drug loaded in the nanoliposome shows a slow release pattern due to its above properties, thereby can enhance patient compliance. In vitro evaluation of this formulation gives kinetics of release rate from the sludge. Its responsiveness to pH- or redox-induced stimuli can be studied by the rat model. In this model, rats receiving oral administration of drugs require fasting before 12 h of study. Rats are divided into five groups containing five rats each. Before giving the formulations, the rat must be anesthetized. Group 1 is the placebo group and received an application of placebo nanoliposomal sludge dermally. Group 2 is the test group here the rats with inflammation receive a particular drug-loaded nanoliposomal sludge dermally. Groups 3 and 4 are the comparison groups; both groups contain rats with inflammation. Rats in group 3 receive a tablet of the drug orally and rats in group 4 receive drug-loaded cream dermally. Group 5 also is a comparison group, but it contains healthy rats and these rats receive an application of drug-loaded nanoliposomal sludge dermally. The dose of the drug used is the same for all the groups except for group 1 because of its placebo group. The blood sample is collected from each group, the concentration of a drug is determined analytically, and drug concentration versus time graph of all the formulation except group 1 is developed. Time of peak concentration of drug released is different for all the formulation and drug released from formulation given to group 2 is higher in a controlled manner as compared to other groups. These in vivo results show higher absorption of the drug under inflammatory condition, thus we can confirm nanoliposomal sludge have pH or redox responsive properties. So, this can be developed for the release of a particular

drug for the treatment of chronic inflammation to achieve better therapeutic efficiency (Mavuso et al. 2018).

The transdermal delivery system is the more convenient and painless route for drug delivery compared to other invasive routes. It can avoid **first-pass metabolism**, other side effects and can also provide controlled release of drugs. But stratum corneum acts as a barrier for the penetration of drugs. Invasomes are the elastic vesicles and they have the ability to increase the absorption of hydrophilic drugs compared to liposomes. This increased absorption of the drug can be evaluated and by using in vivo methods. Rats were grouped into three and must be fasted overnight. Group 1 is the standard group, rats in this group is administered with drug suspension orally. Group 2 and group 3 are positive control and test group, respectively. Rats in group 2 receive transdermal application drug films, while rats in group 3 receive transdermal application of invasomal film. Blood samples were collected at regular interval of time. The collected sample is subjected to centrifugation and analyzed by using high-performance liquid chromatography technique. Invasomal film shows higher bioavailability, this is due to the elimination of the first-pass metabolism. These results show that invasomes are important for enhancing transdermal drug delivery and they can also overcome many problems associated with the oral delivery of drugs (Ahmed and Badr-Eldin 2019).

Nanoemulsions are one of the nanocarriers for the delivery of drugs across the skin layer thereby we can avoid the first-pass metabolism, and can reduce side effects. Nanoemulsions are a clear transparent oil–water mixture. The permeation behavior of these gels can be assessed by in vivo evaluation techniques. So, nanoemulsion gels can be used as a drug delivery carrier for the delivery of drugs across the transdermal area for the treatment of many diseases (Kaur and Ajitha 2019).

4.3.1.5 Oral Drug Delivery System

The oral route of administration is the most convenient route for the delivery of drugs. But, oral bioavailabilities of certain drugs are very low. Hence, different methods have been developed to solve this. Certain drugs used for the treatment of hypertension are having low bioavailability because of its decreased aqueous **solubility** and efflux mechanism by intestinal p-glycoprotein (p-gp). Drugs incorporated into a self-nano emulsifying drug delivery system can be developed and its in vivo performance evaluated in hypertensive male Wistar rats. This formulation is found to increase oral bioavailability by inhibition of intestinal p-gp activity by excipient within the formulation as well as their bioactive effects such as tight junction opening which will enhance intestinal uptake. Hence this approach can be considered as a promising delivery system for enhancing poor oral bioavailability (AboulFotouh et al. 2019).

Encapsulation of drugs into nanostructured lipid carriers (NLC) can overcome limited oral applications of drugs. The development of drug-loaded NLC was done by the ultrasonication technique, which enhances its oral bioavailability by enhancing its solubilization in the intestine and increasing intestinal permeability. A female Wistar rat was used for this study. Here, a comparison of the plasma concentration

profile of drug suspension and drug-loaded NLC formulation is done. This in vivo assessment shows that drug-loaded NLC has more oral availability than suspension (Singh et al. 2019).

Polysaccharides from various natural sources can be used as a nanocarrier for targeted delivery of many drugs, especially anti-cancer drugs, because of their biocompatibility and biodegradability. A polysaccharide micelle is formed from Bletillastriata by the action of stearic acid and histidine. This polysaccharide micelle can be used for targeting an anticancer drug. The effectiveness of polysaccharides in drug delivery can be determined by evaluating the tumor cells. Test samples were injected intravenously to the test animals. The sample treatment is continued for 12 days. Bodyweight of the animal and the tumor volume is noted. Results show these micelles are very useful in tumor accumulation and release of drugs. Therefore, this polysaccharide micelle can be used for targeting anticancer drugs for its effective drug release (Wang et al. 2019).

Entecavir is the drug of choice for hepatitis B disease. Here, entecavir palmitate microcrystals in different particle sizes were developed. In vivo assessments were performed in rats. Rats were divided into different groups. To each of them, this formulation is given dorsally. At regular intervals of time for 46 days, blood samples are collected and analyzed. It proves that this is an alternative therapy for hepatitis B (Ho et al. 2018).

BBB is the main obstacle for the drugs acting centrally. In vivo assessment of drugs in the brain can be done in anesthetized rats. Here, rats are anesthetized and into the right jugular vein catheter inserted for blood sample collection. For drug administration, another catheter was inserted into the caudal vein. Then IV infusion is given. Blood samples are collected at regular intervals of time and analyzed. After that, those animals are sacrificed, skull was removed, and brain tissue was isolated. Brain tissue analysis was done. This study is a simple and an efficient method for in vivo assessment (Andersen et al. 2014).

The bioavailability of many drugs can be improved by mucoadhesive films. This buccal route of drug administration can be used to overcome the first-pass metabolism and irritation due to gastric secretions. Bioadhesive films are more chosen oven tablets because of their comfort and flexibility. In vivo assessment of buccal films can be done by using human subjects. Four male healthy subjects are used for this study. Firstly, each subject's mouth must be rinsed by using distilled water. A small piece of film is placed in the buccal mucosa. Then the residence time of the film is recorded (Mohamed et al. 2011).

4.3.1.6 Sublingual Drug Delivery System

Drugs that require rapid onset of action can be delivered through the sublingual route. It is considered as an alternative to oral administration of drugs. This route possesses more patient compliance, increased bioavailability, and it bypasses the first-pass metabolism. As human mucosa is not keratinized, an animal without keratinized mucosa is most suitable. But, there are only a few laboratory animals in this category.

Rabbit is such an animal without keratinized mucosa. Hence, the in vivo evaluations were performed using the rabbit model. From the plasma data, the peak plasma concentration and time to reach peak plasma concentration are determined. Then area under the plasma concentration–time profile and then is bioavailability determined (Narang and Sharma 2011).

4.3.1.7 Vaginal Drug Delivery System

The human vagina can be considered as a major route for the delivery of drugs to specific sites. The drugs can be delivered both locally and systemically by using special carriers. This route is mainly considered for treating diseases of women. The examples for delivery systems appropriate for vaginal administration include pessaries, vaginal tablets, suppository, hydrogels, etc. In vivo assessments were performed in animal models that determine the spreading efficacy, distribution, and formulation retention within the target site. Many animals can be used for this purpose. Sheep, rabbit, monkey, dog, mice, etc., are commonly used animals.

4.3.1.8 Intravenous Drug Delivery System

Intravenous (IV) delivery system is one of the most important routes of administration, which involves the delivery of drugs directly into the vein either by injection or infusion. In vivo assessment of this IV delivery can be assessed by using different animal models. In vivo assessment of IV delivery is not applicable for bioavailability studies. The bioavailability of IV delivery is considered 100% as the entire drug reaches systemic circulation. Nevertheless, the pharmacokinetics of a drug can be assessed after the administration of a drug by this route.

Pharmacokinetics of nicotinamide was evaluated by in vivo assessment using Wistar rats as an animal model. Fifteen male Wistar rats are used for the study. Nicotinamide weighing 2 mL/kg was administered to five rats via lateral tail vein as a bolus injection. One hundred and fifty microliters of blood samples were collected from each animal from the jugular vein at regular intervals of time. To the collected blood samples were analyzed after processing (Linnik et al. 2019). Similarly, in vivo assessment of marbofloxacin was done in goats after IV administration. For this study, healthy female goats were selected. The drug was administrated to the goats at a dose of 2 mg/kg for 5 days at 24-h interval. The blood sample was collected from the jugular vein at 1, 2, 4, 6, 9, 12, and 24 h. Heparin was used to prevent coagulation. Then collected sample was centrifuged at 3000 rpm at 5 °C for 10 min. Finally, it was separated and stored at −20 °C before drug analysis (Bhardwaj et al. 2018).

4.3.2 Based on the Specialty of the Molecule Delivered

4.3.2.1 Delivery of Gene

Both mammalian and non-mammalian models are used for in vivo evaluation of gene delivery. Non-mammalian species like *Caenorhabditis elegans* and zebrafish are commonly used due to their short life cycle and cost-effectiveness. Moreover, the genomes of these species show homology to human equivalent genes. Transgenic zebrafish are used in research as models of immune disorders, cancer, and cardiac diseases (Spence et al. 2008; Liu and Leach 2011; Drummond 2005; Novoa and Figueras 2012).

C. elegans is a small roundworm having a life cycle of 3–5 days, found to have a 74% match in genome sequence with humans and also in various biological pathways. Transgenic worms can provide data regarding the role of some particular mutations in human disease pathophysiology. *C. elegans* have been used by various researches in Huntington's disease Alzheimer's disease, Parkinson's disease as well as in cancer (The C. elegans Sequencing Consortium 1998; Li and Le 2013; Kirienko et al. 2010).

Wild-type mice, genetically engineered mouse, and some higher disease animal models are the commonly used mammalian models for in vivo studies for drug development and translation research. The selection of the correct background strain is crucial based on pathophysiology before in vivo validation. Some online databases are available to find out the appropriate strains as per the research requirements.

Genetically engineered mouse models are useful in elucidating human disease pathophysiology as well as in vivo target validation. For example, hemophilia mouse models are used very commonly for the validation of pharmacokinetic and pharmacodynamic parameters in gene therapy studies (Cook et al. 2012; Kucherlapati 2012; Crudele et al. 2015; Metzner et al. 2009).

Larger disease animal models are used in research due to limitations of mice models like low homology of some diseases and also limitations in biological sampling, monitoring of vital signs, the difficulty for some surgical procedures, etc. For example, hemophilia A sheep, dog, and pig, hemophilia B dog, etc., are extensively used by various researchers as animal models since they are more closely related to man (Giles et al. 1982; Brinkhous and Graham 1950; Porada et al. 2010; Kashiwakura et al. 2012; Graham and Buckwalter 1949; Mauser et al. 1996).

For the assessment of in vivo gene expression, CCCD imaging techniques (light emission from **bioluminescence** cooled charged-coupled device) was used by Zeira et al. in anesthetized rats after the administration of plasmid gene (Zeira et al. 2003). Andrew and associates used bioluminescence imaging technique for the in vivo evaluation of the dynamic gene expression of plasmid DNA after systemic delivery (Wilber et al. 2005). Peterson and associates studied the luciferase gene expression kinetics by a non-invasive imaging system using a full-thickness injury mice model (Peterson et al. 2009). They placed lyophilized collagen gene-activated matrix containing Ad-lucin on the wound site and observed a 40-fold increase in gene delivery (Fig. 4.4).

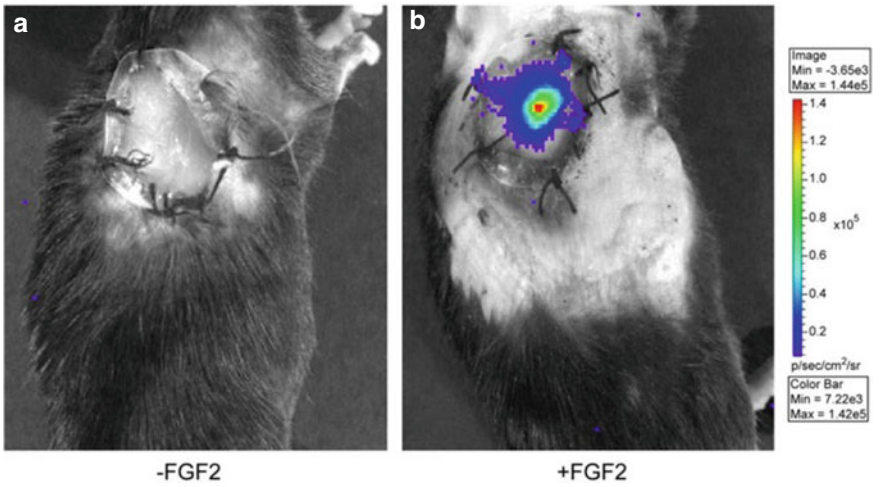

Fig. 4.4 Adenovirus-mediated gene delivery in skin wound **a** images and **b** quantification. Reprinted from (Peterson et al. 2009) with permission from Elsevier

Gene therapy is one of the main approaches used to treat a variety of disorders. It is being evident that Gluc (Gaussia luciferase) a naturally secreted luciferase which earlier, most commonly used in BLI (bioluminescence imaging), is having potential application in the in vivo monitoring of systemic protein delivery. The Gluc has already been investigated for animal immune liposome delivery, gene transfer efficiency, and as a part of a bifunctional fusion protein and now, for this particular application, we are injecting Gluc encoding plasmid into mice by hydrodynamic tail vein injection. Assessment of kinetics of Gluc in non-tumerogenic cell lines shows the involvement of pinocytosis in Gluc internalization into peripheral organs with highest levels formed in kidney/bladder and stomach/intestine and failure to cross BBB. Thus, in vivo tracking of temporal changes and biodistribution of agents, especially proteins following systemic delivery can be done using Gluc-based system.

Gluc can be used as a marker to determine gene delivery in vivo. Here, for the purpose of in vitro modification of spleen lymphocyte, a retroviral vector containing Gluc expression cassette has been used. These cells were implanted into mice and blood collected from the tail vein and monitored. Gluc levels have been found to be correlating with genetically modified spleen lymphocytes. Gluc activity in blood and plasma was found to be identical which results in the overlapping of emission spectra of Gluc and absorption spectra of hemoglobin. Gluc cells disappeared after 6 days of implantation because of the neutralization by antibodies present in the mice. This result shows that Gluc is more useful in immunodeficient animals. Assessment by using Gluc can be completed within a short duration of time. Thus, real-time in vivo monitoring of gene delivery can be done by blood sample collection at a short duration of time (Chen et al. 2010). In vivo assessment of some recently reported other gene delivery systems are provided in Table 4.3 (Ohtsu et al. 1999; Pattabhi et al. 2019;

Table 4.3 In vivo assessment of some recently reported gene delivery systems

Gene/cell	Purpose	Animal model/method	References
Histidine decarboxylase gene	In vivo effects of histamine	Mice model	Ohtsu et al. (1999)
HBB gene	In vivo outcome of homology	Alternative donor template delivery method	Pattabhi et al. (2019)
Brown midrib mutant gene (BMR)	Nutritive value of Sudan grass	Lactating dairy cow	Ledgerwood et al. (2009)
Gonad cells	Secondary sex characteristics and spawning	Casper zebrafish	Brantley and Lessman (2019)
Human hematopoietic progenitor cells	Lysosomal storage disorder	Murine model	Hofling et al. (2003)
P glycoprotein gene expression	Ivermectin resistance	*Haemonchuscontortus*	Maté et al. (2018)
Aromatic L amino acid decarboxylase gene	Parkinsonism	Positron emission tomography	Asari et al. (2010)
2, 3, 7, 8 tetrachlorodibenzo-p-digoxin	Carcinogenic effect	Mouse	Yang et al. (2019)

Ledgerwood et al. 2009; Brantley and Lessman 2019; Hofling et al. 2003; Maté et al. 2018; Asari et al. 2010; Yang et al. 2019).

4.3.2.2 Delivery of Peptide

Peptide drugs are commonly administered through parenteral routes, which are having some difficulties. Within this study, a stomach-targeted delivery system has been developed and the in vivo evaluation was performed. The potential of this particular system was evaluated by combining stomach delivery and thiomer technology. This study shows that stomach targeted oral delivery is a novel approach for systemic peptide delivery (Guggi et al. 2003).

Most of the peptide drugs have poor bioavailability when they are administered through the oral route; this is due to the presence of proteolytic enzyme present in the gastrointestinal tract. These enzymes will degrade peptide drugs, so its bioavailability is reduced. Insulin is one of the important drugs used for the treatment of diabetes mellitus. But it cannot give through the oral route because it undergoes the first-pass metabolism. Enteric-coated pellets of insulin can avoid first-pass metabolism by protecting insulin from proteolytic activity. For the in vivo assessment of these pellets, diabetes is induced in rats. The rats must be fasted before giving insulin. Pellets were applied by force-feeding and then acidified saline solution was given.

Blood samples were collected at regular interval of time. Insulin and glucose levels were determined (Trenktrog et al. 1996).

Glycogenolysis is the main reason for the increased production of glucose which results in type 2 diabetes. The glycogen phosphorylase [GP] enzyme plays a major role in this pathway. GP inhibitors [GPi] act on the glycogenolysis pathway and decrease glucose output. Hence, these can be used for the treatment of type 2 diabetes. The study was conducted in Wistar and Zuker rats and the effect of GPi was observed in both of them. This method allows direct measurement of GPi effect by using glucagon response. Glucagon administered to rats and blood glucose levels were measured before and after administration. The results suggest that the assessment of the in vivo efficiency of GPi is an easy task (Loxham et al. 2007).

The dysfunction of β cells is a major factor contributing to type 2 diabetes. Fuel homeostasis in mammals was controlled mainly by glucose-stimulated insulin secretion (GSIS). This process occurs by the closure of ATP-sensitive potassium channels and thereby activation of calcium channel which results in the secretion of insulin. GSIS diminishes due to β cell dysfunction. Hence, type 2 diabetes can be prevented by protecting β cell function. GSIS can be assessed by an intravenous glucose tolerance test (IVGTT). The investigations were performed in both anesthetized and conscious male Wistar rats. The animals were cannulated through the right jugular vein and left carotid artery and then anesthetized. The animals undergo IVGTT by using increasing doses of glucose or by using Exendin 4 with glucose. Results show a dose-dependent increase in blood glucose and plasma insulin. The plasma C-peptide level is also increasing. Hence, IVGTT can be used as a method for assessing GSIS (Frangioudakis et al. 2008).

Insulin can be loaded with nanoparticles. It can be assessed by in vivo methods. Before the administration of the drug, the rats must have fasted. Then immediately collect blood samples before administration. Then blood glucose level is determined. Then samples are allowed to clot the blood sample. The supernatant liquid is collected and stored. Then the concentration of insulin present in the sample is determined by the ELISA test (Deutel et al. 2008). In a recently reported insulin delivery system based on bioinspired yeast microcapsules, the bioavailability was assessed by blood glucose level (Sabu et al. 2019). In addition, the proposed M-cell mediated uptake of the yeast microcapsule was demonstrated microscopic analysis of the intestinal section (Fig. 4.5).

4.3.3 Based on the Dosage Form

4.3.3.1 Liposome

The pulmonary drug delivery system is an important non-invasive route for the treatment of several diseases. In vivo circulation time of the drug is increased and the toxic effects are eliminated by the use of liposomes. In vivo assessment of pulmonary delivery is done by in vivo absorption and tissue distribution studies using male

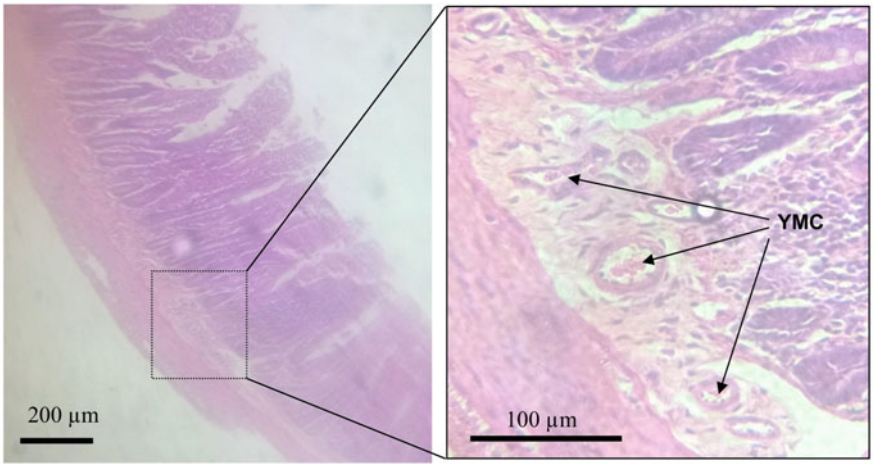

Fig. 4.5 Microscopic image of an intestinal section (ileum) of the rat treated with yeast microcapsules. Reprinted from (Sabu et al. 2019) with permission from Elsevier

rats. Absorption study is conducted in three groups: solution, liposome, and powder groups. The drug is administered to the rats in the solution and liposome group by intravenous injection. At regular intervals of time, the blood sample is collected from the jugular vein, and the collected sample is centrifuged to separate the serum and, finally, its concentration is determined.

Tissue distribution study is done in two groups, powder and liposome group. After administration of the drug, whole tissues like liver, heart, spleen, and lung are homogenized using a homogenizer and the resultant tissue homogenate is used to determine the concentration of the drug after pulmonary delivery (Xu et al. 2019).

Gene therapy is an important strategy for the treatment of many neurodegenerative disorders. This requires the development of a carrier for the particular gene to cross BBB and reaches the brain. Liposome mediated gene delivery can achieve the required concentration of the gene in the brain. A dual functionalized liposome is developed for the transfection of neurons. The transport of dual functionalized liposomes across BBB can be studied by the determination of the transfection efficiency of the liposome containing the gene. For an in vivo transfection study, mice were used. In this study, the liposomal formulation is given to six mice as a single dose. After 5 days, internal organs like liver, brain, kidney, and heart were removed from mice, weighed, and then transferred to a buffer to promote tissue lysis or protein extraction. This solution is then subjected to centrifugation at 40 °C for 15 min. The supernatant liquid is extracted and an equal volume of assay buffer is added to it. Then it is incubated for 60 min. After incubation, measure the absorbance at 420 nm. The result shows that the formulation deposited at high concentration in brain, liver, and kidney, and lesser in the heart. This study can provide evidence in the gene delivery for neurodegenerative disorders by multifunctional liposomes (Santos Rodrigues et al. 2018).

Certain drugs, when administered orally or parenterally, may cause toxicities. Such drugs are administered through the transdermal route. To enhance the penetration of drugs through the skin, the drug is entrapped within liposomes and placed in a hydroxyethyl cellulose gel. In vivo evaluations were performed in rats. They are divided into two groups. The first group is given radiolabeled transdermal nanogel formulation and they are sacrificed at different intervals of time. The second group is treated with the plain radiolabeled drug and they are also sacrificed at different intervals of time. Using the gamma spectrometer, the radioactivity measured. The results show that the formulation is having a high sustained systemic delivery and low organ toxicity (Sadarani et al. 2019).

Celecoxib (CLX) is a drug having anti-cancer activity but its use in cancer treatment is limited because of decreased water solubility. Hence, liposomal formulations of the drug have been developed which allows CLX accumulation in tumor tissue and thereby increased retention and permeability mechanism. In vivo assessments were performed in BALB\c mice. Biodistribution analyses were performed by using radioactivity methods, which show that this particular formulation has greater accumulation in tumor tissue. Hence, this has greater in vivo efficiency (Matbou Riahi et al. 2018).

Tripterine is an anticancer drug but is having low bioavailability and low intestinal absorption. To increase its bioavailability, they are converted to phytosomes coated with nanocarriers of protamine and then loaded to sponges. Evaluations were performed in rabbits. Rabbits are divided into three groups each containing six animals. Group one is given tripterine phytosome sponges and protamine coated with this formulation is given to the second group. Sponges loaded with drug suspension are given to group 3. Then rabbits were anesthetized and a catheter inserted into the marginal vein of the ear for blood sample collection. Sponges were placed inside the mucosal lining and blood is collected at a regular interval of time and analyzed. Results show that the absorption of tripterine got increased (Freag et al. 2018).

4.3.3.2 Nanoparticle

Nanoparticles are promising carriers for drug delivery whose size ranges from 1 to 1000 nm. Selenium is an important trace element available in different protein forms. Nanoform of selenium has several therapeutic uses like chemotherapeutic agent, antidiabetic, and antioxidant. Also selenium nanoparticles can be used as a dietary supplement. Artemia nauplii larvae, 48-h-old, are used for the in vivo toxicity assessment and are placed in a well plate and are tested against selenium nanoparticles. Twenty-four plates were used for this particular study. Toxicity of the selenium nanoparticles on the larvae treated with selenium nanoparticles after 24 h. This in vivo toxicity assessment suggests selenium nanoparticles are harmless. So, they can be readily used against bacterial infections because of their higher biocompatibility (Yazhiniprabha and Vaseeharan 2019).

Gold nanoparticles are used in many areas like medicine, biotechnology, etc. Its usage is now increased, so its toxicity evaluation becomes more important. In vivo

assessment of this toxicity is done by using somatic mutation and recombination test (SMART test). It is one of the cheap in vivo assays, but it is rapid and this can be used to detect any alterations in the gene. The principle of this assay is the comparison of the increase in clone frequency between the test and the control. So, this SMART assay can be used to understand the mechanism of action of gold nanoparticles to ensure their safety (Ávalos et al. 2018).

Carvedilol used for the treatment of hypertension and angina pectoris is having low bioavailability due to lower water solubility and is having the first-pass effect. The mucoadhesive gel of the drug is prepared. For increasing their bioavailability, they are converted to nanoparticles by ionotropic gelation technique. In vivo studies were performed in rabbits. The rabbits were anesthetized and lignocaine solution sprayed on their tongue to prevent the swallowing of formulae. The formulation administered to the buccal mucosa of the rabbits. Blood samples were withdrawn from the eye vein and analyzed (Garhy et al. 2018).

Silica nanoparticles are a class of materials having applications in both therapy and diagnosis. For assessing the toxicity of SiO_2, many in vivo studies were performed, which show that they are causing inflammatory reaction and hepatotoxicity. In vivo assessment was performed in mice. Both $FeSiO_2$ and pure SiO_2 nanoshells were prepared. Animals were divided into three groups; the first group receives $FeSiO_2$, and group 2 receives Sio_2 nanoshell at 4 mg/ml. SiO_2 nanoparticles at 2 mg/ml were given to group 3. Group 4 is the control group. Blood samples were collected and analyzed (Mendez et al. 2017).

Anti-inflammatory agents and antioxidants can be simultaneously delivered to dermis and epidermis. For that, these drugs are loaded to nanoparticles. The in vivo study was performed in NC/Nga mice. Clinical results show that this particular preparation has the ability to reduce the signs and symptoms associated with the disease. This formulation has been compared with others, which shows that this controlled transepidermal water loss, the intensity of erythema, dermatitis. Hence, it can be regarded as a promising approach for percutaneous delivery of anti-inflammatory and antioxidative agents together (Hussain et al. 2013).

Mucoadhesive nanoparticles can be used for the treatment of oral candidiasis since it maintains the drug substance in the oral cavity for a prolonged period of time. This formulation shows increased bioavailability and extended-release profile. In vivo studies were performed in rabbits and yeast-infected rabbits were removed from the study. The animals were made to infect with the disease and infected animals were divided into three groups. No treatment is given to the first group and blank nanoparticles formulation is given to group 2. Drug-loaded formulation is given to group 3. Smears of oral mucosa collected on continuous 7 days and inoculated in the SDA medium. Then it is incubated for 2 days and the efficacy of formulation is determined (Rençber et al. 2016).

Biological molecules, when loaded with nanostructures, act as a good vehicle for systemic delivery and it can cross the biological barriers present in our body easily. Red blood cells can be integrated with luminescence nanophosphorous which is persistent to near IR. This system is coated with a mesoporous silica particle. The in vivo studies were performed in mice. The results of the study show that the

above-mentioned system is a good carrier for long-circulating bioimaging (Liu et al. 2018).

4.3.3.3 Flexosome

Transdermal drug delivery is an important noninvasive drug delivery method. Flexosomes are soft nanovesicles that can be loaded with high soluble drugs. These flexosomes are developed to enhance the permeation of the loaded drugs. In vivo assessment of flexosomes is performed in four groups of rats containing six rats each. Before 5 min of starting the experiment, skin from the dorsal area is shaved for all rats in four groups by using a depilatory product. Samples were collected from each group and it is fixed for 24 h in paraformaldehyde in phosphate-buffered saline. Samples are washed with tap water and ethyl alcohol is used for dehydration. Then it is embedded in paraffin in a hot air oven at 56 °C for 24 h. Tissue sections are then collected on a glass slide, paraffin is removed, and it is treated with eosin stain. It is examined using an optical microscope. Results obtained from this study shows that flexosomes can be used as a carrier for the drugs for systemic delivery (Abdel-Messih et al. 2019).

4.3.3.4 Transferosome

Colchicine used for the treatment of gout is having some side effects and low bioavailability. Hence, they are complexed with beta-cyclodextrin and incorporated into transferosomes. The experiments were performed using two models of gout in rats. The first model involves the administration of monosodium urate crystals in saline and the second involves the administration of uricase inhibitor (UI). The animals were divided into seven groups. Group 1 is given vehicle alone and UI to groups 2 and 3. After 1 h, oral saline was given to group 2 and colchicine to group 3. After injecting with UI, transferosome formulations are applied transdermally to group 4. MSU crystals suspensions are given to groups 5 and 6. After 1 h, group 5 was given oral saline and colchicines to group 6. MSU crystals are given to group 7 and transferosome formulation was given after 1 h. The in vivo assessment results show that the current formation is having more efficiency than plain colchicine tablets (El-Feky et al. 2019).

4.3.3.5 Tablets

Enteric-coated tablets release drug based on the pH level and they can have an impact on when the drug released and where the drug released. The efficacy of drugs with a narrow therapeutic index is dependent on the plasma concentration of the drug. In vivo drug release results in the changes in the plasma concentration of drugs. In vitro and in vivo releases are linked by screening the individual plasma profile. In

vivo assessment is done for the enteric-coated formulations, and a pharmacokinetic in silico model is developed by applying in vitro dissolution as output, and results from in silico model are compared with the fasted in vivo model. Thus, in silico model and in vitro release can be used as a tool for estimating the in vivo release of drugs from the enteric-coated tablets and its plasma concentration (Karkossa and Klein 2019).

The bioavailability of many drugs can be improved by several methods. Many drugs are absorbed only in the stomach. But gastric emptying of such drugs can produce the amount of drug absorbed from the stomach, so its bioavailability decreases. This gastric emptying of such drugs can be reduced by increasing the residence time of the drug in the stomach. This can be achieved by using floating drug delivery systems. The performance of floating tablets can be determined by in vivo assessment techniques. The scintigraphic method is used to determine the residence time of the tablet in the stomach. It is done by determining the residence time of both floating and non-floating tablets. Residence time is noted both in fed and fast state. Residence time is the time required to leave the stomach. It is noted by scanning using a gamma camera (Desai and Bolton 1993).

4.3.3.6 Miscellaneous

Apart from the aforementioned, other dosage forms have been exploited. One example is microparticles. Microparticles of poly(lactide-co-glycolide) (PLGA) modified with phospholipid can be used to regulate the interaction with macrophages in alveoli. Both in vitro and in vivo investigations were performed. In vivo examinations were done in male rats. Here, animals were anesthetized and drug formulation administered to them. At regular interval of time, the animals were killed bronchoalveolar lavage fluid collected and analyzed (Li et al. 2019). Another example is exosomes, which are membrane-derived vesicles released by different cells. Exosomes will interact with the target tumor cell and deliver the toxic substance. Exosomes from macrophage can be loaded with cancer drugs and can be used as a vehicle for drug delivery. Now researchers have developed drug-containing exosomes targeted to cancerous cells for systemic administration. Its evaluations were performed in rats (Kim et al. 2018).

Finally, nanoemulsions of drugs used for the treatment of hypertension can be formulated for increasing bioavailability and therapeutic activity of the drug. Here, the required drugs are loaded to nanoemulsions, and the in vivo study was performed in female rats. Animals are divided into seven groups each containing four animals each. The drug solution is prepared and administered to the control group. Drug-loaded nanoemulsions are given subcutaneously. Blood samples are collected from the jugular vein and analyzed. The result shows that this particular formulation has more than others (Özdemir et al. 2018).

4.4 Summary and Outlooks

In vivo assessment of a drug plays a vital role in the discovery of a drug. A drug is made available in the market only after in vivo studies. These studies determine the safety, efficacy, and toxicological effects of the drug. The in vivo assessment is performed in animals like dogs, monkeys, guinea pigs, rats, etc. depending upon the aim of the study. Many newer models have been developed by the scientific world for assessing the efficacy of the drugs. The zebrafish model is such a new model used for the in vivo assessment of a drug. This model has several advantages compared to the rodent model. Several techniques are also developed for the investigation of drug distribution (e.g., imaging techniques, equilibrium dialysis, and microdialysis). In vivo assessment has a crucial role in the determination of bioavailability of a drug, which can be either a free drug or a drug that has been pre-loaded into a carrier for systemic delivery.

Important Notes

- Different in vivo assessment models are available for systemic delivery.
- Zebrafish is an emerging in vivo assessment model.
- In vivo assessment methods are available of different routes of administration.

Questions for Future Research

- **How to select and use different models for in vivo assessment of systemic delivery?** Over the years, the use of different models (ranging from mice to zebrafish) has been reported for in vivo assessment of systemic delivery. How the models can be properly selected so that data collected can be more accurately reflect the situation in human bodies is a question that is worth contemplation for future research.
- **How in vivo assessment can be done in different routes of administration?** Drugs can be administered to a body via different routes, and the selection of the administration route may affect the ultimate biodistribution profile attained. Proper selection of the route is, therefore, a challenge when a biogerontological intervention is developed and administered.

Glossary

Bioavailability It is the rate and extent of drug absorption to the circulatory system of the body from the site of administration.

Biocompatibility It is the ability of a material being compatible with living tissues.

Bioluminescence It is the production and emission of light by living organisms.

First-pass metabolism It is a process that results in the reduction of drug concentration before reaching the systemic circulation when it is administered orally.

In vitro studies Experiments performed outside of living organisms, such as in glass apparatus.

In vivo studies Experiments for the determination of effects of the drug or other entities in a living organism like cells, tissues, or whole organisms, especially in animals and humans.

Pharmacodynamics It is the study of the physiological and biochemical effects of a drug in the body after its administration.

Pharmacokinetics It is the study of the time course of absorption, distribution, metabolism, and excretion of drugs.

Solubility It is the maximum amount of solute dissolved per unit volume of a given solvent at equilibrium.

Systemic delivery It is the administration of drugs to the body of the patient systemically through intravenous, oral, pulmonary, subcutaneous, etc.

Therapeutic efficacy It is the capacity of a drug or other interventions to produce a therapeutic effect in the body.

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Part II
Systemic Delivery Techniques Based on Prodrug Design and Synthetic Materials

Chapter 5
Prodrug Design to Enhance Bioavailability and Systemic Delivery

Bruna Machado Araújo Sanches and Elizabeth Igne Ferreira

Abstract In the preceding two chapters in Section I, different strategies used to characterize the properties and performance of a drug, which can be either a free drug or a drug that has been loaded into a carrier, have been discussed. From Section II onward, selected strategies to enhance systemic drug delivery will be presented. As the first chapter in this section, we will introduce the concept of prodrug design. In fact, prodrugs have been very important to solve many problems with leads and drugs. The optimization of leads can overcome the "valley of death," allowing the drug candidate to reach clinical phases. On the other hand, drugs can be optimized even though they have been launched in the therapeutics, improving their usage and patient adherence. Pharmaceutical, pharmacokinetics, and, indirectly, pharmacodynamic problems of leads and drugs can be managed by prodrug design. In this chapter, we will present some classical and recent examples related to bioavailability, prolonged action, reduction of toxicity and, mainly, selectivity of action, and will also introduce some of the drugs approved by FDA to illustrate the use of prodrug design in reality.

Keywords Molecular modification prodrug design · Carrier prodrugs · Bioprecursor prodrugs · Targeted drugs · Pharmacokinetic problems

5.1 Introduction

Conceptually, a prodrug is an inactive molecule, or less active compound, which needs to undergo enzymatic and/or chemical **biotransformation**, in vivo, to release the parent drug to be pharmacologically active (Bundgaard 1985) (Fig. 5.1). Unlikely analogs, which act per se, biotransformation is mandatory to prodrug activity. The term prodrug was discovered by Albert in 1958, and prodrug design approach, also

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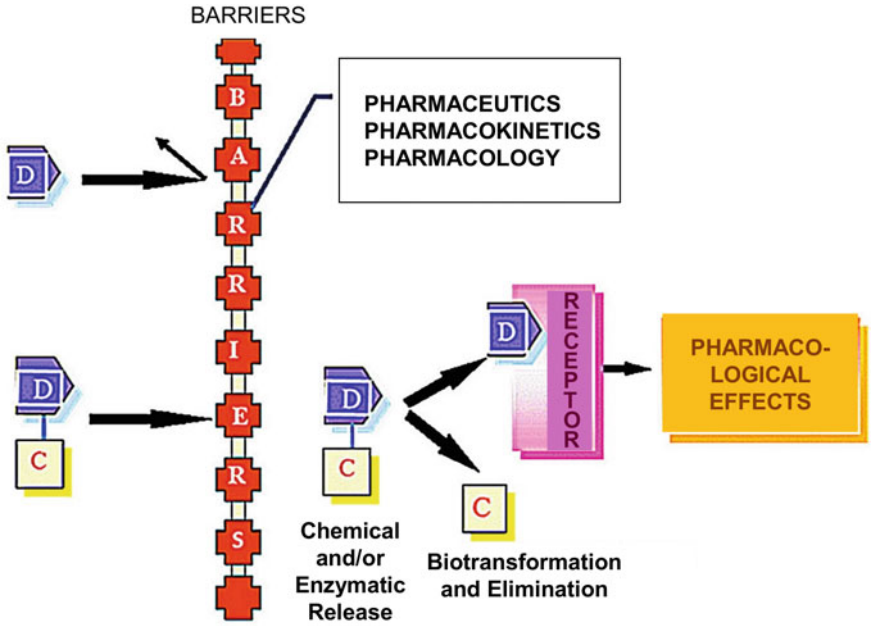

Fig. 5.1 A schematic diagram showing the process of prodrug design

named latentiation, was introduced in 1959 by Harper. In the beginning, the biotransformation was only carried out by enzymatic means, but after Kupchan, the chemical transformation was included (Bundgaard 1985; Choi-Sledeski and Wermuth 2015; Chung et al. 2005; Silva et al. 2005). Currently, the biotransformation in cascade, through enzyme and chemical reaction, is arisen high interest, as it allows achieving a more controlled system (Choi-Sledeski and Wermuth 2015).

Hans Bundgaard, a professor from the University of Copenhagen, contributed significantly to the area of prodrug design (Bundgaard 1985). In 1978, he created the Prodrug Research Group and coordinated until his death in 1992. His seminal book *Prodrug design* was published in 1985 and it comprehends the main principles of this important approach of **molecular modification**. Thanks to his relationship with industries, he disseminated the importance of prodrug design in this environment and many marketed prodrugs were based in his research in the field[(a)].

Prodrug approach has been a versatile strategy to chemists and researchers over several decades, to overcome undesirable properties of a drug **hit** or a **lead** compound under development and/or of clinically approved drugs (Zawilska et al. 2013; Abet et al. 2016; Clas et al. 2014; Ettmayer et al. 2004; Hamada 2017; Huttunen et al. 2011; Huttunen and Rautio 2011; Jana et al. 2010; Rautio et al. 2017; Rautio et al. 2018; Rautio et al. 2008; Testa 2004). It is, generally, applied to improve poor drug-like properties or, even to achieve a site-selective targeted delivery, so, prodrugs are designed to overcome several barriers that limit or diminish the therapeutic effect of the drug or bioactive molecule. These barriers can be low water solubility low bioavailability, several adverse effects, among others (Jana et al. 2010; Sanches and Ferreira 2019; Jornada et al. 2016). Over the past decade, the number of approved

prodrugs is considerable among all drugs launched in the market, emphasizing the importance of this tool on drug design. Huttunen and collaborators, in 2011 (Huttunen et al. 2011) reported that 20% of all marketed drug worldwide could be classified as prodrugs.

It is important to emphasize that prodrugs are not considered "me too", which allowed the recognition of patent in the US (Chao J PTAB says yes to (pro) drugs, The post-grant strategist, wolf greenfield, US 2017). However, it is important to notice that the industry can be used it, as well as polymorphs and salts as secondary patent, to increase the patent life, mostly of the best seller's drugs (Kapczynski et al. 2012). This, in addition to the relative facility of the process, at least for simple derivatives, can make prodrug design interesting to the pharmaceutical industries. The tendency is to use this approach not only in the end of the process of new drugs introduction in the market, named "post hoc" process, but also during lead optimization, named "ad hoc" process, right before the candidate is chosen (Sanches and Ferreira 2019). This can decrease the risk of failure in the "**valley of death**" (Seyhan 2019).

Despite the versatility and the potential for drug/lead optimization, mostly recognized a decade ago, prodrug design had faced many challenges to the pharmaceutical industries environment. They had considered the process as the last option to solve many drug/lead problems. However, this scenario has changed: in 2015, FDA approved eight prodrugs, which represents 20% of small molecules or 15% of the total amount (Rautio et al. 2018). Invasive aspergillosis, heart failure, hereditary orotic aciduria, schizophrenia, HIV infection, multiple myeloma, pulmonary hypertension, and carcinoid syndrome diarrhea are the important indications for these prodrugs. Five out of eight are derived from drugs newly approved: isavuconazole, ixazomib, sacubitril metabolite LBQ6567, selexipag metabolite ACT-333679, and telotristat (Fig. 5.2). It is also worth noting that many prodrugs are in the clinical trials.

In 2018, FDA approved three more prodrugs: baloxavir marboxil, for influenza A and B; tafenoquine, a bioprecursor, for malaria; and fosnetupitant, for nausea and vomiting induced by chemotherapy. In addition, the number of papers published in the last decade, 3.630, testifies the increased interest in this molecular modification approach (Fig. 5.3). From 2011 to 2019, this number has more than doubled, most of them being related to pharmacology and pharmacy and chemistry medicinal, according to the areas of Web of Science (Web of Science, Prodrug design 2011). Moreover, the citations of papers in the last 20 years were 66.851. It is worth to note that prodrug design has been considered among the recent strategy advances in **medicinal chemistry** (Wu et al. 2019; Najjar and Karaman 2019). Modern approaches in prodrug design involve computational methods, as molecular orbital and molecular mechanics (DFT, ab initio, and MM2) (Karaman 2013).

5.2 General Design Principles of a Prodrug

After becoming aware of the problem of a drug/**bioactive compound**, some questions must be answered before rationally designing a prodrug (Clas et al. 2014):

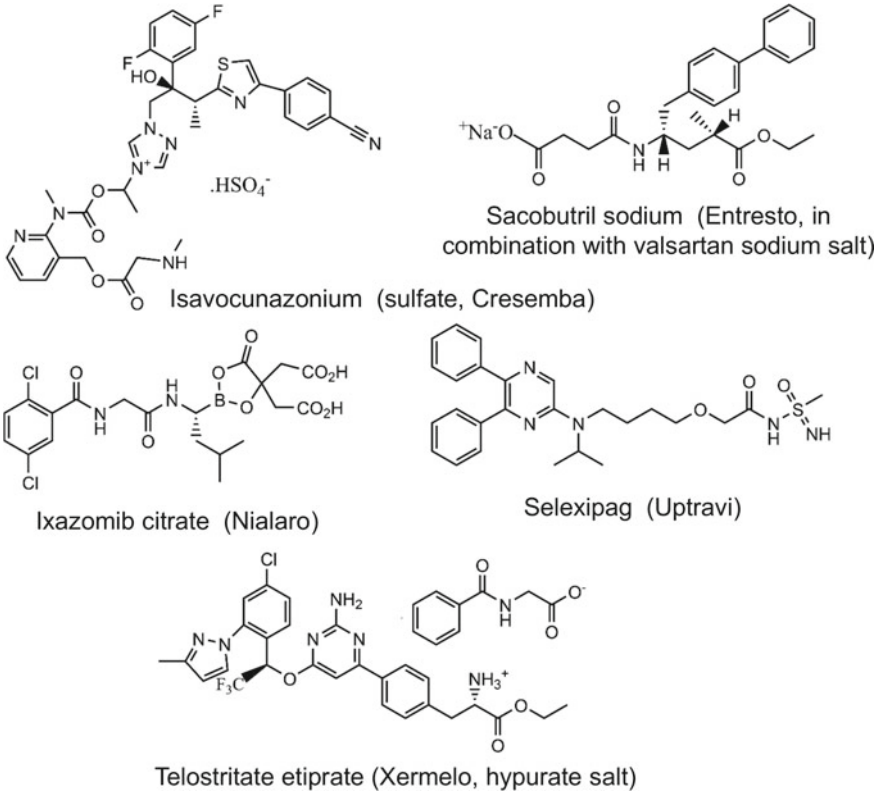

Fig. 5.2 Structures of FDA approved prodrugs

1. Which is the most appropriate class of prodrug to be used to solve the problem?
2. Which are the drug/bioactive compound functional groups available for composing a labile linkage (Table 5.1)?
3. Which is the best carrier to be used (not for bioprecursor)?
4. Which is the most suitable linkage to be used?
5. Which are the enzymes and/or chemical processes involved in the drug/bioactive compound release?

Problems to be solved can be divided according to their nature in pharmaceutical, pharmacokinetics, and, indirectly, pharmacodynamics. As detailed in Fig. 5.4, the target properties to be improved comprehend specific objectives such as pharmaceutical, pharmacokinetic, or, indirectly, pharmacodynamic, bearing in mind that many of these properties are interrelated.

The improvement of pharmacokinetic properties has been the main goal for prodrug design along the years, although pharmaceutical problems, as low solubility, have been an important property to be changed to overpass the "valley of death" (Sanches and Ferreira 2019). Currently, selectivity of action is being a tendency

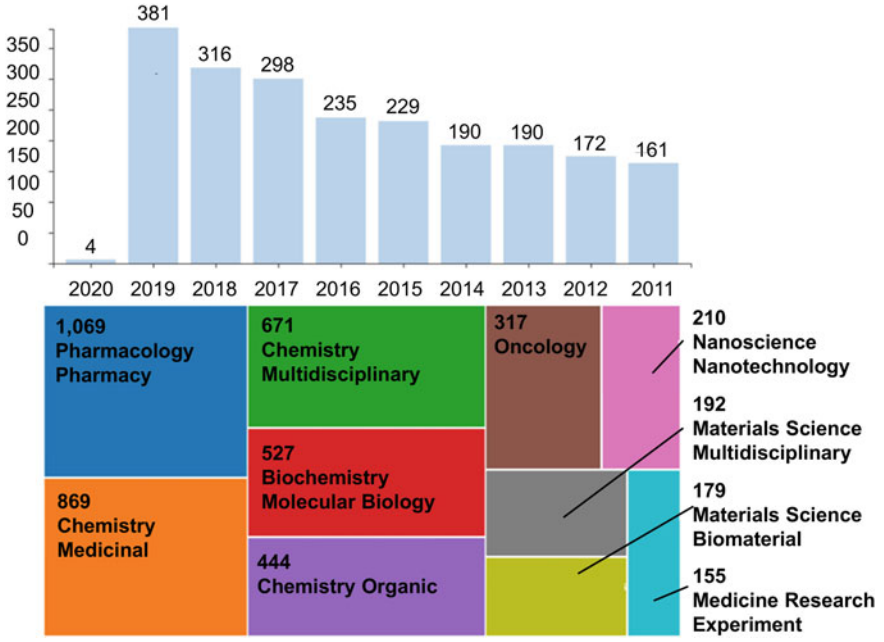

Fig. 5.3 Number of publications and respective areas according to the Web of Science—2011–2020

in prodrug design, mainly in the search for better antineoplastic and antiviral drugs (Delahousse et al. 2019; Lesniewska-Kowiel and Muszalska 2017). Other chemotherapeutic agents can also be modified through prodrugs to target the tumor or infections, decreasing their side effects. There are several types of prodrugs and they were classified as follows by Wermuth, in 1984 (Wermuth 1984):

- *Classic prodrugs*: are defined as carrier linked prodrugs and bioprecursors. *Carrier linked prodrugs* present a moiety linked temporarily to the pharmacologically active compound, generally, by a covalent linkage and need to suffer biotransformation, in vivo, to be released. The carrier has no activity (Fig. 5.5).
- *Bioprecursors* are prodrugs without a carrier moiety, but also must suffer biotransformation to be active as a drug. Some blockbusters are bioprecursors, as lovastatin (Fig. 5.6), whose active form is the open-ring derivative.
- *Mixed prodrugs*: in this type of latent form, the drug carrier has similar characteristics of a bioprecursor, which means, it must be biotransformed before releasing the drug. A classic example of this type of prodrug is CDS (Chemical Delivery System) (Fig. 5.7).
- *Mutual prodrugs*: in contrast to classic prodrugs, the carrier moiety is pharmacologically active. The prodrug can be designed by having either different or similar therapeutic activities, acting by different or equal mechanisms of action. The main goal is to obtain synergism of action (Fig. 5.8).

Table 5.1 Most common functional groups on parent drugs

Functional group	Moiety	Prodrug
–COOH	–COOR	Ester
	–COOCH(R)OOCR	α-acyloxyalkyl ester
	–CONHR	Amide
–OH	–OOCR	Ester
	–OOOCR	Carbonate ester
	$-OPO_3H_2$	Phosphate ester
	–OR	Ether
	–OCH(R)OOCR	α-acyloxyalkyl ether
–SH	–S-R	Thioether
	–SCH(R)OOCR	α-acyloxyalkyl thioether
	–SCOR	Thioester
	–S-S-R	Disulfide
–C = O	$RRC(OR')_2$	Ketal
	–HC = N-R	Imine
	–C = C-OOCR	Enol ester
		Oxazolidine
		Thiazolidine
$-NH_2$	–NHCOR	Amide
	–NHCOOR	Carbamate
	–N = CRR	Imine
	–NHCH = CRR	Enamine
	$-NH-CH_2N(R)COR$	*N*-Mannich bases
	–NHCOOCH(R)OCOR	*N*-acyloxyalkoxycarbonil
Tertiary–N	–+NC(R)CHOCOR	*N*-acyloxyalkyl derivative
$-SO_2NH_2$ or $-SO_2NH-$	$-SO_2N = C(OR)R$	*N*-sulfonyl imidate
	$-SO_2NHCH_2OR$	*N*-alkoxymethyl derivative
Acidic–NH	$-CON(R)CH_2-NR_1R_2$	*N*-Mannich bases
	$-CONRCH_2OH$	*N*-hydroxymethyl derivative
	–CONH-COR	*N*-acyl derivative
	$-CONRCH(R_1)OCOR_2$	*N*-acyloxyalkyl derivative

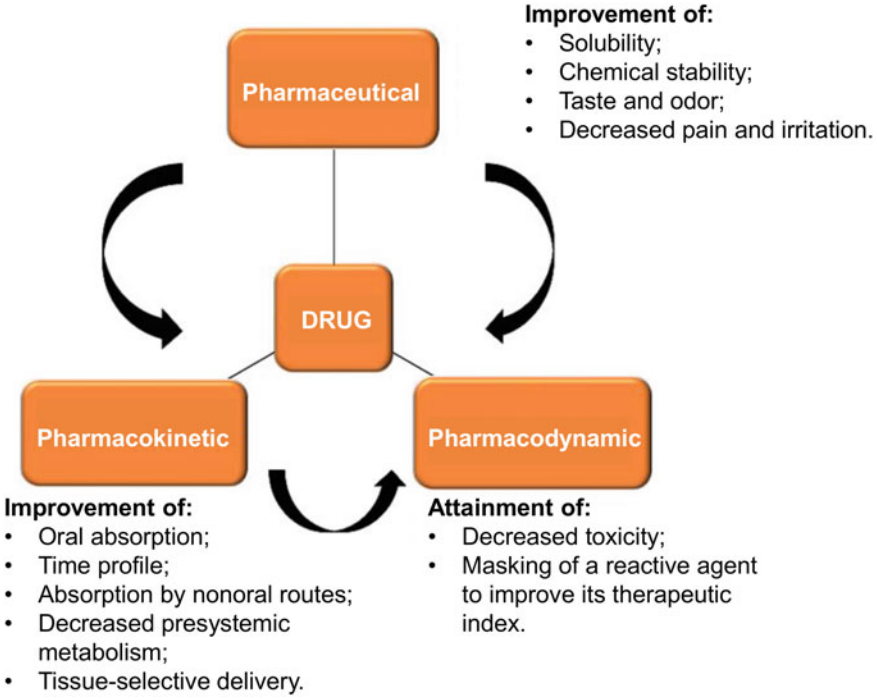

Fig. 5.4 Objectives of the prodrug approach

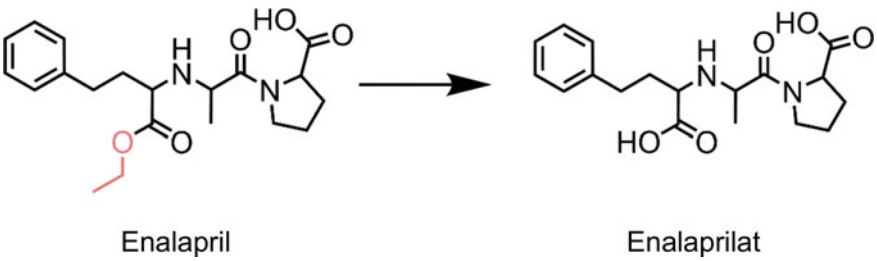

Fig. 5.5 Classic prodrugs. Example of angiotensin converting enzyme (ACE) inhibitor prodrug and the respective drug. The carrier is highlighted in red

- *Targeted drugs*: are characterized by having a specific carrier linked to the drug or bioactive molecule, normally presenting a directing group to interact with specific **receptors** or enzymes in the site of action, avoiding interaction with other tissues, thus increasing the molecule efficacy, the selectivity of action, and reducing the adverse effects of the drug (Fig. 5.9).

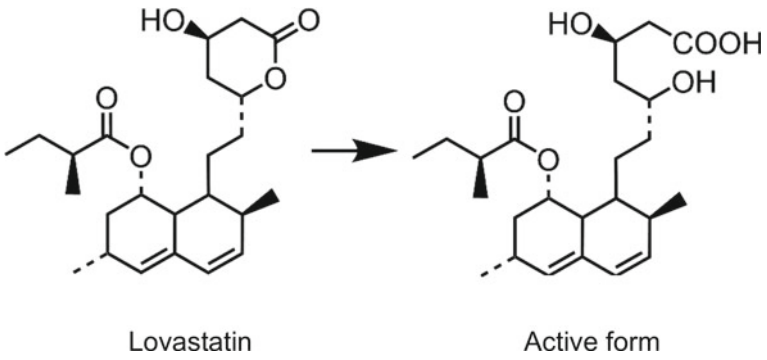

Fig. 5.6 Blockbuster lovastatin, a hypocholesterolemic drug

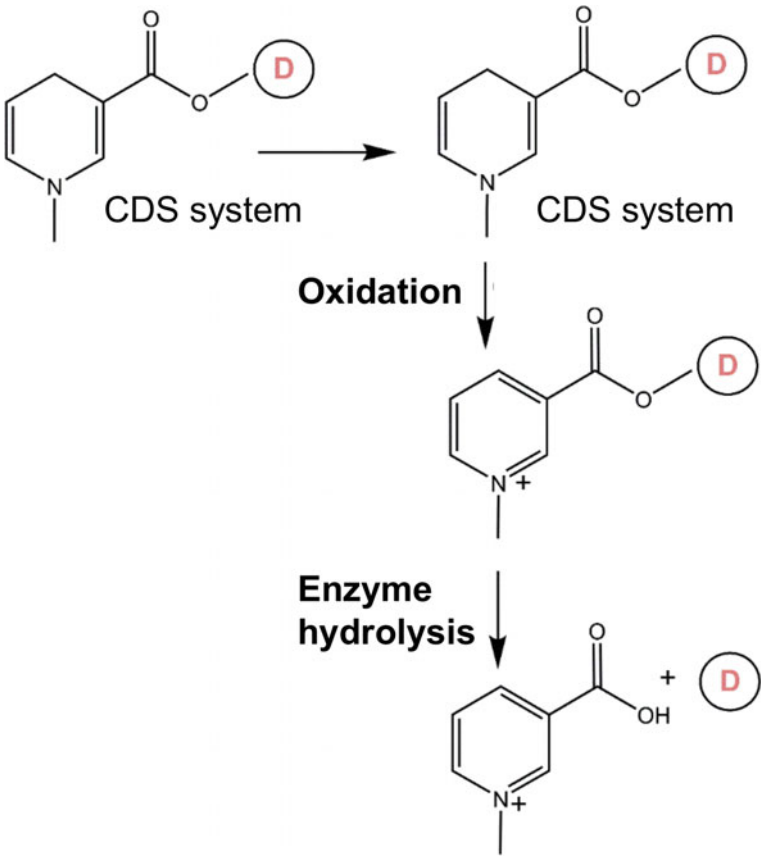

Fig. 5.7 The working principle of a chemical delivery system. Abbreviations: BBB, blood brain barrier; D, drug

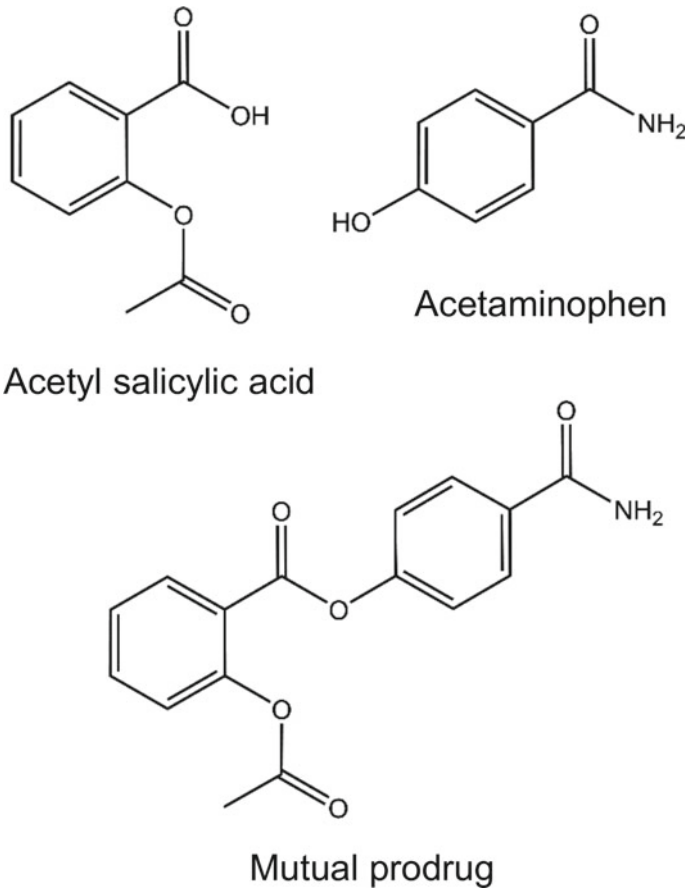

Fig. 5.8 Mutual prodrug of acetyl salicylic acid and acetaminophen

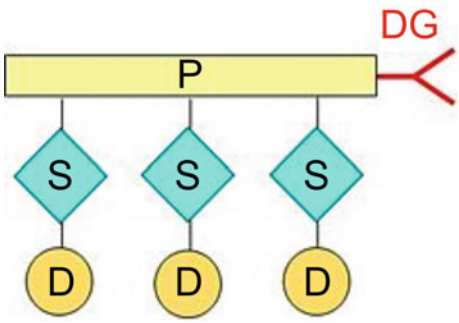

Fig. 5.9 A general type of targeted drug. Abbreviations: P, polymer; S, spacer group; D, drug; DG, directing group

Besides this classification, a simple one, based on the presence or absence of carrier moiety, is found in the literature (Abet et al. 2016). By that, prodrug design comprises carrier prodrugs and bioprecursors prodrugs. Some functional groups are amenable to prodrug design such as carboxylic, hydroxyl, amine, phosphate/phosphonate, and carbonyl groups, and the most commons forms produced via modification of these groups include esters, carbonates, carbamates, amides, phosphates, oximes, among others. Table 5.1 presents this information.

Alkoxy- and acyloxyalkyl derivatives, for example, both provide the way to have a prodrug in cascade, which means, an enzymatic hydrolysis in the beginning followed by a chemical rearrangement. This leads to a more controlled release of the drug from the carrier (Choi-Sledeski and Wermuth 2015). Independently on the problem to be solved, the carrier must be devoid of toxicity. They can be chosen either among simple structures or more complex ones, like polymers, dendrimers included (Mariyam et al. 2018; Santos et al. 2016), with or without targeting groups, which lead to specific (Huttunen and Rautio 2011). Simple carriers may be used for several situations, mainly for pharmaceutics and pharmacokinetics purpose. On the other hand, polymers/dendrimers are used either to obtain prolonged action or to have specific delivery, which means pharmacokinetics and pharmacological objective, this latter, indirectly.

The type of linkage is also depicted in Table 5.1. Ester bond is the most common, as it is, in general, easily hydrolyzed by esterases, as well as amides and thioesters (Fukami and Yokoi 2012). However, carbamate and amide can be used to achieve prolonged action, as they are less labile to enzyme reactions. Both alkoxy- and acyloxayalkyl bonds, mentioned before, need at least two drug-releasing steps, thus allowing longer action. On the other hand, some linkages, as Mannich bases, just require chemical delivery at physiological pH (Bundgaard 1985).

It is worth noting that choosing the bond between the drug and the carrier, except for bioprecursors, implies the knowledge about the most suitable enzyme for releasing the bioactive part of the prodrug (Choi-Sledeski and Wermuth 2015). In this sense, bioprecursors require a more complex study about the biological/physiological system. Considering the selectivity of action, the choice depends on which enzyme predominates in the specific organ/tissue and this is extensively applied for chemotherapeutic agents, mainly for tumors. Rautio and colleagues (Rautio et al. 2018) present a Table with the enzymes and their predomination in the organs/tissues. For instance, carboxylesterase 1 predominates in liver, lung, and gallbladder, albeit carboxylesterase 2 is found mostly in the intestine, liver, and kidney. Prodrugs designed for being activated by those enzymes are oseltamivir and irinotecan, respectively (Fig. 5.10). Recent examples of prodrugs, at least from the last decade, are given as follows. They are related to some of the main problems to be solved with prodrug design.

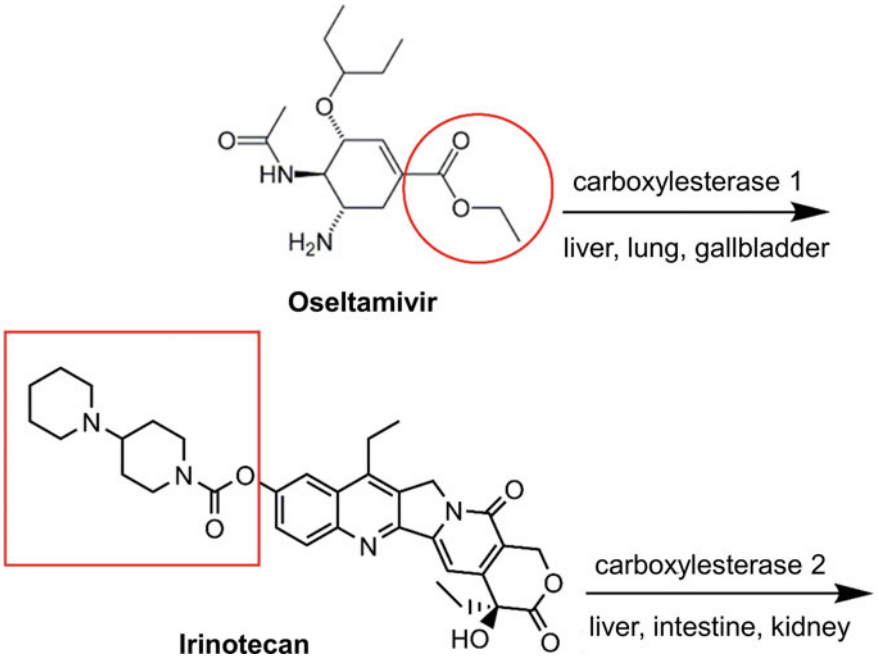

Fig. 5.10 Oseltamivir and irinotecan, activated by carboxylesterases 1 and 2, respectively

5.3 Designing Prodrugs for Increasing Oral Bioavailability

Achieving the most suitable bioavailability is one of the main objectives of designing prodrugs. US Food and Drug Administration (FDA) definition of bioavailability corresponds to "the rate and extent to which the active drug, ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action" (Allam et al. 2011). It has received high interest not only in the drug development but also in the drug discovery. Most of the candidates failed in the clinical phase because of the undesirable or insufficient bioavailability.

Many factors could interfere with drug bioavailability (Allam et al. 2011). For oral administration, the pharmaceutical phase, which means drug dissolution and drug disintegration, is important as it depends on drug/bioactive compound solubility, a factor that affects the bioavailability of more than 40% of the drugs that are already in the therapeutics. Hence, water solubility is an important factor to deal with when designing a drug and the prodrug approach can be a useful approach to correct this unwanted property (Sanches and Ferreira 2019).

Pharmacokinetics phase for oral dosages comes further and comprehends absorption, biotransformation (metabolism), distribution, and excretion. After reaching the gastrointestinal tract, the drug must be absorbed from the gut and, in some cases, depending on the glycoprotein P efflux mechanism, goes back to the intestinal lumen.

This, in turn, depends on the physico chemical properties of the drug (Jana et al. 2010; Jornada et al. 2016; Gandhi et al. 2019). Intestinal enzymes, as well the hepatic portal system, can biotransform the drug before entering in the systemic circulation. This means, the drug can be excreted prior to exert its function and it is called first-pass metabolism effect or pre-systemic elimination (Allam et al. 2011). It is worth noting that biotransformation is not only a way of transforming the drug into an inactive metabolite but also to turn it into its active form, as occurs in prodrugs (Bundgaard 1985).

Generally, the problem with passive drug absorption is related to lipophilicity (Jana et al. 2010). Drug partition coefficient, represented in its log form, LogP, can be inadequate, lower or higher, than that needed for membrane permeation. A prodrug can increase the drug lipophilicity using a lipophilic carrier (Gandhi et al. 2019). The classical example of this approach is ampicillin prodrugs (Fig. 5.11). Ampicillin is broad-spectrum penicillin with high polarity, which interferes with its absorption and hence with its bioavailability. Carriers providing an acyloxyalkyl group, as pivaloic acid and acetic acid, lead to double esters, which can release the antibiotic by two steps, one enzymatic and the other chemical. The bioavailability of

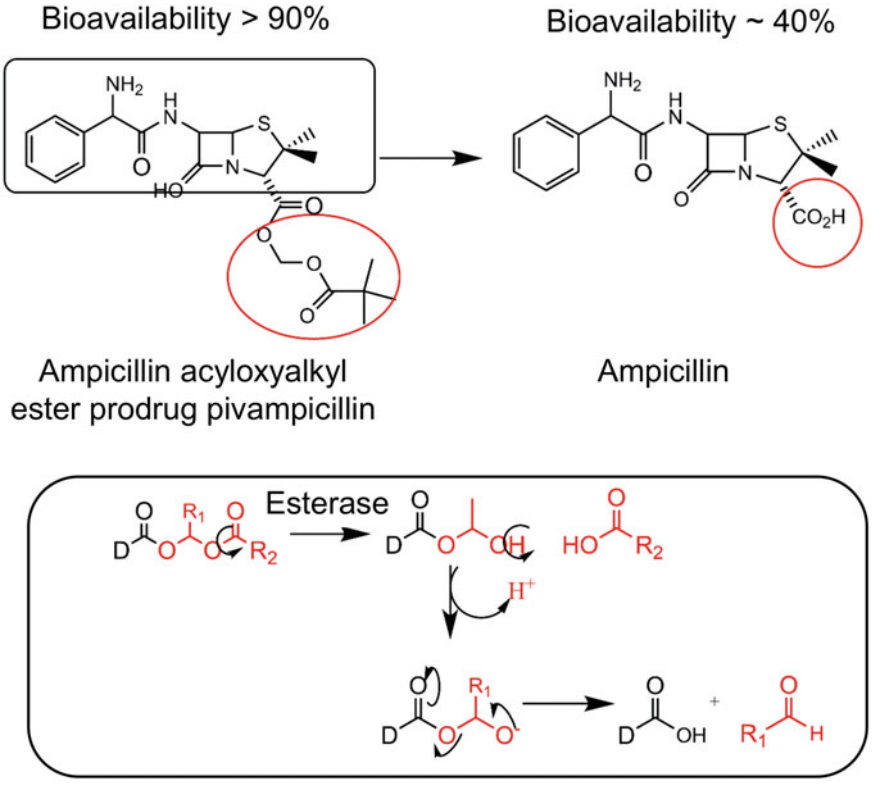

Fig. 5.11 Ampicillin prodrugs with higher bioavailability

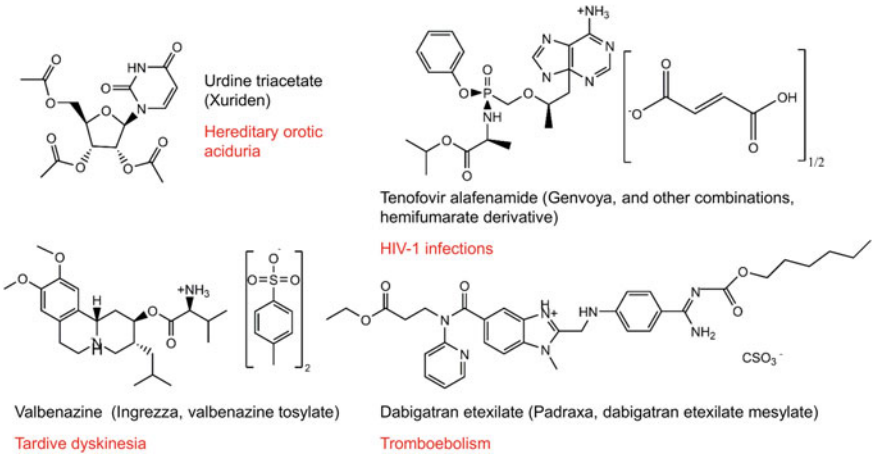

Fig. 5.12 Prodrugs with improved bioavailability approved by FDA in the 2008–2017 decade

ampicillin is around 40%, while its prodrugs have about 98% of bioavailability. After absorption, the prodrugs release ampicillin in 15 min, exerting its antibiotic activity. The mechanism of drug release is shown in Fig. 5.11. Among the 30 FDA prodrugs approved from 2008 to 2017 (Rautio et al. 2018), at least 30% were designed to achieve higher bioavailability through a lipophilicity increase, which improves their absorption. Some of these structures are depicted in Fig. 5.12. For some of them, other effects besides an increase in the absorption and bioavailability were also obtained.

Other approaches to increase the absorption of drugs can be the use of peptidomimetic carriers, through the PepT1, intestinal peptide transporter 1, discovery in 1994, to enhance membrane permeability (Wang et al. 2017; Vig et al. 2013). This transporter is responsible for the absorption of around 400 small different types of dipeptides and 8000 tripeptides from the dietary proteins. Figure 5.13 shows examples of valacyclovir and valagancyclovir prodrugs, which can be converted by a valacyclovirase (Xu et al. 2008) into the respective drugs, before their phosphorylation to the virus and cellular kinases (Vig et al. 2013).

When low bioavailability is due to high hepatic first-pass metabolism, it is important to identify the groups prone to suffer this biotransformation. The carriers should protect these sites and, not rarely, they are very simple ones. Many anti-hypertensives undergo the first-pass metabolism as calcium-channel blocker (Dhaneshwar et al. 2011), for example (Fig. 5.14). Although they show to be well absorbed, the high level of this biotransformation leads to the real bioavailability, which varies depending on the extent of the metabolism. Many beta-blockers, in general, present a high level of first-pass metabolism because of the catechol ring. To solve this problem, normally esters are designed to obtain prodrugs with better bioavailability and also higher half-life time.

Lipid prodrugs are currently arousing interest with the objective of improving oral delivery by interfering with many steps (Markovic et al. 2019). The perspectives point

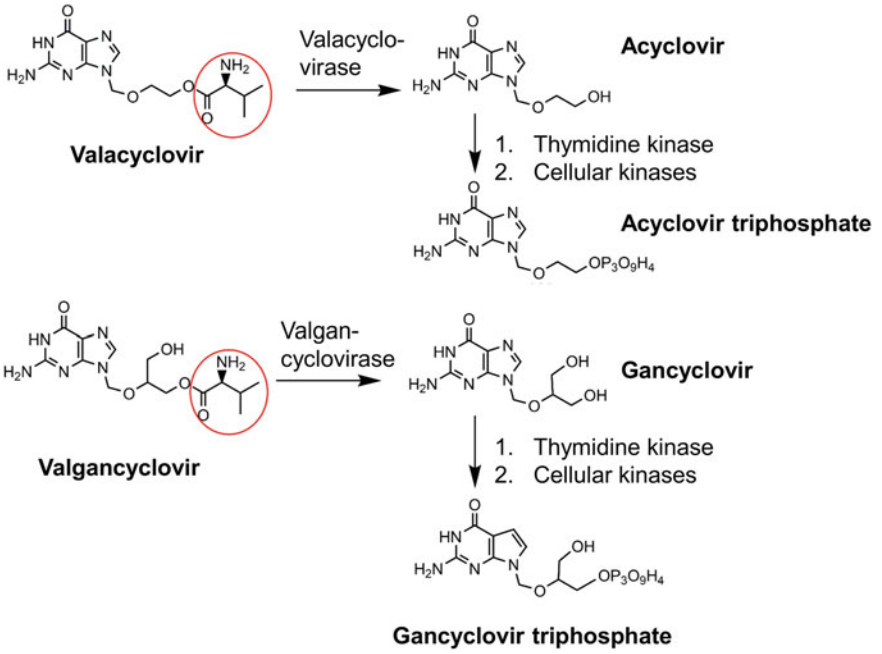

Fig. 5.13 Mechanism of activation of valacyclovir and valganciclovir

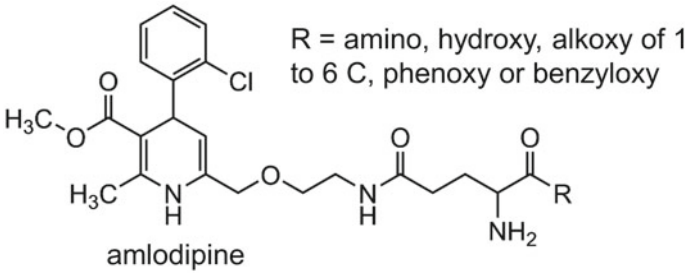

Fig. 5.14 Prodrugs of amlodipine, a calcium channel blocker

out for significant growth in the use of this approach. Lipid prodrugs can be built using fatty acids, glycerides, phospholipids, and cholesterol as carriers. As for example, linking a drug to glyceride and fatty acids, as carriers, leads to "trygliceride." In the gastrointestinal lumen, the prodrug is hydrolyzed to a "monoglyceride" prodrug. This compound, after entering the enterocyte, is re-synthesized to a "trygliceride" prodrug, which in turn is packed as a lipoprotein and transported, preferentially, to the lymph. This process avoid the first-pass metabolism and the compound access the lymphocytes (Markovic et al. 2019).

5.4 Prolonged Duration of Action

The half-life time of a drug is a pharmacokinetics parameter and represents the time the plasma concentration or the total amount of the drug in the body is reduced to 50% (Allam et al. 2011). Some drugs with short half-life time need more doses a day to achieve the effect and this can be the reason for lack of patient adherence. Although sustained plasma drug level can be accomplished by formulations using polymers, prodrugs have also been used to attain this objective (Chung et al. 2005; Silva et al. 2005)

Prolonged duration of action can be achieved by many ways through prodrug approach, depending on the factor restricting the length of drug activity (Rautio et al. 2018). The use of a proper carrier can modify drug/bioactive compound aqueous solubility and dissolution, interfering on the rate of drug release, oral absorption and distribution in the tissues. This can be reached through subcutaneous and intramuscular prodrugs in which the carrier moiety is a fatty acid, which is linked to the drug by an ester bond. Generally, these forms use an oil-based vehicle, leading to deposit formulations, allow the drug to be slowly released. In addition, controlled bioconversion might lead to sustained release for drugs in which the half-life time is short. In that case, the bonds between the drug and the carrier must be more stable, as amides and carbamates, for instance. Figure 5.15 presents a prodrug of sustained release and prolonged duration of action, to be used in intramuscular administration (Rautio et al. 2018). It was approved in October 2015 for schizophrenia.

Polymers can be used to prolong drug action, although they are also used to get specific delivery (Ali et al. 2019). The perspectives for the next decades are that will have their contribution increasing in the context of practical medicine (Duncan and Vicent 2013). Dendrimers (Mariyam et al. 2018; Santos et al. 2016; Santos et al. 2017; de Araujo et al. 2018; Dias et al. 2020) and other polymeric matrices, including polysaccharides, which are normally complex structures, convey to slow release of drugs, besides targeting to specific organs. Prodrugs from polymers are also called polymer–drug conjugates or polymeric prodrugs (Duncan and Vicent 2013). Polymeric matrices have aroused interest in the last 20 years. Ringsdorf, in

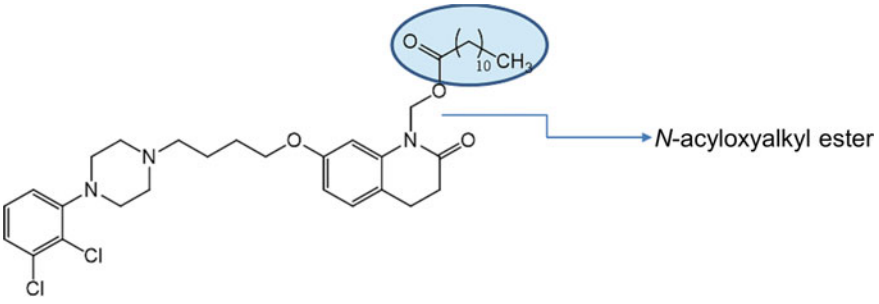

Fig. 5.15 Arilpiprazole lauroxil (Aristada)

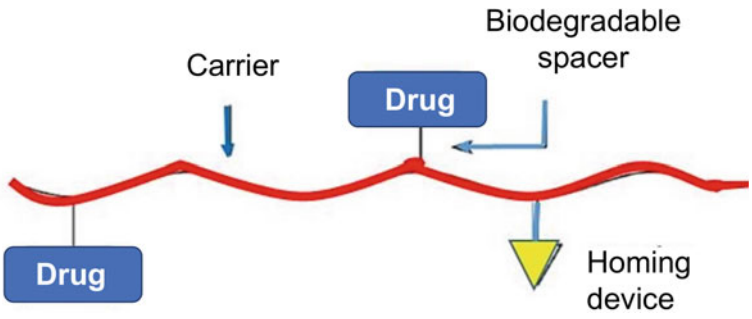

Fig. 5.16 Ringsdorf's model of a polymer prodrug. Homing device is a directing group. Reproduced from (Hoste et al. 2004) with permission from Elsevier B.V.

1975, created a rational model for designing polymeric prodrugs (Fig. 5.16). This model is versatile, constituted of a polymer backbone, a drug, a solubilizing agent, and a spacer group, with the possibility of linking also a directing group, or homing device, if the objective is to make up a targeting drug (Hoste et al. 2004).

The drugs are linked to the polymer through a covalent bond. Functional groups either of the drug or the polymer carrier must allow the labile bond to be formed. If the polymer does not present a suitable group to have this link formed, a derivatization is needed by means of a proper spacer group. Besides being a way to conjugate the drug in the polymer, spacer groups make it easier to hydrolyze the bond. It is worth noting that a combination of drugs can also be used to get synergism of action, especially in the case of chemotherapeutic agents. Besides this requirement, polymers need to present some other important properties to be considered as a carrier for a polymeric prodrug (Hoste et al. 2004): 1. Biocompatibility, which means lack of toxicity and immunogenicity; 2. Biodegradability or a molecular weight below the renal excretion limit, as this contributes to prolong the action, and availability.

Depending on their nature, the polymers useful for conjugating drugs can be synthetic, like polyethylene glycol (PEG) (Pasut and Veronese 2012), divinylether-maleic anhydride/acid copolymer, polyethyleneimine, vinyl polymers; natural, like dextran, chitosan, which are polysaccharides and proteins; and semisynthetic, as synthetic poly(amino acids): poly(L-glutamic, poly-L-lysine), poly(*N*-hydroxyalkyl)glutamines, and polyglycolic or polyglycolide acid. Gandhi et al. (2019) presented many examples of polymeric prodrugs available in the market. A polymer prodrug of adriamycin can be viewed in Fig. 5.17. An interesting example of polymer used to get sustained release of a drug is TransCon hGH (Höybye et al. 2017). This is a prodrug of human growth hormone, used in children with hGH deficiency (NCT02781727). It comprehends a methoxypolyethylene glycol carrier and a linker, named Transcon, to bind hGH. The hormone releasing from the linker is one-week long and it is carried out by temperature and physiological pH, through the auto-hydrolysis of the linker. As a prodrug, only after its release the hormone interacts with the receptor. This prodrug was in clinical Phase 3, under subcutaneous injection administration. Especially in the case of polymeric/dendrimer prodrugs

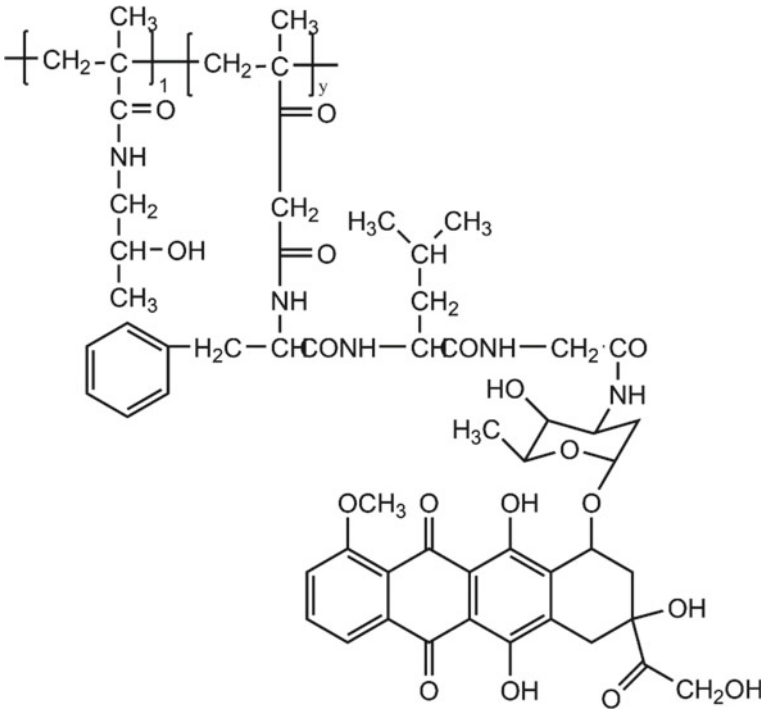

Fig. 5.17 Polymer prodrug of adriamycin. Reproduced from (Hoste et al. 2004) with permission from Elsevier B.V.

with prolonged action, a reduction of toxicity is also accomplished. The gradual drug release avoids the effect of "peak and valley" observed for many drugs with short-time action.

5.5 Decreasing Toxicity of Drugs/Bioactive Compounds

Reduction of systemic toxicity can be achieved by several manners depending on the drug molecule and the site of action, though, sometimes can be accomplished just by modulating one or more of the **ADME** properties, such as solubility or duration of action. However, in most of the cases, there are two main approaches to reach the goal, the masking of a reactive agent to improve its therapeutic index, and the in situ activation of a cytotoxic agent (Chung et al. 2005; Silva et al. 2005).

The non-steroidal anti-inflammatory drugs (NSAIDs), are among the most widely used and prescribed medications to treat anti-inflammatory diseases in a short- and long-term therapeutic uses. For long-term use, mainly, the NSAIDs showed several

serious adverse effects, the most common is related to gastrointestinal (GI) toxicity generating, for example, gastritis, ulcerogenicity, and mucosal hemorrhage. The mechanism of action of NSAIDs consists in the inhibition of cyclooxygenases (isoforms COX-1 and COX-2) which converts arachidonic acid into prostaglandins present in inflammatory conditions. However, this inhibition is not selective to COX-2, which is the one responsible for the response of the inflammatory stimuli. The side effects of non-selective NSAIDs are generally believed to be caused by two different mechanisms: undesirable inhibition of COX-1, which synthesizes physiological prostaglandin in the stomach, responsible for gastroprotection from the secreted acid, and local irritation in the gastric mucosa due to the acidic group present in NSAIDs (Sehajpal et al. 2018; Halen et al. 2009; Bandgar et al. 2011).

Taking this information into account, Bandgar and collaborators (Bandgar et al. 2011), synthesized orally active prodrugs of indomethacin, a well-known NSAID, aiming molecules with high enzymatic bioconversion rate, desirable physicochemical properties, and decreased GI toxicity properties. Four ester prodrugs of indomethacin were developed, as shown in Fig. 5.18, following the principle of masking the carboxylic acid group. Besides the evaluation of ulcerogenicity (GI toxicity), all prodrugs were also evaluated by partition coefficient (log P), aqueous solubility, aqueous stability, in vitro metabolic stability in rat liver microsomes and rat plasma. With the exception of the prodrug 1b, all the other molecules showed lower ulcer index (UI) and a safer profile in comparison with the parent compound indomethacin. However, due to the metabolic stability in the in vivo evaluation, prodrugs a and c were selected, as they presented rapidly enzymatic transformation to the parent drug in both models, rat liver chromosomes and rat plasma (Bandgar et al. 2011).

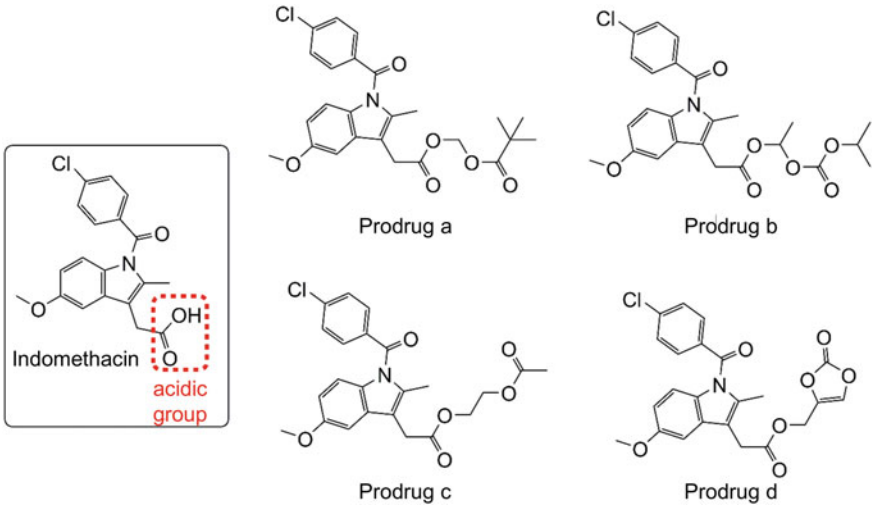

Fig. 5.18 Chemical structures of indomethacin and prodrugs **a–d**

Following the same principle as the previous example, nine ester prodrugs of ketoprofen were designed to obtain derivatives with increased bioavailability, analgesic, and anti-inflammatory properties with lesser ulcerogenicity than the parent drug. Among the nine prodrugs synthesized, all of them presented evident anti-inflammatory activity ranging from 91.8 to 113.3% in comparison with ketoprofen. The ulcerogenic index of the ester prodrugs was significantly lower than the one exhibited by the parent drug. Some of the prodrugs exhibited no ulcerogenicity at all, as shown in Table 5.2. The results demonstrated that the ethyl, isopropyl, *sec*-butyl, and propyl esterification of ketoprofen are a viable strategy to reduce GI toxicity and give origin to safer medications (Ahmed et al. 2016). In both examples cited above, the reduction of toxicity obtained by the prodrug approach is attributable to avoid the direct contact of the carboxyl group with the gastric mucosa that leads to gastric damage. The therapeutic class of antineoplastic drugs must be mentioned when it comes about toxicity, once many conventional chemotherapeutic agents cause severe side effects due to undesirable ADME properties and, mainly, lack of selectivity.

To reduce severe side effects and overcome poor aqueous solubility of the potent anticancer drug paclitaxel (PTX), Ayalew and collaborators (Ayalew et al. 2017) designed a conjugation of PTX to a hybrid collagen–cell-penetrating peptide carrier (COL–CPP) (Fig. 5.19). Due to the hydrophobic character of PTX, the best synthetic modification was attaching hydrophilic moieties to it and peptides are becoming a popular choice when the issue is enhancement of water solubility. The benefits in the use of peptides as carriers is due to their biocompatibility, producing safe byproducts to the organism, the small molecular weight, facilitating cell membrane penetration, and the possibility to select the best modular design according to the desired property

Table 5.2 Acute anti-inflammatory and ulcerogenic effects of the ester derivatives (1–9) in comparison to the parent drug ketoprofen. The data in the table are retrieved from (Ahmed et al. 2016)

Compound	Comparative anti-inflammatory activity (%)	Ulcerogenic activity (Severity index ± SEM)[a]	Comparative ulcerogenic potential (%)
1	95	2.4 ± 0.11	30
2	96.1	0.5 ± 0.20	6.3
3	91.8	0.5 ± 0.2	6.3
4	96.7	0.0 ± 0.0	0
5	93.6	0.5 ± 0.13	6.3
6	95.7	0.0 ± 0.0	0
7	92.1	1.2 ± 0.3	15
8	92.9	0.5 ± 0.2	6.3
9	113.3	7.5 ± 1.5	93.8
Ketoprofen	100	8.0 ± 1.5	100

[a]Tested in gastric mucosa of rats after 5 h of injection
Data are expressed as mean ± SEM (n = 5)

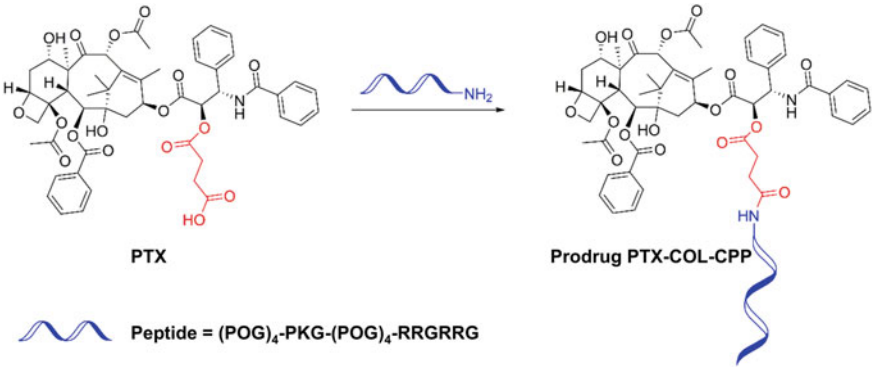

Fig. 5.19 Fabrication of the paclitaxel conjugated prodrug PTX-COL-CPP

improvement. To give origin to the prodrug of PTX, the C2'-OH position is esterified with a succinate linker, and then, via an amide bond, reacted with helical peptide, which forms a nanoparticle that carries three PTX molecules. The prodrug PTX-COL-CPP presented a great improvement in solubility and exhibited higher IC_{50} values in vitro studies on Jurkat (human T lymphocyte of acute T cell leukemia), compared to the free parent drug (Ayalew et al. 2017).

Amino acid promoieties were chosen by Lee and collaborators (Lee et al. 2014), to decrease severe toxicity of the novel and promising anticancer (*S*)-1-[1-(4-aminobenzoyl)-2,3-dihydro-1*H*-indol-6-sulfonyl]-4-phenyl-imidazolidin-2-one (DW2282). Despite its great therapeutic potential, in vivo preclinical tests on beagle dogs revealed significant GI toxicity, probably due to poor aqueous solubility. The basic aromatic amine attached to the indoline moiety of the parent compound was the site chosen to be linked to amino acids (Fig. 5.20). All synthesized amino acids prodrugs went through aqueous solubility, pharmacokinetic profile, reconversion rate in human plasma, cytotoxicity against SW620 human colon cancer, and in vitro and in vivo anti-tumor activity tests.

Compounds e and g presented good results in all performed tests, including good reconversion rate in human plasma. Especially attention was given on the anti-tumor activity test that showed remarkable tumor growth inhibition against SW620 xenograft tumor model in mice at single-dose experiment (Dose: 70 mg/kg, b.i.d., 5 days, p.o.) and decreased toxicity due to low body weight changes. Therefore, compounds e and g were selected as promising prodrugs candidates (Lee et al. 2014).

Modifying pharmaceutical and/or pharmacokinetic barriers may be a successful strategy to decrease toxicity, but currently, the prodrug approach is being focused on site-selective delivery, which has become the most promising strategy to develop safer medications. Methotrexate (MTX) was first developed as a folic acid antagonist to high-dose antitumor therapy, but currently is the standard treatment for rheumatoid arthritis (RA) due to the potent anti-inflammatory effects at low doses. However, MTX presents low patience compliance due to high variability in efficacy and, several adverse effects such as GI toxicity, hepatotoxicity, renal insufficiency,

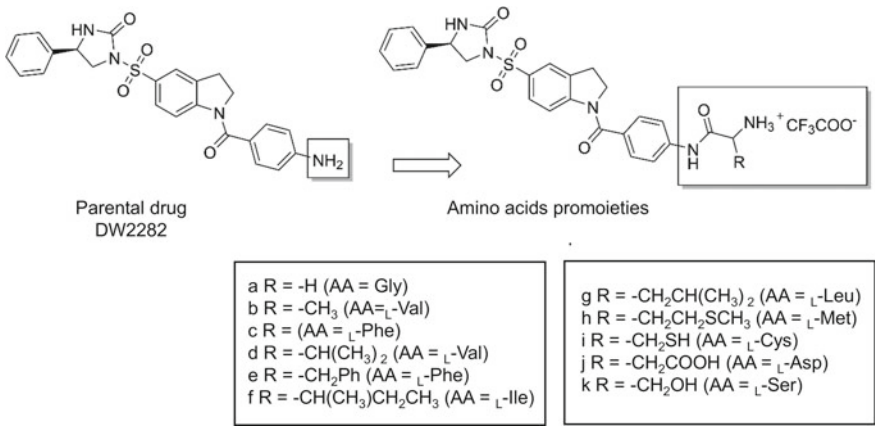

Fig. 5.20 Structure of parental drug DW2282 and designed amino acid prodrugs (A–K). Reproduced from (Lee et al. 2014) with permission from Elsevier B.V.

anemia, and neutropenia. Aiming improvement of safety profile and potentiation of the efficacy of MTX, MTX-prodrugs were proposed utilizing thiazolidinone (TZ), an H_2O_2-sensitive promoiety, once reactive oxygen species (ROS) are present in high levels in inflammatory processes, obtaining a site-selective delivery of the drug in inflammatory tissues. Three novel H_2O_2 sensitive MTX-prodrugs were synthesized (Fig. 5.21) and two of them showed promising properties such as high chemical and metabolic stability, excellent aqueous solubility, and proper activation when submitted to patho physiological concentrations of H_2O_2. The MTX-ϒ-TZ prodrug exhibited even better results in vivo, comparable efficacy to MTX and reduced toxicity, in a murine collagen type II-induced arthritis (CIA) model, compared to the parent drug (Andersen et al. 2018).

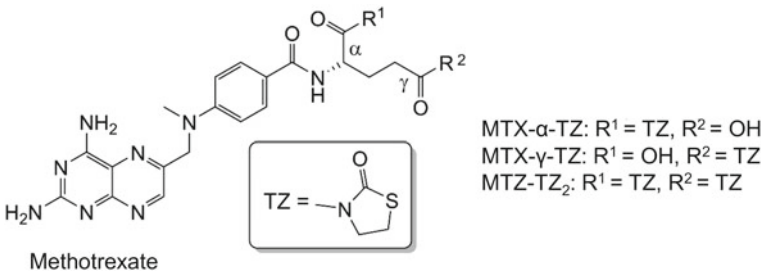

Fig. 5.21 Chemical structure of the three targeted thiazolidinone (TZ) based MTX prodrug candidates. Reproduced from (Andersen et al. 2018) with permission from Elsevier B.V.

5.6 Targeting Drugs to Specific Tissues/Organs

Targeted-prodrug design has become widely employed on drug discovery and development, aiming high efficacy with minimal adverse effects by site-selective delivery of drugs. Targeting can be achieved by two manners: site-specified drug delivery and site-specific drug bioactivation. In this context, the active drug is attached to a carrier-specific to certain receptors or enzymes present in the site of action (Fig. 5.22). So, the drug is inactive during transport and is activated only when released in the specific target organ without affecting healthy cells, thus reducing severe side effect (Abet et al. 2016; Lesniewska-Kowiel and Muszalska 2017; Mahato et al. 2011). The designing of a successfully targeted prodrug must fulfill some important requirements (Mahato et al. 2011):

1. Precisely transport to the site of action;
2. Selective drug releasing in enough quantity;
3. Therapeutic effect production in the desired target. Besides that, site-selective drug delivery can be achieved by targeting:

    a. Tissue or cell-specific enzymes;
    b. Tissue or cell-specific transporters;
    c. Tissue or cell-specific enzyme antibodies (for instance, in the case of antibody-directed enzyme prodrug therapy, ADEPT) or enzyme encoding genes (for instance, in the case of **gene-directed enzyme prodrug therapy**, GDEPT).

Tumor-targeted prodrugs are, probably, the main class developed by this approach, although, currently, colon, liver, kidney, and brain-targeted prodrugs are also gaining ground in site-selective drug delivery (Mishra et al. 2018). Most cancer cells often overexpress certain enzymes, proteins or membrane transporters, turning them possible to specific drug targeting. In this context, the enzyme β-glucuronidase was found to be present in a wide variety of cancer types, such as breast, lung, ovarian, and gastrointestinal tract carcinomas as well as melanomas. This enzyme is secreted in the extracellular environment by inflammatory cells (monocytes/granulocytes) in necrotic areas, while in normal cells its activity is within lysosomes. Taking this into account, a self-immolative dimeric glucuronide prodrug of doxorubicin was designed

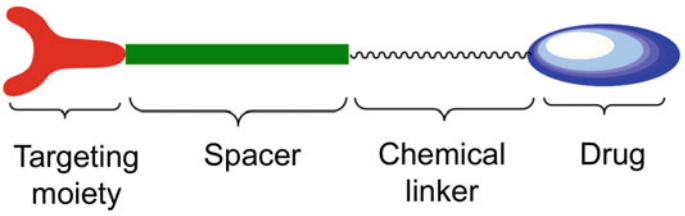

Fig. 5.22 General design of a targeted prodrug

and tested for its antiproliferative activity against H661 lung cancer cell line. The prodrug structure is constituted by a glucuronide trigger, a linker, and two molecules of the active drug articulated around a chemical amplifier. Through enzymatic hydrolysis of the glycosidic bond, the phenol intermediate will induce the release of the aniline via 1,6 elimination, then the amplifier unit conduct the release of the two doxorubicin via 1,4- and 1,6- elimination, as shown in Fig. 5.23. The cytotoxicity of the glucuronide prodrug against H661 lung cancer cell line was, approximately, two-fold higher compared to the parent drug (Tranoy-Opalinski et al. 2014).

The low permeability in the blood–brain barrier (BBB) protects the brain from free access to many unspecific drugs and other xenobiotics. On the other hand, effective drug therapies the central nervous system (CNS) disorders and diseases are a major challenge to develop. For this very reason, targeted prodrugs to the CNS are an

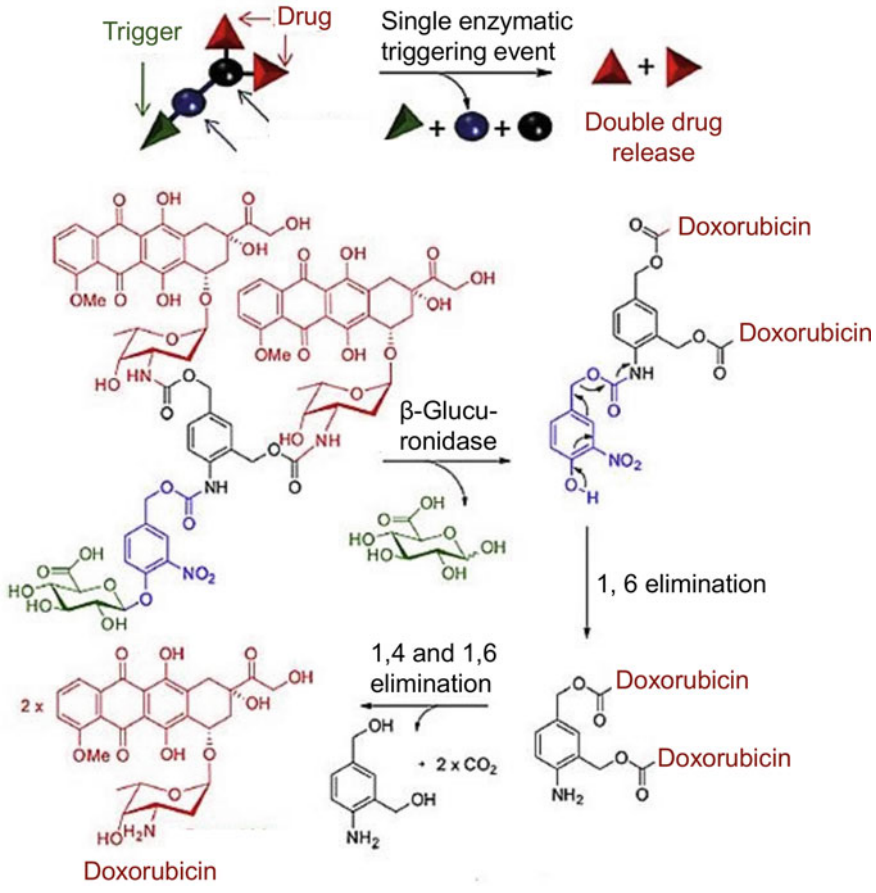

Fig. 5.23 Chemical structure of the dimeric glucuronide prodrug. Reproduced from (Tranoy-Opalinski et al. 2014) with permission from Elsevier B.V.

amenable strategy to overcome this obstacle and delivery bioactive compounds to the target site at effective concentrations (Melisi et al. 2011).

For that purpose, brain-targeted prodrugs were developed through the esterification of the parent compound with D-galactose. D-galactose and other hexoses can be transported across the BBB by the carrier GLUT and its isoforms, which are present on the membrane of endothelial cells. Several drugs that have been conjugated with D-galactose such as, 7-chlorokynurenic acid, nipecotic acid, and dopamine, exhibited increased activity in the CNS (Lee et al. 2014).

In this context, an N^{ω}-nitro-L-arginine prodrug, a non-selective nitric oxide synthase (NOS) inhibitor, was synthesized with D-galactose (Fig. 5.24), which was named as NAGAL, to target GLUT-3 transporter which is overexpressed in spinal cord microglia and astrocytes. The prodrug increased the anti-nocifensive efficacy of the parent drug in the spared nerve injury (SNI) pain model, at lower doses, reaching the main goal of a specific carrier-mediated transport. Besides that, NAGAL can be used to treat neuropathic pain with higher potency of the parent drug, thus low toxicity profile due to the ability of action in the specific target site, lowering the adverse effects commonly associated with long periods of neuropathic treatments (Melisi et al. 2011).

Meinig and collaborators (Meinig et al. 2017), also opted for the targeted-prodrug approach to increase the brain delivery of the parent compound sobetirome by targeting fatty acid amide hydrolase (FAAH), which is expressed in the brain. Sobetirome is in clinical trials, developed for the treatment of hyperlypidemia, but became

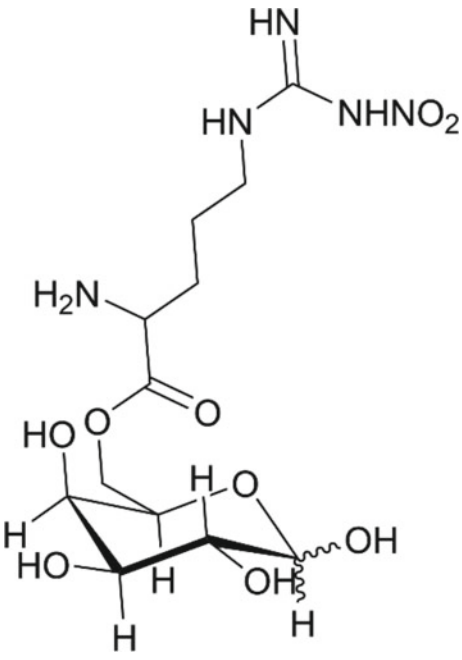

Fig. 5.24 Chemical structure of the prodrug NAGAL

an attractive alternative to thyroid hormone due to its structural analogy. The thyroid hormone agonists could be used for the treatment of CNS disorders, for example, the genetic diseases MCT-8-deficiency and X-linked adrenoleukodystrophy (X-ALD), as well as multiple sclerosis (MS), once it stimulates myelin development and repair. However, thyroid hormone lacks therapeutic index (TI) between the beneficial and the thyrotoxic effects on several tissues such as heart, bone, and skeletal muscle. Taking this information into account, two amide prodrugs of sobetirome (Fig. 5.25) were synthesized and showed to be efficient substrates of FAAH. In the in vivo studies, the sobetirome amide prodrug Sob-AM2, exhibited a 60-fold increase in brain distribution leading to a 30-fold increased potency, compared to the parent drug (Meinig et al. 2017).

Colon-targeted prodrugs to treat ulcerative colitis (UC) were researched by Markovic and group (Markovic et al. 2019) employing the enzyme phospholipase A_2 (PLA_2) as a prodrug-activating enzyme, once it is overexpressed in the inflamed colonic tissues. This novel colon-targeted approach, using phospholipase (PL) prodrugs was first studied with PL-fluorenylmethyloxycarbonyl (Fmoc) conjugates in vitro, to understand the activation and releasing process, and then, link to an active drug. Previous reports of the group established that the linker length between the PL and Fmoc moieties, plays an important role in the activation of the

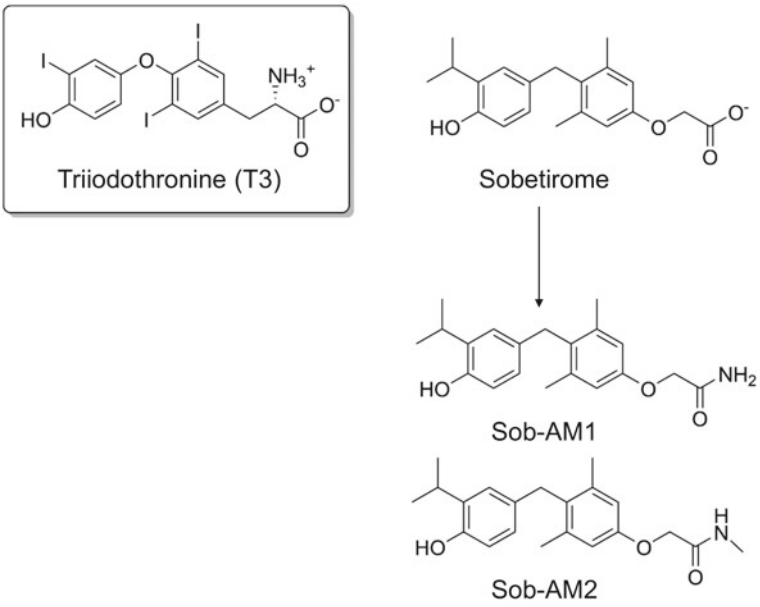

Fig. 5.25 Chemical structures of thyroid hormone (T3), sobetirome, and the FAAH-targeted sobetirome amide prodrugs

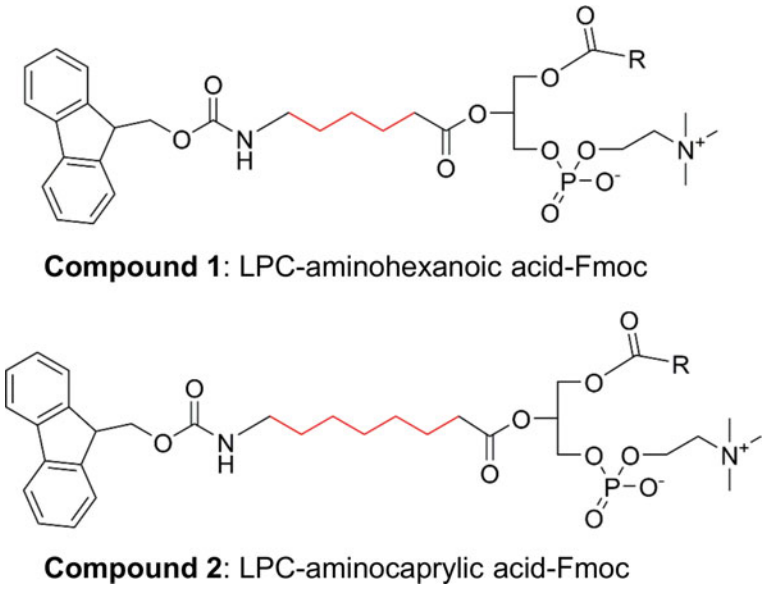

Fig. 5.26 Structures of the two LPC-Fmoc conjugates with different linker lengths, 5–CH_2 units (compound 1) and 7–CH_2 (compound 2) units marked in red. Reproduced from (Markovic et al. 2019) with permission from MDPI

PLA_2-prodrug, thus determining the quantity of drug at the target site. So, two PL-conjugates with Fmoc moieties were synthesized, with carbamate linkers of different lengths (Fig. 5.26).

The PL-Fmoc conjugate with a 7-carbon linker demonstrated a higher activation profile than the conjugate with a 5-carbon linker. After analyses of several tests, the group hypothesized that the free drug would be released in a significant amount in the compromised tissues exclusively, due to PLA_2 overexpression in the inflamed colonic tissue. Besides that, it was observed that the conjugate exhibits very low bioavailability and does not penetrate the intestine leading to an efficient release of the drug only in the colonic inflamed tissues. Finally, the design of the PL-Fmoc conjugates may provide a novel colonic drug-targeting approach in UC therapy, and can also be employed in other conditions with PLA_2 overexpression, such as colon cancer (Markovic et al. 2019).

5.7 Summary and Outlooks

Prodrug design is an approach of molecular modification introduced in the 1950s. Despite this, it has gained more interest in this century, not only in the academy but also in the pharmaceutical industry. Considering the possibility of overcoming

many important problems faced either by leads or by drugs, prodrug design has been chosen by improving different properties of bioactive compounds. Pharmaceutical, pharmacokinetics and, indirectly, pharmacodynamics profiles can compromise the efficacy of these compounds. Managing pharmacokinetics unwanted properties can solve other problems and many examples herein presented use this approach to accomplish the goals.

Carrier prodrugs have been mostly explored in this chapter. Thanks to the advance of carrier types, as many different polymers, which provide different labile linkages with the bioactive compounds, the interest of prodrug design has raised. The interface with nanotechnology, especially in the case of dendrimers as carriers, has brought a multidisciplinary vision of the process. On the other hand, the mechanism of releasing and delivering lead/drug is a challenge, in some cases, preventing the success of the design and therapeutic application. It is worth noting that some emphasis has been given to the application of computational methods, as molecular orbital and molecular mechanics, in prodrug design, to better understand the mechanism of action of these latent forms either of leads or drugs. The perspective is that this approach can raise further interest in both academy and pharmaceutical industry in the near future. The tendency in prodrug design today and, possibly, in the future is to explore systems that lead to the selectivity of action. For this very reason, most examples of targeted drugs are related to cancer chemotherapy. We glimpse that some highly selective transport forms of drugs, as ADEPT and GDEPT, for instance, will broaden their application provided their bottlenecks be settled.

Important Notes

- Prodrugs have arisen much interest to the pharmaceutical industry.
- Around 15% of the total drugs approved by the FDA are prodrugs.
- Prodrug approach can be used ad hoc, for bioactive compounds, or post hoc, for drugs already in the market.
- Prodrugs can solve pharmaceutical, pharmacokinetics, and, indirectly, pharmacodynamic problems either of bioactive compounds or drugs.
- Targeting drugs to specific organs/tissues has triggered the interest in prodrug approach.

Questions for Future Research

- **How advances in dendrimers can benefit the development of prodrugs?** Research on dendrimers as carriers for prodrugs should be increased towards targeted drugs. Targeted drugs provide a selective system to deliver drugs and dendrimers have been a versatile approach to achieve this goal. It allows linking many drug molecules besides directing groups, or homing devices. In addition, they allow to use drug combinations in the same dendrimer

architecture, which is very important in chemotherapy. Despite these advantages, there are few examples that have been studied so far, and for this very reason, the interest should be increased.

- **What roles should be played bying in prodrug development?** Molecular modeling should be applied more often to provide means to explore the stability based on the energy of final prodrugs/targeted drugs structures. This approach is a good tool for investigation not only about the possibility of having structures that can be synthesized but also to have the stability to reach the targeted. In addition, molecular modeling can estimate the possibility of drug releasing after reaching the target through the interaction with enzymes. This process has not been explored so far.
- **How the release and delivery processes experienced by prodrugs in complex carrier/prodrug systems can be more effectively determined?** Some systems, polymers, and dendrimers as well, for their complexity introduce difficulties in establishing the best method to prove the drug has been properly released. This has been a challenge and analytical methods should be studied towards solve this problem. The knowledge of the drug release is intrinsically related to the biological activity and to set the best dosage scheme for further in vivo assays.
- **How research on prodrugs can be more effectively translated from the laboratory into practical applications?** The main bottlenecks in the targeted drugs, especially Virus-Directed Enzyme Prodrug Therapy (VDEPT) and GDEPT, must be managed to make them available for therapeutics, as none are applicable so far. Those advanced forms of targeted drugs, although implying high selectivity, have the difficulty in reaching only the infected or tumorous cells. The transference of genes that encode the expression of some enzymes, involved in the release of the drug from the prodrug, has been a great problem. However, with the development of the genetic area, this problem could be more easily faced.
- **How works on selective advanced targeted drugs can be applied to the treatment of cancer, which is regarded as one of the most important age-associated diseases to be tackled in the next several decades?** The interest in applying selective advanced targeted drugs should be extended to other chemotherapeutic classes, like those for neglected diseases. The most studied therapeutic class so far has been the antitumor agents, which essentially needs the reduction of toxicity, and, hence, selectivity. However, neglected diseases are urgently needing to foster more effective and selective alternative as well to face this challenge. Although those advanced delivery forms are, not rarely, expensive, their effectiveness in only doses, for instance, could be interesting to minimize the problem of these diseases, which compromised more than one billion people in the world.
- **How the versatitlty and functionality of prodrug development can benefit from the emergence of theranostics?** Theranostic approach with

targeted drugs could be more explored in order to have more advanced conjugated structures. Theranostic systems have been studied for some groups of drug delivery as they can be useful to diagnose the disease while treating it. In some cases, the diagnostic is not so easy, but when confirmed with some fluorescence, for instance, it allows the drug to treat selectively the disease, saving time and increasing the efficacy. In spite of being a complex matter so far, the advance in the areas involved in this approach could help the researchers to explore it successfully.

- **How could the popularity of the use of prodrugs in pharaceutical indistries could be enhanced?** Pharmaceutical industries should broaden the use of prodrugs in pre-clinical phase to reduce the percentage of compounds in the "valley of death". Although some pharmaceutical industries have been using prodrugs in preclinical analysis, there still have a kind of resistance to put effort in this usage. However, the tendency of some companies is to increase their interest in using ad hoc prodrug design. This can be seen in this chapter by the number of new chemical entities launched recently. Notwithstanding, the drug repurposing approach, which have been stimulated ultimately, could increase the post hoc use of the design of prodrugs.
- **How could the development and use of products be facilitated at the research and development (R&D) level?** Much efforts must be directed to consider prodrugs as the first patent. Being the first patent can foster mainly the interest from pharmaceutical industries to use prodrug design as a mean for innovation. Despite this, the fact that prodrugs are being applied as the second patent for some pharmaceutical industries to extend the time protection of a drug, whose patent has expired, can be a bottleneck to change the scenario. However, the battel towards the goal must persist.

Glossary

ADME An acronym referring to absorption, distribution, metabolism and excretion of a compound upon administration to a biological body.

Bioactive compound A compound that presents biological activity but is not a drug yet.

Biotransformation The conversion of a chemical entity from one form to another form by means of a biological system.

Gene-directed enzyme prodrug therapy A two-step process in which a gene encoding an enzyme is first delivered to a target cell, followed by the administration of a nontoxic prodrug that can be converted to a cytotoxin under the action of the enzyme.

Hit A molecule which has a known structural identity and at the same time shows reproducible activity above a defined threshold in a biological assay.

Lead A compound (or compound series) which on one hand display desirable biological activity in a relevant cell-based assay and on the other hand demonstrates proper activity, selectivity, and tractable structure-activity relationship. It is a candidate for further structure-activity optimization.

Medicinal chemistry A discipline in chemistry which concerns with the invention, discovery, identification, design, and preparation of bioactive compounds. In addition, the metabolism and the mode of action of those compounds at the molecular level, along with the structure-activity relationships, will be studied in medicinal chemistry.

Molecular modification Processes that give rise to changes in some characteristics of a compound/drug towards optimization.

Receptors Molecules or polymeric structures present on the cell surface or in the cell. They can recognize and bind other agents which serve as molecular messengers (e.g. neurotransmitters, hormones, and drug molecules) in a body.

Valley of death The occurrence of problems (e.g. o lack of solubility and high toxicity) that avoid the innovative molecule to pass through the development phase and prevent it from finally reaching the clinical phase as a drug candidate.

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Chapter 6
Development of Biodegradable Polymeric Nanoparticles for Systemic Delivery

Nandita G. Das and Sudip K. Das

Abstract In the previous chapter, the possible use of prodrug design in the enhancement of systemic delivery has been discussed. As the technique applicable to one drug may not be generalized to another drug, such an approach is comparatively labor intensive, and requires the structure of the agent designed to be manipulated case by case. As an alternative to, or as a complementary strategy for prodrug design, extensive efforts have been devoted to the development of diverse types of carriers over the last several decades. These carriers on one hand enable the delivery of multiple chemical entities and on the other hand allow for functionalization to enhance versatility and working performance. Due to their high structural flexibility, polymers have emerged as one of the most extensively studied materials for fabrication of such carriers. In this chapter, we will discuss different approaches to prepare polymeric particulates and will highlight the parameters to be characterized for optimal delivery performance in systemic delivery.

Keywords Non-biodegradable · Biodegradable · Polymers · Particulate drug delivery systems · Particle size · Surface charge · Hydrophobicity

6.1 Introduction

Delivery of therapeutics is a burgeoning field of research in biomedicine (Lai et al. 2017; Pottanam Chali and Ravoo 2019; Lai and Lin 2015; Ferrari et al. 2018). Its promising potential has been demonstrated in a variety of treatment of diverse age-associated disorders, ranging from cancer and diabetes mellitus to cardiovascular diseases (Lai et al. 2016; Calzoni et al. 2019, 2013; Wen et al. 2018; Lai 2011, 2012; Battistella and Klok 2017). Over the years, different types of carriers have been developed for therapeutics delivery, including liposomes, exosomes, viral vectors, and polymeric nanoparticles (Lai et al. 2019; Narayani and Rao 1995; Lai and Wong 2018; Lai and Rogach 2017). Among them, polymeric nanoparticles have attained

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great interest because of the high flexibility and ease of functionalization of polymers, as well as the availabilities of mature technologies for polymer synthesis. Based on their structural and chemical properties, polymers can be biodegradable or non-biodegradable. The latter has been extensively used in animals for preclinical studies of tissue distribution. This is because non-biodegradable particles can avoid overlapping of organ distribution of particles associated with biodegradation effects. The most commonly used non-biodegradable particulate systems are made of polystyrene. Suspension polymerization of stabilizer free styrene can generate polystyrene particulate systems in a stirred system containing potassium persulfate as the initiator (Goodwin et al. 1974). Colloidal gold can be incorporated in these particulate systems during nanoparticle fabrication (Chainey et al. 1982), and in this occasion, it can be used as an electron-dense marker for TEM investigations. In recent years, several in vitro gene transfection studies have been reported to use non-biodegradable particles too. This can be exemplified by the case of poly ((2-dimethylamino)-ethyl methacrylate) nanoparticles, which have been used for transfection of plasmid DNA in a cell culture model (Cherng et al. 1996).

The major advantage of using nanoparticle systems generated from non-biodegradable polymers is that the surface of those particles can be hydrophobic in nature. This enables the nanoparticles to adsorb coating polymers, and to be easily labeled with fluorescence and other radioactive materials for determination of organ distribution. In addition, non-biodegradable polymeric nanoparticles are commercially available in several size ranges. This makes the application of these nanoparticles easy and convenient. Despite these advantages, non-biodegradable polymers are not used for internal human administration, partly because of the difficulties of removing the nanoparticles from the body, accumulation in major organs, and the potential toxicity caused by the nanoparticles. This explains the fact that, compared to non-biodegradable polymeric nanoparticles, most of the nanoparticle systems intended for human use are generated from biodegradable polymers. In this chapter, we will focus only on the generation and optimization of biodegradable polymeric nanoparticles.

6.2 Advantages of Biodegradable Polymeric Nanoparticles in Biomedical Use

Biodegradable polymers are one of the important components used in the development of polymeric particulate systems. Gref et al. (1994) listed the desired features of biodegradable long-circulating polymeric particulate systems: (i) that the loading of bioactive agent is quite high (e.g. more than 30%), (ii) that the encapsulation efficiency is reasonably high (e.g. more than 80%), (iii) the ability to be freeze-dried and reconstituted in solution without aggregation, (iv) biodegradability, (v) small size, and (vi) specific properties to prevent rapid clearance of the particles from the bloodstream. Although literature reports reveal the use of natural polymers such

as albumin (Burger et al. 1985), gelatin (Yoshioka et al. 1981), or polysaccharides (Artursson et al. 1987), they are not being used in recent years as the characteristics of the materials vary depending on the sucrose content of the latter, and due to their chance of producing immunological reactions.

In vivo degradation is a complex phenomenon where water molecules penetrate a **hydrophilic polymeric matrix** and degradation becomes a bulk phenomenon and occurs fast, whereas the degradation of hydrophobic polymers is much slower and occurs from the surface. Moreover, hydrolytically unstable bonds (e.g. ester bonds) degrade fast. Polymer molecular weights normally affect degradation parameters, with smaller molecules degrading faster than the larger ones. Highly crystalline polymers are usually degraded slower than the amorphous ones as water can penetrate easily through the amorphous regions. Polymers that possess **glass transition temperature** higher than the body temperature behave like glassy solids having slow degradation rate while those with glass transition temperature lower than the body temperature behave like rubbery solids having higher degradation rate. Geometrical parameters like size, shape, and surface to volume ratio affect the degradation of polymers. A high surface area to volume ratio indicates high water penetration leading to high degradation rates. Monomers and drugs in certain situations can plasticize the matrix, leading to change in the glass transition temperature and degradation rates (Piskin 1994). Enzymes and some degradation products, such as carboxylic acid end groups produced during the hydrolysis of the ester bond, can significantly enhance the degradation rate through autocatalytic or enzymatic pathways (Vert et al. 1994). In addition, environmental factors such as pH, ionic strength, and temperature can also affect the degradation rate.

6.3 Use of Lactide and Glycolide Polymers in Drug Delivery

Aliphatic polyester copolymers based on lactic and glycolic acids became popular in drug delivery research in the 1970s after major reports of their use as surgical sutures and implantation products were published. Poly (D, L-lactic-co-glycolic acid) has a long history of safe use in veterinary and medical applications. It is used for the manufacture of implants and internal sutures and is known to be biocompatible, degrading to produce the natural products lactic acid and glycolic acid (Visscher et al. 1985). In an early study, 65/35 polylactide/glycolide microspheres implanted subcutaneously in rats were biocompatible from 15 until 150 days of implantation. Microsphere implantation sites were gradually replaced by collagenous tissue during microsphere degradation. At the microsphere tissue interface, the predominant cell type changed from the macrophage to the foreign body giant cell and thereafter the macrophage was predominant at later time points (Yamaguchi and Anderson 1993).

Monomer stereochemistry, co-monomer ratio, polymer chain linearity, and polymer molecular weight could achieve a wide spectrum of characteristics of the lactide and glycolide polymers (Lewis 1990). The racemic poly (DL-lactide) is less

crystalline and has a lower melting point than the two stereo-regular polymers, D-polylactide and L-polylactide. The copolymers of lactide and glycolide are less crystalline than their homopolymers. Due to the presence of the methyl group in the lactide polymers, they are more hydrophobic than the glycolide polymers. Also, the water uptake increases as the glycolide ratio in the copolymer increases (Gilding and Reed 1979).

Biodegradation of lactide and glycolide polymer occurs by bulk erosion of the polymer, due to hydrolysis to monomeric acids and elimination through **Krebs cycle**. Pitt et al. demonstrated that molecular weight of the polymer decreases in the first stage of degradation due to random hydrolytic cleavage of the ester linkage followed by the onset of weight loss and a change in the rate of chain scission in the second stage (Pitt et al. 1981). The degradation of these polymers differs in vivo and in vitro mainly due to the reason that although in vivo there is no major influence of enzymes during the glassy state of the polymer, the enzymes can play a significant role when the polymer becomes rubbery (Holland et al. 1986). Degradation of lactide/glycolide copolymers has been studied by a number of investigators (Miller et al. 1977; Pitt et al. 1981; Chu 1985). A review by Holland et al. described the details of the biodegradation of polyesters (Holland et al. 1986). Normally, the 50:50 lactide/glycolide copolymers have the fastest half-life of degradation, around 50–60 days, while 65:35, 75:25, and 85:15 have progressively longer degradation half-lives in vivo. As poly (l-lactide) is more crystalline and hydrophobic, their in vivo degradation rate is the slowest. Jalil and Nixon (1989, 1990, 1990) demonstrated that although the physical properties of the microparticles were not seriously affected by the molecular weight of poly (D, L-lactide), the swelling properties (that are a function of hydrophilicity of the polymer) can be affected due to the variations in the molecular weight and core loading.

6.4 Nanoparticle Fabrication from Lactide/Glycolide Polymers

Microparticles and nanoparticles of lactide/glycolide polymers are the most popular amongst researchers worldwide for delivery of small molecule drugs as well as macromolecules. Lactide/glycolide microparticles can be classified based on the technique of preparation.

6.4.1 Phase Separation of Polymer

This technique relies on the decrease of solubility of the coating polymer by addition of a third compound to the polymer solution in an organic solvent. The point has to be reached where two liquid phases are formed: the rich-in polymer coacervate

and the supernatant liquid phase depleted in polymer. If a drug is initially dispersed in the polymer solution, it can be coated by the coacervate. The use of the **coacervation technique** to develop polyester microspheres was first reported by Fong in 1979. Nihant et al. reported the effect of processing parameters on the microcapsule characteristics (Nihant et al. 1995; Stassen et al. 1994). As summarized in Table 6.1 (Leelarasamee et al. 1988; Kent et al. 1980; Kent et al. 1987; Ruiz et al. 1989; Batcheller et al. 2020; Johnson et al. 1991; Mandal 1996; Wang et al. 1990; Nihant et al. 1993; Nihant et al. 1994; Boswell and Scribner 1973; Nakano et al. 1980; Hazrati et al. 1993; McGee et al. 1994; Wang and Wu 1998; Lapka et al. 1986), a number of organic solvents such as dichloromethane, isopropanol, and heptane have been used as solvent, coacervating agent, and hardening agent, respectively. As mentioned by Ruiz et al. microencapsulation using phase separation coacervation

Table 6.1 Phase separation coacervation of lactide/glycolide polymers

Solvent	Coacervating agent	Hardening agent	Active ingredient	References
DCM	Mineral oil	Mineral oil	peptide	(Leelarasamee et al. 1988)
DCM	Silicone oil	Heptane	LHRH analogs	(Kent et al. 1980, 1987; Ruiz et al. 1989)
DCM	Silicone oil	Heptane	Antisense	(Batcheller et al. 2020)
DCM	Polybutadiene in DCM	Hexane, cold	Atriopeptin III	(Johnson et al. 1991)
DCM	DCM/silicone oil	Petroleum ether	Zidovudin	(Mandal 1996)
DCM	Silicone oil with Span 85	Petroleum ether	Bovine serum albumin	(Wang et al. 1990)
DCM	Silicone oil	Heptane	Thrombin and bovine serum albumin	(Nihant et al. 1993; 1994)
DCM	Hexane	Hexane	Fertility control agents	(Boswell and Scribner 1973)
Ethyl acetate	Diethyl ether	Diethyl ether	Sulfamethizole	(Nakano et al. 1980)
Ethyl acetate	Silicone oil	Heptane	Vaccine	(Hazrati et al. 1993)
DCM	Silicone oil	Heptane	Ovalbumin	(McGee et al. 1994)
DCM	Silicone oil	Heptane	Insulin with agarose gel	(Wang and Wu 1998)
Toluene	Polybutadiene in toluene	Heptane	Calcitonin	(Lapka et al. 1986)

DCM: dichloromethane

of lactide/glycolide polymers involves four steps, using methylene chloride as the solvent and silicone oil as the coacervating agent (Ruiz et al. 1989):

1. The phase inducer, silicone oil is added in a low concentration (1–5%) to the solution of the polymer in dichloromethane that produces a pseudo emulsion,
2. On adding more coacervation-inducing agent, the start of phase separation is noticed. The coacervated liquid polymer droplets are unstable and merge together to produce larger droplets that expand and finally rupture,
3. On continuation of adding more coacervation inducing agent, a stable dispersion of the coacervated droplets are formed,
4. Aggregation of coacervated droplets leads to precipitation of microparticles.

The function of hardening agent is to extract the solvents from the liquid polymer droplets and finally produce solid microparticles. If the active drug is dispersed in the polymer solution at the beginning, it will produce drug entrapped in the microparticles depending on the solubility of the drug in the solvent, however, in most situations it forms monolithic systems. The porosity of the surface of microparticles depends on the rate of solvent extraction, while the shape is typically spherical. A number of drugs have been incorporated in these systems and the drug release rate varied based on the matrix structure and the solubility of the drug in the physiological medium. The main advantage of phase separation method is that it protects active drugs from partitioning out into the dispersed phase. Also, the residual solvent content is a major concern, especially when heptane is used as the hardening agent (Muller et al. 1993).

6.4.2 Solvent Evaporation and Extraction Methods

This is the most popular method for development of microparticles of lactide/glycolide and has been used by numerous researchers. The technique is based on the formation of an emulsion of an organic solvent solution of the polymer and evaporation or extraction of the solvent in a non-solvent of the polymer, resulting in the formation of microparticles. Different types of microparticles may form based on the nature of the active agent in the organic solution of the polymer, nature of the external phase, and solvent elimination procedure.

6.4.2.1 Water in Oil in Water (W/O/W) Solvent Evaporation Method

One of the popular techniques relies on the emulsification (W/O) of aqueous solution of the active agent in the water-immiscible organic solutions of lactide/glycolide polymers, followed by emulsification (W/O/W) of the system in an aqueous phase and removal of the organic solvent while stirring the system. This method was first reported by Ogawa et al. (Ogawa et al. 1988a, b) and has been applied by a number of researchers. Kawashima et al. also reported the development of mini-depot tablets made of nanoparticles of poly (DL-lactide-co-glycolide) prepared by this method

(Murakami et al. 2000). The problems associated with this method could be (Conti et al. 1992):

- aggregation of microparticles due to the organic solvent or temperature used,
- loss of drug in aqueous phase with formation of free crystals in the aqueous phase,
- formation of drug crystals on the particle surface.

The most common systems use dichloromethane as the solvent for the polymer and the primary emulsion (W/O) is dispersed in an aqueous medium containing polyvinyl alcohol. Although a few literature reports claim that this method is suitable for water soluble drugs, due to partitioning of the drugs through the oil phase to the bulk aqueous phase, often the loading of water soluble drugs like proteins and peptides is exceptionally low. In addition to the concern of inherent toxicity of dichloromethane, stability of protein structures in presence of dichloromethane and processing conditions may impose obstacles to successful entrapment of proteins drugs (Ghaderi and Carlfors 1997; Lu and Park 1995; Tabata et al. 1993). It was demonstrated with bovine serum albumin, ovalbumin, and lysozyme that emulsification induced adverse events that were detrimental to the integrity of proteins and the importance of preserving protein stability toward microencapsulation (Sah 1999). Moreover, the use of ultrasound during the preparation of lactide/glycolide microparticles could cause pronounced changes in the molecular weight distribution leading to changes in drug loading efficiency, initial drug release, hydration, and degradation pattern of the polymer (Reich 1998). Table 6.2 lists representative reports on the W/O/W solvent evaporation technique (Ghaderi and Carlfors 1997; Chattaraj et al. 1999; Sturesson and Carlfors 2000; Scholes et al. 1993; Alonso et al. 1993; Mehta et al. 1994; Aso et al. 1994; Kofler et al. 1996; Shenderova et al. 1997; Morlock et al. 1997; Gasper et al. 1998; Witschi and Doelker 1998; Zambaux et al. 1998; Takahata et al. 1998; Jeffery et al. 1993; Ferdous et al. 1998; Cleland et al. 1997; Sansdrap and Moes 1993; Jalil and Nixon 1990; Singh et al. 1997; Izumikawa et al. 1991; Iwata and McGinity 1992; Prieto et al. 1994; Pavanetto et al. 1994; Sah et al. 1995; Eldridge et al. 1991; Coombes et al. 1998; Lukowski et al. 1992; Park et al. 1995; Sah et al. 1996; Gander et al. 1995; Eyles et al. 1997; Hora et al. 1990; Gupta et al. 1997; Okada 1997; Uchida et al. 1996a, b; McGee et al. 1997; Yan et al. 1994; Soriano et al. 1995; O'Hagan et al. 1994a, b; Okumu et al. 1997; Conti et al. 1995; Cleland et al. 1997; Pean et al. 1998).

6.4.2.2 Oil in Water (O/W) Solvent Evaporation Method

This technique is economical and very suitable for hydrophobic drugs but not preferable for hydrophilic drugs. It involves the emulsification of an organic phase (drug dispersed/dissolved in it) in an aqueous solution containing a surfactant (Alex and Bodmeier 1990; Fong 1988). A number of drugs have been encapsulated using this method, including antineoplastic (Deyme et al. 1992; Mestiri et al. 1993), hormones (Rosilio et al. 1991; Beck et al. 1983), and anti-inflammatory drugs (Leelarasamee et al. 1986; Bodmeier and Chen 1989). In order to increase the drug

Table 6.2 Solvent evaporation and solvent extraction technique using W/O/W emulsion

Objective	Solvent	Condition	Active agent	References
Immune response	DCM	Evaporation in PVA solution	Influenza viral vaccine	(Chattaraj et al. 1999)
Retention of bioactivity	Ethyl acetate or DCM	Evaporation in PVA solution	Urease	(Sturesson and Carlfors 2000)
Site specific-delivery	DCM	Evaporation in PVA solution	None	(Scholes et al. 1993)
Release rate	DCM	Evaporation and extraction	Tetanus vaccine	(Alonso et al. 1993)
Parenteral delivery	DCM and methanol	Controlled temp evaporation and controlled dilution	Salmon Calcitonin	(Mehta et al. 1994)
Mechanism of drug release	DCM	Reduced pressure solvent evaporation	Progesterone and superoxide dismutase	(Aso et al. 1994)
Characterization of microspheres with antigen	DCM	Solvent evaporation	Pneumotropic bacterial antigens	(Kofler et al. 1996)
Stabilization	DCM	Solvent evaporation	10-hydroxycamptothecin	(Shenderova et al. 1997)
Biological activity	DCM	Solvent evaporation	Lysozyme	(Ghaderi and Carlfors 1997)
Protein stability	DCM	Solvent evaporation	rh-erythropoietin	(Morlock et al. 1997)
Enzyme loading and activity	Ethyl acetate	Solvent evaporation	L-asparaginase	(Gasper et al. 1998)
Polymer degradation	DCM	Solvent evaporation	Tetracosactide	(Witschi and Doelker 1998)
Characteristics of nanoparticles	DCM	Solvent evaporation	Human serum albumin	(Zambaux et al. 1998)
Distribution of proteins	DCM	Solvent evaporation	Ovalbumin	(Takahata et al. 1998)
Entrapment of protein	DCM	Solvent evaporation	Ovalbumin	(Jeffery et al. 1993)
Role of polymer nanoparticles	Acetone	Solvent evaporation	Monensin	(Ferdous et al. 1998)
Long-lasting effect	DCM	Solvent evaporation	r-Human growth hormone	(Cleland et al. 1997)

(continued)

Table 6.2 (continued)

Objective	Solvent	Condition	Active agent	References
Influence of manufacturing parameters	DCM	Solvent evaporation	Nifedipine	(Sansdrap and Moes 1993)
Effect of Span and Brij	DCM	Solvent evaporation	None	(Jalil and Nixon 1990)
Evaluation of immunogenicity	DCM	Solvent evaporation	Hepatitis B vaccine	(Singh et al. 1997)
Effect of crystalline morphology of drug release	DCM	Solvent evaporation	Progesterone	(Izumikawa et al. 1991)
Multiphase microspheres	Acetonitrile	Solvent evaporation	Chlorpheniramine, Procainamide, Promazine	(Iwata and McGinity 1992)
Peptide loaded small microspheres	DCM	Solvent evaporation	V3 BRU—immunogenic fraction of GP120 of HIV	(Prieto et al. 1994)
Evaluation of process conditions	DCM and chloroform	Solvent evaporation	Diazepam	(Pavanetto et al. 1994)
Effect of shear force to make primary emulsion on morphology & protein release	DCM	Solvent evaporation	Bovine serum albumin	(Sah et al. 1995)
Adjuvant properties	DCM	Solvent evaporation	Staphylococcal enterotoxin B	(Eldridge et al. 1991)
Control of protein release by modification of external aqueous phase	DCM	Solvent evaporation	Ovalbumin	(Coombes et al. 1998)
A comparison of O/O, O/W, and W/O/W methods	DCM	–	Bovine serum albumin	(Lukowski et al. 1992)
Protein release kinetics, stability, and polymer degradation	DCM	Solvent evaporation	Carbonic anhydrase and bovine serum albumin	(Park et al. 1995)

(continued)

Table 6.2 (continued)

Objective	Solvent	Condition	Active agent	References
Solvent as methyl ethyl ketone (MEK)	MEK	Solvent evaporation	Progesterone	(Sah et al. 1996)
Spontaneous emulsion solvent diffusion	Acetone and DCM	Solvent diffusion/evaporation	5-FU	(Gander et al. 1995)
Oral delivery of encapsulated interferon	DCM	Solvent evaporation	Interferon	(Eyles et al. 1997)
Delivery of large proteins	DCM	Solvent evaporation	Human serum albumin	(Okada 1997)
Protein loading	DCM	Solvent evaporation	Tetanus toxoid	(Gupta et al. 1997)
Review of leuprorelin acetate microspheres	DCM	Solvent evaporation	Leuprorelin acetate	(Okada 1997)
Microspheres of water soluble dye	DCM	Solvent evaporation	Water soluble dye	(Uchida et al. 1996)
Process reproducibility	DCM	Solvent evaporation	Ovalbumin	(McGee et al. 1997)
Morphological analysis	DCM	Solvent extraction	Ricin	(Yan et al. 1994)
Effect of surfactants	DCM	Solvent evaporation	Bovine serum albumin	(Soriano et al. 1995)
Antibody response after oral administration	DCM	Solvent evaporation	Ovalbumin	(O'Hagan et al. 1994)
Microparticle/polymer degradation rate	DCM	Solvent evaporation	Ovalbumin	(O'Hagan et al. 1994)
Peptide properties on its release from microspheres	DCM	Solvent evaporation	Linear and cyclic hexapeptides	(Okumu et al. 1997)
Process parameters	DCM	Solvent evaporation	Indomethacin	(Conti et al. 1995)

(continued)

Table 6.2 (continued)

Objective	Solvent	Condition	Active agent	References
O/O/O method	Acetonitrile	Solvent evaporation	Ovalbumin	(Uchida et al. 1996)
Stability of r-human growth hormone	DCM	Solvent evaporation	r-human growth hormone	(Cleland et al. 1997)
Optimization of encapsulation	DCM	Solvent evaporation	Human serum albumin coencapsulated with nerve growth factor	(Pean et al. 1998)

loading attempts have been made to saturate the external phase with the drug (Jalil and Nixon 1989; Bodmeier and McGinity 1987) or the formation of hydrophobic prodrugs (Seki et al. 1990). Herrmann et al. (J. Herrmann, R. Bodmeier, 1995) reported that an increase in the volume fraction of the internal aqueous phase in the primary W/O emulsion resulted in lower encapsulation efficiencies. Replacement of the dichloromethane as an organic solvent with ethyl acetate reduced the encapsulation efficiency and increased the porous nature of the microparticles.

6.4.2.3 Oil in Oil or Non-aqueous Solvent Evaporation Method

This method apparently looks similar to the phase separation method, however, it differs from the phase separation method in the aspect that the continuous phase does not produce the coacervate but the solvent of the dispersed phase evaporates while the polymer droplets serarate from the medium. An organic solution of the lactide/glycolide polymer is poured in a medium of immiscible, non-volatile organic solvent (mostly an oil) and the microparticles are produced on slow evaporation of the solvent from the medium. While this method is suitable for hydrophilic drugs it is often challenging to control the particle size to nanometer range. This technique has not been popular among the researchers and only a few drugs have been formulated using this technique (Ike et al. 1992; Sato et al. 1988).

6.4.2.4 Emulsion Solvent Extraction Method

This modification of the solvent evaporation process has seldom been used for the preparation of microparticles with some early exceptions (Gupta and DeLuca 1989). The first part (emulsification) is common for the solvent evaporation and extraction processes, however, the solvent extraction method involves the introduction of a diluent phase that does not solubilize the polymer but is miscible with the emulsion's continuous and dispersed phases. The advantages of the method are that it is possible to extract the solvent even at low temperatures, and it is suitable for drugs that degrade at high temperatures.

6.4.3 Spray Drying Method

The technique of spray drying is based on the **atomization** of a solution of an active agent by nitrogen or compressed air through a drying chamber or drying using warm air. Large-scale production of a number of microparticles involves this technique. The distinct advantages over the other methods are; (1) drugs can be efficiently encapsulated irrespective of their affinity to water (Bodmeier and Chen 1988), (2) drying times are very short at around 50–70°C that prevents thermal degradation of the drug (Wang et al. 1990; Pavanetto et al. 1991), and (3) the quality of particles

are monodisperse, mainly depending on the viscosity of the solution sprayed and atomization parameters. In most of the situations, dichloromethane has been used as the solvent, sometimes with chloroform (Pavanetto et al. 1991). A number of drugs including bromocriptine (Montini et al. 1993), theophylline and progesterone (Bodmeier and Chen 1988), and piroxicam (Wagenaar and Muller 1994) have been encapsulated using this method. Although there are disadvantages of handling chlorinated solvents in the nozzles, the active ingredient loss is minimum in this process. Clogging of nozzles is the major concern in producing nanometer-size particles. Gander et al. (Gander et al. 1995) reported the formulation of bovine serum albumin loaded microspheres of D, L-polylactide prepared by spray drying technique using ten different solvents including dichloromethane, acetone, and ethyl acetate. Also, the polymer type, molecular weight, and its concentration in the spraying solution affect microsphere characteristics (Pavanetto et al. 1993).

6.4.4 Formation of Microparticles Using Supercritical Fluids

The production of particles with specific physicochemical properties for delivery of drugs and cosmetic agents is a constant challenge in the pharmaceutical industry (Lai and Shum 2015; Lai and He 2016), especially for micron and submicron-sized particles. The application of a **supercritical fluid** (SF) as a means of particle formation offers promise (Bleich et al. 1993). This method involves atomizing an organic polymer solution into a vessel containing compressed CO_2 (Bleich et al. 1993). More specifically, the formulation of microparticles using supercritical fluids involves spraying of polymer/core solution into the supercritical gas phase (state of a fluid at temperatures and pressures above its critical point) where the polymer precipitates and incorporates the core material (Fischer and Muller 1991). There are two main reasons for using this technique: (1) the selective solvating power of SF makes it possible to separate a particular component from a multicomponent system, (2) solvent (including residual) and anti-solvent gas involved in the process can be removed very efficiently. Depending on the degree of crystallinity and the molecular weights of lactide/glycolide polymers, different types of microparticle formation have been reported. Nanoparticles are formed after precipitation of the polymer caused by extraction of the organic solvent into CO_2 and by CO_2 diffusion into the droplets. This process, which has been termed the aerosol solvent extraction system (ASES) or precipitation with a compressed fluid antisolvent (PCA), has great versatility as the properties of CO_2 may be adjusted over a continuum throughout the gaseous, supercritical, and liquid states by varying temperature and pressure. Therefore, this process has benefits over the existing particle formation methods in terms of improved control, flexibility, and operational ease.

Conventional nanoparticle processing techniques as solvent evaporation, spray drying, and organic phase separation techniques can prepare matrix and reservoir type microparticles and nanoparticles (Schubert 1996; Deasy 1984; Kondo 1979) but have several disadvantages. Most of these methods suffer from drawbacks such

as the use of organic solvents for the solubilization of polymers, the hazards and environmental concerns associated with these solvents, residual organic solvents, non-uniformity of particle size, and low encapsulation efficiencies because of poor drug partitioning. The application of supercritical fluid technology to develop particle systems will open newer avenues to explore the application of nanoparticles in several pharmaceutical as well as non-pharmaceutical approaches. A few research groups are currently working on the development of the process of using supercritical fluids for production of micron- and submicron particles. The research is still in rudimentary stages and needs a lot more systematic study to bring perfection as is proposed in this application.

6.5 Potential of Polyanhydride in Systemic Delivery

The major goal to precisely control the release of drugs has evolved the development of polyanhydrides. Polyanhydrides are a class of bioerodible polymers that were developed specifically for controlled release drug applications and display certain features characteristics of surface erosion (Tamada and Langer 1992) that is because the carboxylic acid anhydrides are among the functional groups that hydrolyze rapidly. Since the polyanhydrides have been developed in the 1980s (Rosen et al. 1983), they have become popular in drug delivery and a number of biocompatible analogs have been proposed. Polyanhydrides can be manufactured as aliphatic or aromatic homopolymers and copolymers as well as cross-linked or branched polymers. One class of aliphatic polyanhydrides that proved to be useful for drug delivery is p(FAD-SA) (Domb and Maniar 1993), which is a copolymer of poly fatty acid dimer (FAD, liquid) and poly sebacic acid (SA, crystalline and brittle). Erosion of aromatic polyanhydrides is much slower than the aliphatic ones (Leong et al. 1985) due to the increased hydrophobicity of the aromatic polymer and inaccessibility of water to anhydride bonds (Tamada and Langer 1992). Aromatic polyanhydrides have low solubility in organic solvents and have high melting points. A copolymer made of 1,3-bis-(p-carboxyphenoxy)propane (CPP) and sebacic acid (SA) erodes within a few weeks while the erosion of homopolymer CPP could take a few weeks. Unlike the lactide/glycolide polymers, polyanhydrides are normally custom synthesized from carboxylic acid monomers. The melt condensation process has been widely used in the synthesis of aliphatic as well as aromatic poly (anhydrides) (Leong et al. 1987; Domb and Langer 1987). Although solvent evaporation methods have been reported in the literature, it is not suitable for hydrolytically unstable polyanhydrides. On the other hand, proteins cannot be encapsulated using the holt-melt method. Spray drying processes have been used to produce microparticles of this polymer, but the quality of the product was aggregated and resulted in irregular particle morphology when amorphous polymers like P(CPP-SA) were used (Mathiowitz et al. 1988, 1990).

The biocompatibility and safety of the polyanhydrides have been established as per the FDA guidelines (Table 6.3) (Leong et al. 1986). Polyanhydrides, their copolymers, and degradation products have been found to be non-cytotoxic, non-mutagenic,

Table 6.3 Physical properties of *p* (CPP-SA) polyanhydrides. Data in this table was adopted from 146

Polyanhydrides	Transition glass temperature T_g °C	Melting point T_m °C	Erosion rate μg/cm^2/hr
P(CPP-SA) 85:15	85	230	6.0
P(CPP-SA) 45:55	<30	192	80.0
P(CPP-SA) 21:79	<30	67	160

and non-teratogenic (Braun et al. 1982). Except for fibroblast tissue growth at the site of administration, they do not possess any inflammatory response. Polyanhydride polymer (CPP-SA, 20:80) containing carmustine (BCNU), a chemotherapeutic agent, has been approved for clinical use (as Gliadel® wafers) for glioblastoma multiforme, a fatal brain cancer.

Polyanhydrides exhibit a unique erosion profile due to the chemical properties of the fast hydrolyzing functional groups. Bulk eroding or homogeneously eroding polymers are distinguished from surface eroding or heterogeneously eroding polymers by the process that bulk eroding polymers erode over its entire cross section while the surface eroding polymers erode from its surface. For degradable polymers, two different erosion mechanisms have been proposed, surface or heterogeneous and bulk or homogenous (Langer and Peppas 1983). In the surface eroding polymers, degradation is faster than the influx of water into the matrix, therefore, the major degradation is confined to the surface. In bulk eroding polymers, the degradation is slow due to slow influx of water into the bulk and the erosion is not limited to the surface. Although surface erosion is the predominant method of degradation in polyanhydrides, in reality both processes can occur simultaneously. An essential condition for a water-insoluble polymer to undergo surface erosion is the fast degradation of its polymer backbone (Gopferich 1996). The erosion kinetics of p (CPP-SA) is different from p (FAD-SA) due to the nature of FAD (fatty acid dimer), which is an insoluble liquid (Gopferich et al. 1996). The erosion of polyanhydrides by hydrolysis of carboxylic anhydride bonds by water as well as formation of amides from drugs carrying primary amine groups could be a potential threat to the stability of the drug (Gopferich and Langer 1993). However, in the case of certain enzymes, it has been reported that encapsulation in polyanhydride microspheres can protect them from degradation. While 90% of the enzymatic activity of heparinase was lost in solution within 24 h at 37°C, only 47% activity was lost when heparinase was encapsulated and subjected to the same conditions (Tabata et al. 1993). Since polyanhydrides are prone to degradation in contact with water, the preparation of microparticles should be carried out only in non-aqueous solvent systems (Table 6.4) (Tabata et al. 1993; Chickering et al. 1997; Mathiowitz et al. 1997; Mathiowitz and Langer 1987; Mylonas et al. 1995; Bhagat et al. 1994; Howard et al. 1989; Tabata and Langer 1993).

Table 6.4 Microparticles of polyanhydrides

Polyanhydride	Technique	Active ingredient	References
Poly(fumeric acid-co-sebacic) anhydride	Ionic gelation, hot-melt microencapsulation and phase inversion	Barium sulfate and dicoumarol	(Chickering et al. 1997)
Poly(fumeric acid-co-sebacic) anhydride	Phase inversion of polymer solution in DCM into excess of petroleum ether	Dicoumarol, insulin and plasmid DNA	(Mathiowitz et al. 1997)
P(CPP-SA)	Hot-melt microencapsulation	p-nitroaniline and myoglobin	(Mathiowitz and Langer 1987)
P(FAD-SA)	Solvent evaporation	Gonadotropic releasing hormone	(Mylonas et al. 1995)
P(CPP-SA) 20:80	Solvent evaporation and hot-melt microencapsulation	Methotrexate	(Bhagat et al. 1994)
P(CPP-SA) and P(FAD-SA)	Solvent evaporation	Chicken egg lysozyme, bovine pancreatic trypsin, ovalbumin, bovine serum albumin, and bovine immunoglobulin	(Tabata et al. 1993)
P(CPP-SA) 50:50	Solvent removal	Bethanechol	(Howard et al. 1989)
P(FAD-SA) and P(CPP-SA)	Solvent evaporation	Acid orange, acid red, and p-nitroaniline	(Tabata and Langer 1993)

6.6 Parameters for the Design of Polymeric Particulate Systems

According to classical polymer chemistry, average chemical compositions and morphologies of surfaces of solid polymers are usually different from those in the interior. With air as a contact partner, the polymer segments with the lowest Gibbs surface energy enrich surfaces in equilibrium. As surface energies depend on the partner (air, water, etc.) and are influenced by the kinetics (thermal history), surfaces of the same polymer can have various compositions and different properties under different conditions (Elias 1997). In the following parts of this section, parameters that have to be carefully considered and fine-tuned during the design of polymeric nanoparticulate systems for systemic delivery will be highlighted.

6.6.1 Particle Size

As particle size is one of the major factors that determine the clearance of particles from the body, it is absolutely important to control the particle size of colloids for bypassing the uptake by mononuclear phagocyte system. Since the upper limit for intravenous administration of colloidal systems is 5 μm, the widely used technique of laser light scattering may not be a suitable method for particle size measurement as its accuracy is debatable for particles <1 μm in diameter. Photon correlation spectroscopy (PCS) that determines mean particle size and size distributions (polydispersity index) in the range of 1 nm to 1 μm is more applicable for particles meant for intravenous administration. When a light beam illuminates a particle with a **dielectric constant** different from unity, depending on the wavelength of the light and the nature of the particle, the light will be absorbed, reflected, refracted, or scattered. In PCS, the particles undergoing random Brownian motion are detected and analyzed by illuminating the particles with a laser and measuring the scattered light with a photomultiplier tube. As most materials exhibit strong absorption in the UV and IR ranges of light, which reduces scattering intensity, scattering experiments are performed in the visible wavelength. The details of the PCS principles are covered elsewhere (Finsy 1994). PCS measurements enable the determination of the **diffusion coefficient**, D, of a particle or droplet in solution. By assuming spherical aggregates, the measured D can be used to calculate the hydrodynamic radius, d, of the particle/droplet using the Stokes–Einstein equation:

$$D = \frac{kT}{3\pi\eta d} \tag{6.1}$$

where k, T, η, and d denote the Boltzmann constant (1.38×10^{-16} erg/°K), temperature (°K), diluent viscosity (poise), and equivalent spherical diameter, respectively.

A PCS apparatus consists of a laser, a sample cell, and a photomultiplier (PMT) that is capable of detecting the scattered light at a certain angle (90°) or multiple angles. PMT signal is transferred to a correlator for calculation of correlation function, which is used by a microprocessor for calculation of diffusion coefficient of particles and correlated mean particle size. PCS does not use absolute intensity of the scattered light, rather fluctuations in the intensity. Particle size measurement at small scattering angles will be heavily weighted by the scattering from the larger particles in the sample and a measurement performed at large scattering angles will be weighted more by the scattering from the smaller particles in the sample. Therefore, a multiangle PCS instrument is desirable for polydisperse particles. A PCS measurement of particle size is immune to normal analog-type drifts, like changes in laser power or detector sensitivity. Also, the measured diffusion coefficient is unaffected by either the composition of the particles or their concentration, provided the suspension is sufficiently dilute that interparticle interaction is negligible.

6.6.2 Surface Charge

Surface charge of particulate systems determines whether they will be cleared by the mononuclear phagocyte system (MPS) fast or will be retained in the body for longer periods of time. A study by Roser et al. demonstrates that surface charge on albumin nanoparticles induced by covalent coupling of different amines significantly increased the uptake of particles by primary mouse peritoneal macrophages and human hematopoietic monocytic cells (Roser et al. 1998). Increased immunological recognition due to the charge causes a consequent increase of receptor mediated uptake of particulates in vivo. It is possible that an increased number of surface charged groups (especially –COOH and hydrophilic functional groups like –OH, $–NH_2$) reduce the surface hydrophobicity. Most of the researchers working in the area of surface-modified particulate systems routinely determine the surface charge of polymeric or lipid particles. Moreover, non-viral delivery of DNA or oligonucleotides using particulate systems would absolutely necessitate the study of surface charge of colloids to facilitate the delivery of anionic charged DNA or antisense oligonucleotides.

Particulate systems are normally charged in an aqueous medium either due to the charged groups on the surface or from adsorbed ions from the dispersion medium. Majority of the particle surface charge is measured in terms of *zeta potential* ζ which is defined as the electrical potential between the bulk solution and the shear plane around the particle. The shear plane is an imaginary sphere around a particle inside which the solvent moves with the particle as the particles move through the solution. Particle charge is measured using the principle of electrophoretic light scattering that combines two principles, electrophoresis (a technique that characterizes particles by their movement in an applied electric field) and laser Doppler velocimetry (a method for measuring the speed of particles by analyzing the Doppler shifts of the scattered light). Electrophoretic mobility is defined as the terminal velocity of a particle in a liquid that is subjected to an applied electric field and is experiencing a viscous drag. Thus, electrophoretic mobility, U, is represented as:

$$U = \frac{v}{E} \tag{6.2}$$

where v and E denote the terminal velocity and the applied electric field, respectively.

Electrophoretic mobility depends on pH, ionic strength, viscosity, temperature, and dielectric constant of the suspending liquid. Zeta potential is a parameter that can be derived from the electrophoretic mobility using the equations of Helmholtz, Henry, or Debye-Huckel (James 1979):

$$\zeta = \frac{4\pi\eta U}{\varepsilon} \tag{6.3}$$

where η, U, and ε represent the viscosity of the dispersion medium, the electrophoretic mobility of the particle, and the dielectric constant, respectively. In case of surface-coated particles, the adsorbed coating layer shifts the plane of shear to a greater distance from the particle resulting in a reduction of the zeta potential. If the coating layer is very thick, it can shift the diffuse layer to a large extent leading to a zero zeta potential that is favorable for MPS bypass.

6.6.3 Hydrophilicity/Hydrophobicity

As mentioned earlier, hydrophobicity of the particle surface also determines the clearance of the particles by macrophages. Normally, an increased hydrophobicity leads to increased opsonization, thus leading to increased phagocytosis and clearance of the particulate systems from the body. Although a relationship between surface hydrophobicity of coating film, as determined by contact angle measurement, and the MPS clearance of IV injected polymers was reported by Troster et al. (Troster and Kreuter 1988), contact angle measurement cannot be applicable to hydrated polymers in an aqueous medium. This is due to the problem that we need a stable surface to measure the contact angle. Moreover, the adsorption on a coated curved surface is not comparable to a flat polymer film, and this technique is not realistic for adsorption on colloids in a dispersion medium.

One common technique for measurement of hydrophobicity uses a hydrophobic dye, Rose Bengal, that is adsorbed onto the surface and the kinetics of adsorption is determined at 542.7 nm. A major problem with this technique is that Rose Bengal adsorbs onto any surface indiscriminately and, therefore, it has to be accounted for completely in order to accurately determine the surface hydrophobicity. Briefly, the particulate systems are incubated with Rose Bengal dye solution in 0.1 M phosphate buffer (pH 7.4) for 3 h. The particles are then centrifuged for 1–2 h at 20,000 rpm to obtain the concentration of free Rose Bengal dye in the supernatant that is determined spectrophotometrically at 542.7 nm. The binding constant is calculated from the Scatchard plot for the equation (Davis et al. 1986):

$$\frac{R}{a} = KN - Kr \tag{6.4}$$

where r, a, K, and N represent the amount of Rose Bengal adsorbed, the equilibrium concentration of Rose Bengal, the binding constant, and the maximum amount bound, respectively. As the study of adsorption isotherm is a time-consuming process, Muller et al. (Lukowski et al. 1992; Muller et al. 1986) suggested a method to identify Rose Bengal adsorption and partition quotient, PQ:

$$PQ = \frac{A_s}{A_d} \tag{6.5}$$

where A_s and A_d represent the amount of Rose Bengal bound to the surface and the amount of Rose Bengal in dispersion medium, respectively.

For partitioning experiments, the concentration of Rose Bengal is kept constant while the concentration of particles in dispersion is changed. A plot of PQ versus the particle surface area yields a slope that can be taken as a measure of hydrophobicity. The major problem of Rose Bengal method is that it is just an average hydrophobicity value and the subpopulations might have a quite different hydrophobicity. Most of the experiments were reported with polystyrene particles which are fairly hydrophobic in nature, however, the most commonly used particles in recent years such as poly (lactide-co-glycolide) are not that much hydrophobic thereby the Rose Bengal method may not be applicable to them. Moreover, this method cannot measure the hydrophobicity of O/W emulsions and liposomes as they adsorb negligible amount of Rose Bengal.

An alternative to the above method is hydrophobic interaction chromatography (HIC) (Hofstee 1973; Pahlman et al. 1977). HIC is a liquid chromatographic technique that is widely used in biochemistry, microbiology, and immunology to separate proteins or particulates based on their hydrophobicity on hydrophobic gel matrix. The particles are characterized in their original dispersion and elution profile provides information on the subpopulation in the sample. Mostly alkyl-agarose is used as the stationary phase and the alkyl chain determines the hydrophobicity of the matrix. This method has been successfully used for determination of surfactant coated nanoparticles (Carstensen et al. 1991), and it was observed that the reduction in surface hydrophobicity is directly related to the increasing length of polyoxyethylene chain for poloxamer and poloxamine polymers on a polystyrene surface. It has also been shown that a number of factors can affect the hydrophobic interaction, pH (Hjerten et al. 1986), temperature (Jennissen 1978), and buffer concentration (Melander and Horvath 1977). The instrument is simple and uses a pump, a large-scale chromatography column filled with alkyl-agarose, and an online UV detector. The method reported by Stolnik et al. using biodegradable colloidal particles coated with poloxamer and poloxamine (Stolnik et al. 1994). In addition, Wallis and Muller (Wallis and Muller 1993) reported a rather inexpensive version of the HIC that uses a Pasteur pipette as the column, plugged with cotton wool and filled with 1 ml hydrophobic alkyl-agarose gel. The sample is directly layered on the gel and distinct fractions (e.g. 0.33 ml) collected and detected using UV/Vis spectrophotometer. To control the eluted volume, the UV/Vis cuvette may be placed on a digital balance.

In addition, surface analysis methods such as X-ray photoelectron spectroscopy can be used for determination of poloxamer and poloxamine surfactants on the surface of nanoparticles (Das et al. 1995). The adsorption of proteins can be identified and quantitated using 2-D electrophoresis that can play a major role in the establishment of correlation of the surface characteristics, adsorbed proteins, and in vivo distribution of the drug carriers (Blunk et al. 1993). Recently, a study explored alternative methods for determination of protein adsorption due to hydrophobicity and concluded that polycaprolactone nanoparticles are prone to higher level of opsonization compared to polylactide-co-glycolide nanoparticles due to their comparative hydrophobicity (Ndumiso et al. 2020).

6.6.4 In Vitro and In Vivo Study

In order to study the in vitro phagocytosis, surface-coated particles are evaluated for uptake by cells against a control of non-coated particles. Kupffer cells appear to be the best for this purpose but are difficult to obtain. The cell lines that are reported to be useful for these studies are peritoneal macrophages (Illum et al. 1987) and human granulocytes (Rudt and Muller 1993). De Jaeghere et al. (De Jaeghere et al. 2000) studied PLA nanoparticles, surface modified with PEO, using the phagocytic human U937 cell line and confocal fluorescence microscopy. It was found that the premature capture of nanoparticles by the cells of the MPS can be avoided by sterically stabilizing their surface with a layer of PEO. The best protective coating was obtained using the "loop conformation" (PLA-PEO6000-PLA triblock copolymer) or the "brush conformation" (PLA–PEO 6000 diblock copolymer). Although the earlier studies report the use of particles labeled with ^{131}I, later reports mostly use the chemiluminescence method (Rudt and Muller 1992). Muller et al. (1997) observed that HL60 cell lines, differentiated to phagocytic cells, can be used as an alternative to human granulocytes for phagocytosis study.

The distribution of surface-modified particles in animals has been reported by a number of investigators. Roser et al. used magnetic resonance imaging (MRI) techniques to track the particles and concluded that in vivo fate of albumin nanoparticles is significantly influenced by factors not reflected in the in vitro cell culture models (Roser et al. 1998). Zambaux et al. studied the involvement of neutrophil granulocytes in the uptake of monomethoxypoly (ethylene oxide)-poly (lactic acid) nanoparticles in guinea pigs and observed the important role of neutrophilic granulocytes as particle-capturing cells and that of the spleen as a degradation organ of stealth biodegradable nanoparticles (Zambaux et al. 2000).

6.7 Summary and Outlooks

Polymers are one group of materials that have been extensively used in the development of carriers for therapeutics delivery (Lai and He 2016; Lai and Shum 2015; Lai 2011, 2013; Lai and Lin 2009). In this chapter, we have introduced the use of polyanhydride, as well as lactide and glycolide polymers, in drug delivery. Representative strategies of nanoparticle synthesis have been also been presented, followed by a discussion of important parameters to be considered for optimization. Regarding the rapid advances in polymer chemistry and engineering, it is expected that the development and use of polymeric nanoparticles will continue to play an important role in research on systemic delivery in the forthcoming decades. Despite this, currently large-scale methods for production of polymeric particles are lacking. This may become a barrier to the clinical transition of the use of polymeric nanoparticles from research to practice in the future. Development of technologies for large-scale production of polymeric nanoparticulate systems is in dire need.

In addition, due to the lack of suitable large-scale methods for production of polymeric particles at the moment, lipids or lipid-based carriers have gained a lot of popularity over the last several decades. In fact, literature in the early eighties first reported the concept of drug delivery using lipid vesicles. In the context of surface modification, this area has advanced far ahead of the other carrier systems. A text edited by Lasic and Martin (Lasic and Martin 1995) discusses the major issues of surface-modified liposomes in great detail, for which reason they will not be repeated here. A number of researchers have also reported the efficacy of solid lipid nanoparticles (SLN) as drug carriers compared to other types of carrier systems (Muller 1991). One important advantage of SLN is the ease of modifying its surface characteristics by adsorption of nonionic surfactants. As an important class of materials alternative to polymers, lipids have been playing an important role in the progress of drug delivery research over the last several decades. Advances in the design and optimization of lipid-based carriers will be discussed in detail in Chap. 8.

Important Notes

- Biodegradable polymers offer several advantages over non-biodegradable counterparts in developing nanoparticles for systemic drug delivery.
- The process for drug entrapment should be selected based on the solubility of the active ingredient in water. Most popular approach for the development of nanoparticles involves W/O/W solvent evaporation method. However, drug entrapment might be low for highly hydrophilic drugs.
- Method of preparation significantly affects the size range of the nanoparticles produced. The biggest challenge is to produce particles in the small size range, reproducibly.
- Hydrophilicity of the surface is determined by the nature of the entrapping polymer and additives used.
- Essential physicochemical parameters to be evaluated for every nanoparticle batches are size distribution, surface charge, and hydrophilicity of the surface.
- Careful selection of polymer is essential to achieve bulk versus surface erosion and release of active ingredients.

Questions for Future Research

- **How to produce nanoparticles, in large scale, in small particle size range with narrow size distribution?** Nanoparticles made by biodegradable polymers show high potential for applications in systemic delivery of therapeutic agents to mediate biogerontological interventions. To translate the use of these nanoparticles from concept to reality, large-scale production is required. Development of strategies to enable large-scale production of

nanoaprticle with a narrow size distribution is, therefore, one of the future directions that warrant research efforts.

- **How to increase loading of active drugs in the nanoparticles?** Although nanoparticles can increase the efficiency of systemic therapeutics delivery, their poor loading efficiency is one of the major problems that impeded their practical use. Solving this problem will help streamline the applications of nanoparticles in intervention execution.
- **How to optimize the production of nanoparticles with minimal residual solvents and additives?** Nanoparticles generated from biodegradable polymers generally raise fewer safety concerns compared to those generated from non-biodegradable synthetic polymers. However, organic solvents and additives may sometimes be adopted during the nanoparticle fabrication process. This will result in toxicity if those solvents and additives fail to be removed completely. Effective techniques to detect residual solvents and additives, and to separate any unreacted agents from the resultant nanoparticles, is in dire need before clinical use.

Glossary

Atomization A process of breaking bulk liquids into small droplets.

Biodegradable polymers Polymers that degrade in the human and animal body and their degradation products are non-toxic.

Coacervation technique A technique that involves the separation of a liquid phase of coating material from a polymeric solution and precipitation with the suspended core particles.

Dielectric constant A measure of a substance's ability to insulate charges from each other. Taken as a measure of solvent polarity, higher ε means higher polarity, and greater ability to stabilize charges.

Diffusion coefficient A proportionality constant between the molar flux due to molecular diffusion and the gradient in the concentration of the species.

Glass transition temperature The temperature at which certain polymers are rubbery (flexible or soft) but not in a completely molten state.

Hydrophilic polymeric matrix A homogeneous aqueous soluble polymeric section.

Krebs cycle The sequence of reactions by which most living cells generate energy during the process of aerobic respiration. It takes place in the mitochondria, consuming oxygen, producing carbon dioxide and water as waste products, and converting ADP to energy-rich ATP.

Supercritical fluid A substance at a temperature and pressure above its critical point. It can behave like a gas, and dissolve materials like a liquid.

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Chapter 7
Use of Nanoparticulate Systems for Tackling Neurological Aging

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Abstract In the last chapter, Das and Das have presented the advantages of biodegradable polymeric nanoparticles in biomedical use, and have also provided an overview of general methods of fabricating and designing nanoparticles. In this chapter, we will continue to talk about nanoparticles as carriers of therapeutics, but will narrow down the focus of the discussions to the context of neurological aging. In fact, neurological disorders such as Parkinson's disease, Alzheimer's diseases, neuropathic pain, and cerebrovascular accidents affect approximately 1.5 billion people globally. Over the years, an array of bioactive molecules have been found to be effective for the treatment of neurological conditions but not to be clinically effective due to the presence of the blood–brain barrier (BBB). The BBB is primarily responsible for the separation of extracellular fluid and blood within the CNS, generating a selectively permeable barrier restricting the passage of an array of substances, such as drugs, biomolecules, and potentially pathogenic substances. Nanomedicine is an attractive non-invasive technology that can be employed to circumvent this barrier. In this chapter, we will review the current advancements and limitations for the employment of nanomedicine to treat neurological diseases, and will also delineate the clinical and regulatory requirement for market entry of these products.

Keywords Nanomedicine · Neurological disorders · Drug delivery · Clinical applications · Blood–brain barrier · Legislative requirements

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7.1 Introduction

The incidence and prevalence of central nervous system diseases and disorders are significantly increasing globally, with a 6–8% global socioeconomic burden attributed from neurological diseases and disorders (Nair et al. 2016). There is significant evidence that isolates neurological diseases as a one of the largest threats to public health. While there is inadequate information for understanding the underlying mechanisms contributing to the incidence of neurological diseases, there is well-established data supporting therapeutic approaches toward its treatment (Albert 2007). Advancements in current therapeutic approaches have significantly contributed to the treatment and management of neurological diseases, however, the complete cure for a vast majority of these diseases (example: stroke, Parkinson's disease, Alzheimer's disease, and neuropathic pain) are unavailable (Kaushik et al. 2014). The blood–brain barrier and its restrictive transport of biomaterials to the brain are the main hurdles in improving treatment efficacy for neurological diseases. Other challenges include the invasive nature of some therapies such as deep brain stimulation and direct local application of drugs into the brain (Kaushik et al. 2016). To overcome some of the limitations researchers have focused on the development of novel drugs and drug delivery technology for improved clinical outcomes. Over the past decade, nanomedicine has received significant attention, to circumvent some of the limitations associated with conventional treatment. Integrating the techniques and concepts of nanotechnology with biological medicine, pharmaceutical scientists have coined the term "nanomedicine". The size of nanomaterials demonstrates close structural resemblance to biological molecules and structures, which directs nanomedicines as an attractive technology for biomedical applications and research. This integration of nanomaterials with biology has led to interesting and novel applications such as tools for enhanced diagnosis, contrast agents, and drug delivery technology (Peluffo et al. 2015; Reynolds and Mahato 2017; Carradori et al. 2016).

Nanomedicine demonstrates an array of advantages attributed from their unique physicochemical properties and phenomena exhibited at their smaller particle size. However, the need for research to be directed and focused on the fate and distribution of nanoparticles in biological systems is imperative. The risk and safety profiles should be clearly outlined and support the overall application of nanoparticles in biomedicine (Xie et al. 2019; Furtado et al. 2018).

7.2 Nanosystems for Neurological Drug Delivery

Nanosystems have received considerable attention over the past decade as an attractive material for drug delivery technology and commonly termed "nanomedicine". Nanomedicine is nanoparticle-based formulations intended to provide a therapeutic outcome exploiting the nanoparticle size range of 1–100 nm. The application of nanomaterials provides an array of advantages to modify fundamental properties

such as solubility, blood circulation time, drug release properties, and immunogenicity as demonstrated by conventional therapeutic approaches. Nanoparticles allow for an increased efficacy, less invasive routes of administration, decrease drug toxicity, improve product lifecycle thereby reducing the overall healthcare costs (Brannon-Peppas and Blanchette 2004; Hwang and Kim 2014).

Drug delivery to the CNS has been vastly impeded by the transport of therapeutically effective and an optimal quantity of drug across the BBB, without disruption of the BBB that is required for the maintenance of normal CNS hemostasis (Daneman et al. 2010). Integrating the techniques and concepts of nanotechnology with biological medicine, pharmaceutical scientists have coined the term "nanomedicine". The size of nanomaterials demonstrates close structural resemblance to biological molecules and structures, which directs nanomedicines as an attractive technology for biomedical applications and research. This integration of nanomaterials with biology has led to interesting and novel applications such as tools for enhanced diagnosis, contrast agents, and drug delivery technology (Xie et al. 2019; Furtado et al. 2018). Commonly employed nanosystems for neurological drug delivery include nanoliposomes, nanoemulsions, carbon nanotubes, and nanohydrogels.

Nanosystems exhibit interesting properties that allow for novel and enhanced therapeutic applications. Firstly, nanosystems allow for the incorporation of an array of different biological molecules, genes, and active pharmaceutical molecules that allow for the sustained delivery of these molecules by conferring stability during circulation. Second, **surface functionalization** of the nanosystem derived particle for the site-specific delivery of drug to the desired pathological cell, which allows for a decrease in the overall drug consumption and adverse reactions associated with the drug of choice. Thirdly, the distinct optical and electrical properties of nanosystems can be tailored to meet the specific requirements in certain biomedical applications. Integrating these unique properties of nanosystems, it can be tailored and formulated specifically to cross the BBB for the treatment of an array of neurological conditions such as neuropathic pain, Parkinson's and Alzheimer's disease or cerebrovascular accidents (Singh et al. 2016; Ljubimova et al. 2017; Das et al. 2016).

The systemic administration of nanoparticle-based nanosystems, is a common technique employed, that involves the administration of the nanosystem into the patient's circulatory system (example: intravenous administration). Systemically administered nanoparticle-based nanosystems circulate throughout the bloodstream and at a sufficient concentration exert a pharmacological effect at the site of the diseased tissue or cell. Prior to the nanoparticle accumulation at the diseased tissue, an array of critical physiological processes occurs (Narum et al. 2020). Exposure of nanoparticle-based nanosystems within the bloodstream results in the formation of a **protein corona**. The protein corona provides a surface coating to the nanoparticles, which is responsible for interacting with opsonins, which direct nanoparticles to phagocytic cells of the mononuclear phagocyte system (MPS). The MPS is comprised of several organs that include the liver and spleen. The Kupffer cells of the liver metabolizes and removes nanoparticles from circulation, therefore, preventing the sufficient accumulation of nanoparticles at the site of diseased tissue. The MPS serves as a significant barrier that needs to be overcome for the successful systemic

delivery of nanoparticles (Tavares et al. 2017; Zhang et al. 2016; Shen et al. 2018; Iyer et al. 2006; Maeda 2001).

The fate and journey of nanoparticles-based nanosystems across complex biological membranes such as the blood–brain barrier requires the optimal formulation and design of nanoparticles (Fig. 7.1). The physicochemical properties of nanoparticles-based nanosystems, such as size, shape, surface morphology and functionality, and stiffness (the '4S parameters of nanoparticles) significantly contribute to the pharmacokinetic responses (Table 7.1) (Liu et al. 2012; Lee et al. 2009; Gentile et al. 2008; Anselmo and Mitragotri 2017; Merkel et al. 2011; Molineux 2002), affecting nanoparticle circulation and distribution (Fig. 7.2). The nature of these parameters can be modified and precisely controlled during chemical synthesis, to overcome biological limitations (Iyer et al. 2006; Maeda 2001).

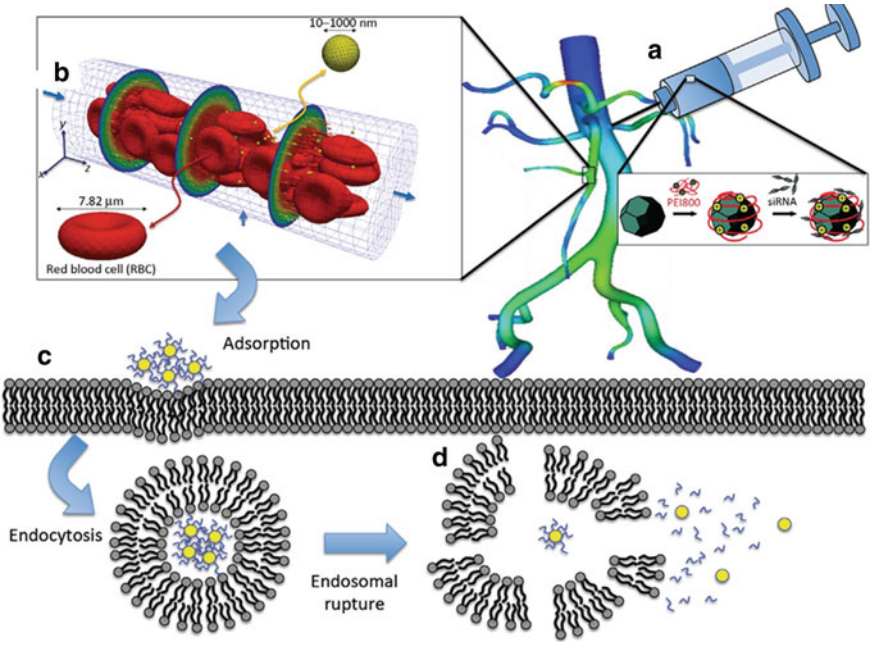

Fig. 7.1 Diagrammatic representation of the fate of nanoparticle-based nanosystems. **a** Systemic administration of nanoparticle-based nanosystems. **b** Microvascular representation of interaction between red-blood cells and the administered nanoparticles. **c** Nanoparticles diffusion into the extracellular matrix followed by absorption onto the surface membrane of the targeted cell. **d** Rupture and disassociation of the nanoparticle releasing the therapeutic agent. Reproduced from (Shen et al. 2018) with permission from Elsevier

Table 7.1 Parameters affecting the pharmacokinetic profile of nanoparticles

Parameter	Pharmacokinetic effect on nanoparticle
Size	Nanoparticles with a particle size diameter below 10 nm are subjected to renal extravasation and removed from bloodstream circulation, whereas nanoparticles with a particle size diameter greater than 200 nm are significantly filtered by the spleen, liver, and bone marrow. Ideally, nanoparticles should be formulated in the particle size range of 10 nm-200 nm (Liu et al. 2012)
Shape	While nanoparticles can be formulated and designed into an array of different structures such as cubes, spheres, rods, and discs. Drug or biomaterial-loaded nanoparticles should demonstrate the ability of margination, or the ability to drift laterally toward the vessel walls (Lee et al. 2009). In a study, it was determined that nanoparticles with a discoidal shape demonstrated greater lateral drift as compared to nanoparticles of different shapes under identical hydrodynamic forces (Gentile et al. 2008)
Stiffness	The mechanical properties of nanoparticles such as stiffness or deformability is a critical parameter that contributes to the density of nanoparticles for drug delivery (Anselmo and Mitragotri 2017). The elastic modulus is one of the principal properties that govern the biodistribution of nanoparticles and blood circulation time. It was demonstrated that an eightfold decrease in the hydrogel microparticles demonstrated a 30-fold increase in the elimination of these particles. Decreasing the modulus of nanoparticles modifies their biodistribution profile, allowing for an enhanced EPR effect (Merkel et al. 2011)
Surface functionality	Surface functionality is regarded as the most critical parameter that needs to consider for nanoparticle formulation and design. For example, the hydrophobic nature of inorganic nanoparticles (Au or Ag-based nanoparticles) aggregate in an aqueous medium and adhere to serum proteins, consequently these nanoparticles will be subjected to immune degradation. However, the incorporation of poly(ethylenegycol) grated onto the surface significantly circumvents the aforementioned problem (Molineux 2002)

7.2.1 Nanoliposomes

Nanoliposomes (Fig. 7.3) demonstrate the ability to circumvent the limitations associated with traditional brain-drug delivery which is attributed to the array of unique physical and chemical properties. Optimizing the ideal nanoliposome candidate for CNS drug delivery creates promising applications for the treatment of neurological diseases. Over the past decade, significant advances and formulations have emerged for enhancing drug delivery across the BBB (Vieira and Gamarra 2016).

The positive charge of cationic nanoliposomes can be exploited for improved neurological drug delivery resulting from the electrostatic interaction between the negatively charged mucous membrane and the cationic nature of the nanoliposome. This electrostatic interaction promotes adsorptive-mediated endocytosis of the nanoliposome (Joshi et al. 2014a, b).

The surface functionalization of nanoliposomes can significantly improve the biodistribution and pharmacokinetic properties of drug molecules for delivery across the BBB. The common polyether, polyethylene glycol (PEG) forms an armored

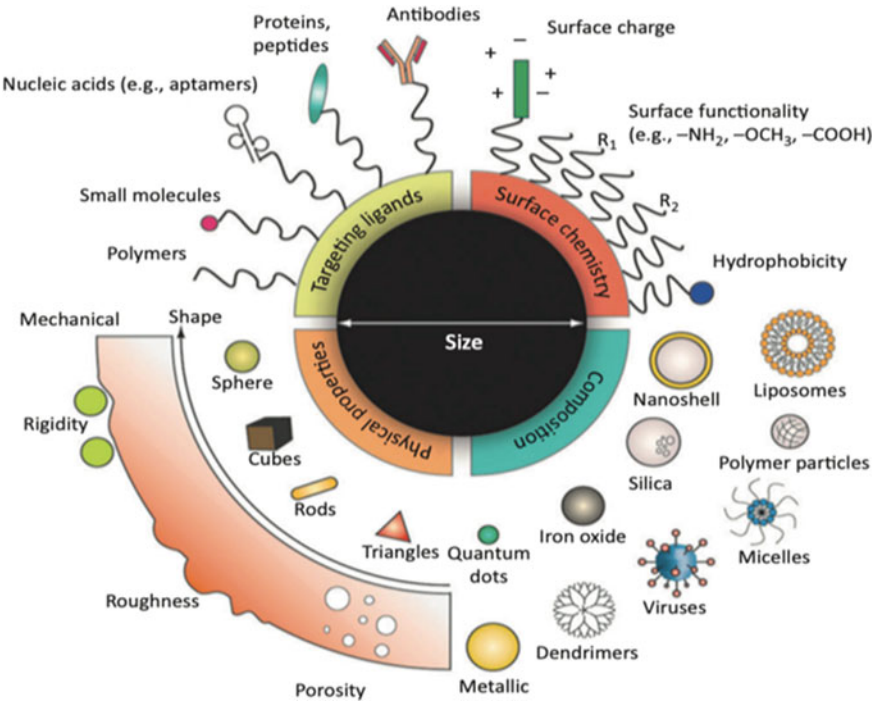

Fig. 7.2 The 4S parameters (size, strength, shape, and surface modifications) critical of the formulation and design of nanoparticles. Reproduced from (Shen et al. 2018) with permission from Elsevier

coating around the surface of the nanoliposomes. Polyethylene glycol is responsible for conferring the ability of nanoliposomes to evade hepatic and immunological degradation, decreasing the clearance of the drug vehicle (Charoensit et al. 2019). While PEG is responsible for increasing circulation time, it also enables nanoliposomes to cross the BBB (Kauscher et al. 2019).

Surface functionalization of nanoliposomes is an effective modification that allows drug transport across the BBB, however, the incorporation of stimuli-responsive properties can further improve drug delivery to the targeted site. Engineering nanoliposomes with bio-responsive nanomaterials, which respond to physiological changes such as changes in pH, redox environment, and the presence of enzymes can address the challenge of drug delivery (Kauscher et al. 2019). For example, the formulation of thermo-responsive nanoliposomes can be produced upon the modulation of the lipid concentration to alter the phase transition temperature. This results in conformational changes in the lipid bilayer, where around the melting point temperature of the lipid, the fatty chains develop into a gel-like state contributing to a "leaky" membrane (Shah 2016). Nanoliposomes can be engineered to exhibit multifunctional capabilities within a single structure for effective disease

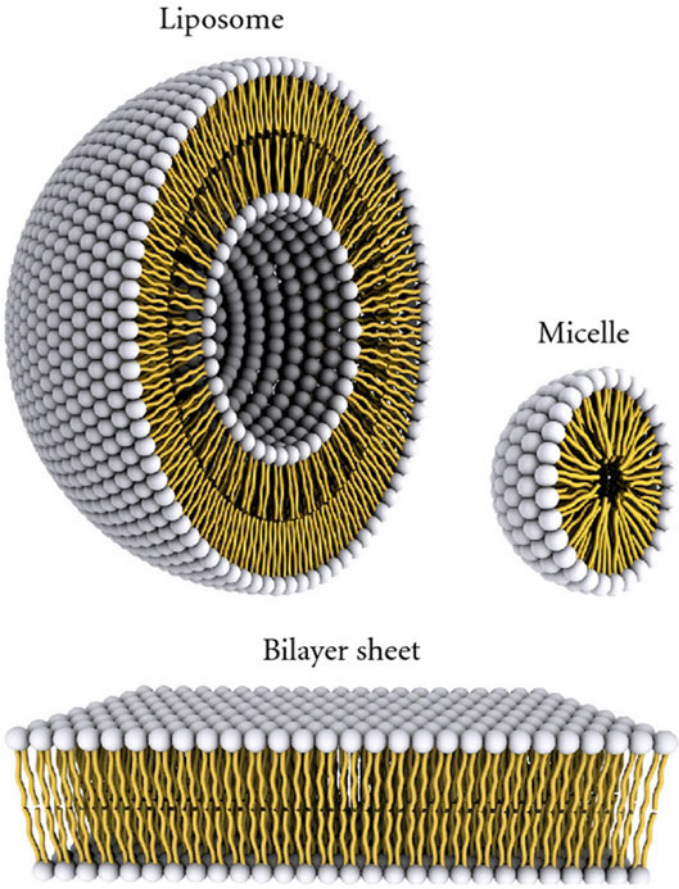

Fig. 7.3 Amphiphiles, which are chemical compounds that exist with the presence of hydrophobic and hydrophilic moieties, exhibit self-assembly to reduce unfavorable interactions between its hydrophobic moiety and surrounding aqueous environment into well-defined structures such as nanoliposomes, micelles, or lipid bilayers. Reproduced from (Shah 2016) with permission from Springer Nature

treatment, diagnosis, and maintenance. Theranostic nanoliposomes are single vesicles that incorporate diagnostic and therapeutic agents (Charoensit et al. 2019). An example of theranostic nanoliposomes was recently formulated. Nanoliposomes were formulated to incorporate **quantum dots** and docetaxel for tissue imaging and brain tumor therapy (Kauscher et al. 2019). More examples of nanoliposome applications for the treatment of neurological diseases are shown in Table 7.2 (Zhao et al. 2014; Liu et al. 2014; Luca et al. 2015; Rashed et al. 2017).

Table 7.2 Nanoliposome applications for the treatment of neurological diseases

Targeting moiety or Target tissue	Biological or Drug molecule	Nanoliposomes attributes	References
Magnetic field	Amphotericin B	Amphotericin B-loaded magnetic nanoliposomes provide an enhanced targeted neurological drug delivery system and reduced side-effect profile for drugs administered via the carotid artery under the influence magnetic field	Zhao et al. (2014)
R8-RDG tandem peptide (conjugation of ligand cyclic RDG to cell-penetrating peptide R8)	Paclitaxel	The conjugation of the R8-RDG peptide improved cellular uptake up to 30-fold, as compared to the individual peptides. This conjugated nanoliposome demonstrated improved permeability across glioma spheres and the blood–brain barrier	Liu et al. (2014)
Lactoferrin and Anti-transferrin	NK3 receptor agonist, Senktide	The delivery of the hydrophilic peptide and NK3 receptor agonist, Senktide (peptide unable to cross the blood–brain barrier), demonstrated superior transport across the blood–brain barrier when subjected to stealth liposomal encapsulation as compared to pure drug administration. Surface-modified anti-transferrin liposomes compared to lactoferrin liposomes showed superior brain-drug accumulation	Luca et al. (2015)
Transferrin	Docetaxel	The theranostic ability of transferrin-receptor targeted nanoliposomes improves the permeability of docetaxel across the blood–brain barrier	Kauscher et al. (2019)
^{99m}TC	Nimodipine	The ^{99m}TC radiolabelled nimodipine-loaded nanoliposome demonstrated superior drug-targeting efficacy to the brain following an intranasal administration	Rashed et al. (2017)

7.2.2 Nanogels

The movement toward nanogel technology is primarily directed because nanogels inherently bare properties that can be induced for the site-specific delivery of drugs and bioactive molecules to achieve excellent therapeutic outcomes with reduced adverse drug reactions (Vashist et al. 2018). The production of nanogels can be demonstrated by the hybridization of conventional or bulk hydrogels with nanosized materials, which include carbon nanotubes, polyaniline nanosticks, and inorganic clays (Vashist et al. 2017).

Decreasing hydrogel particle size into the submicrometer range these particles can then be referred to as nanogel particles. Nanogel particles are hydrogel particles that are chemically or physically crosslinked hydrophilic polymeric networks that demonstrate advantages such as an effective and attractive interior network for the encapsulation of bioactive molecules, and modifiable size range that allows for an increased surface area which is effective for multivalent **bioconjugation**. Nanogels, which are mostly often hydrophilic, offer the ability to encapsulate guest molecules such as biomacromolecules (DNA and proteins), drugs, or inorganic nanoparticles which confer little to no limitations to the gel-like performance of the drug delivery vehicle. Nanogels also allow for the encapsulation of multiple different guest molecules within the same carrier (Vinogradov et al. 2004,2002; Vinogradov 2010). Nanogels confer exciting attributes such as stimuli-induced behavior, softness, and swelling which allows its network to offer protection of the entrapped bioactive molecule or drug from biopharmaceutical degradation and elimination. Such attributes contribute to the overall controlled and desired trigger responsive activity that results in drug release at the target tissue (Kabanov and Vinogradov 2009). Stimuli such as pH, temperature, magnetic attraction, oxidation and reduction ability, functional molecules such as enzymes, glucose, or a combination of them are useful for the controlled and targeted release of guest molecules such as bioactive molecules and drugs. These changes result in the compartments of the nanogel particle to interact, exchange chemicals, receive energy, produce mechanical work with the resultant changes in particle dimensions, structure and interactions which contribute to the overall drug release profile of the drug delivery vehicle (Vinogradov et al. 2004,2002; Vinogradov 2010; Kabanov and Vinogradov 2009).

Nanogels like any other nanoparticulate drug delivery system demonstrate superiority by exhibiting an array of advantages (Fig. 7.4). However, like any nanoparticulate drug delivery system, there are significant limitations associated with nanohydrogels such as the optimization of biodistribution, degradation and elimination and clinical toxicity.

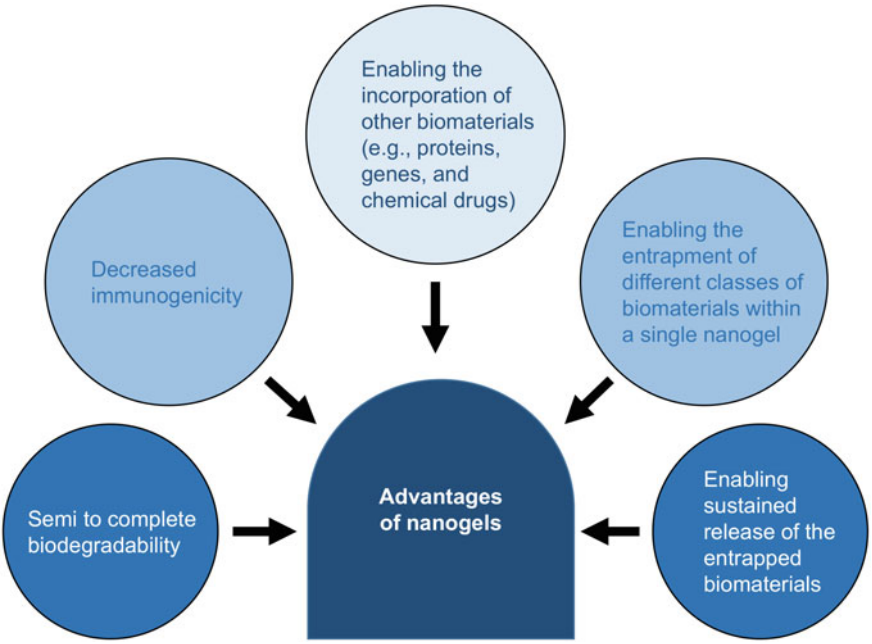

Fig. 7.4 Diagrammatic representation of an array of advantages demonstrated by nanohydrogels as an attractive novel drug delivery technology

7.2.3 Carbon Nanotubes (CNTs)

Carbon nanotubes represent the allotropic form of carbon which belongs to the fullerene family. CNTs exist as elongated and cylindrical molecules that conform to a hexagonal arrangement of hybridized carbon atoms. The cylindrical shape of CNTs is attributed from its fabrication of rolling up a single sheet of **graphene** (single-walled carbon nanotubes, SWCNT) or rolling up multiple sheets of graphene (multi-walled carbon nanotubes, MWCNT) (Fig. 7.5) (Holban et al. 2016; Elhissi et al. 2012). Owing to the advantageous ultrahigh surface area, CNTs are suitable for the delivery of cargo molecules across the blood–brain barrier. The susceptibility of CNTs to undergo surface-engineering (incorporation of organic functional groups to the surface of the CNTs) allows for the manipulation of its physical and biological properties allowing for targeted drug delivery in biomedical applications (Chen et al. 2007). The delivery of drugs is accomplished by the incorporation of the desired molecule onto the surface or the tip of the CNT (Soni et al. 2019). The formulation of polyethylene glycol and lactoferrin customized SWCNT demonstrated significant cellular permeation for the delivery of dopamine to the brain. This study has also reported the attractive ability to surface coat SWCNTs with polyethylene glycol to increase residence time of the drug delivery vehicle within the brain (Guo et al. 2017).

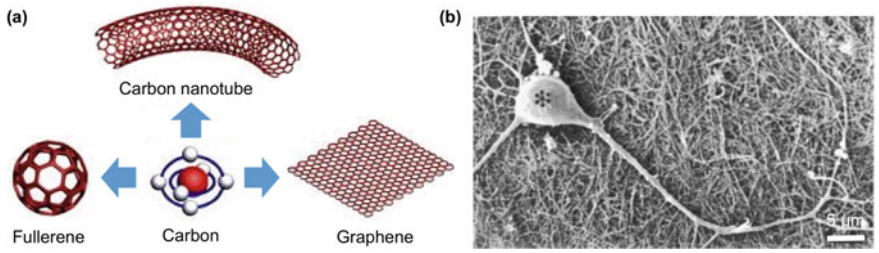

Fig. 7.5 **a** Carbon-based nanotechnology including the formulation of carbon nanotubes, fullerene, and graphene. **b** A scanning electron microscope image of the growth of a hippocampal neuron of embryonic origin on dispersed multiwall carbon nanotubes. Reproduced from (Shah 2016) with permission from Springer Nature

7.2.4 Nanoemulsions

Nanoemulsions are defined as a kinetically stable biphasic colloidal dispersion comprising two immiscible liquids and an emulsifier. The emulsifier is a critical component of the nanoemulsion as it is primarily responsible for decreasing interfacial tension between the oil and water phases of the nanoemulsion for the promotion of decreased particle size and maintaining the stability of the nanosystem through repulsive electrostatic interactions and steric hindrance (Soni et al. 2019). The minute particle size range exhibited by nanoemulsions confers attractive and useful properties such as tunable rheology, optically transparent appearance and increased surface area per unit volume (Gupta et al. 2016). Oil in water nanoemulsions demonstrate significant usefulness in the delivery of hydrophobic drug molecules. Poor water-soluble **risperidone** loaded-nanoemulsions increases the uptake of the active drug moiety to 1.3 times greater as compared to a risperidone solution, across the blood–brain barrier, contributing to the enhanced anti-psychotic activity of risperidone (Đorđević et al. 2017). Quetiapine loaded-nanoemulsions demonstrate superior drug release and drug transport efficacy as compared to the pure drug molecule. This nanosystem is an attractive drug delivery vehicle for targeted delivery to the central nervous system (Boche and Pokharkar 2017). More examples of nanoemulsion applications for the treatment of neurological diseases can be found in Table 7.3 (Đorđević et al. 2017; Boche and Pokharkar 2017; Abdou et al. 2017; Tan et al. 2017).

7.3 Dendrimers

Dendrimers are well-defined three-dimensional, hyperbranched, multivalent, spherical polymeric nanocarriers that exist within the particle size diameter of 1–10 nm. The well-defined spherical architecture of dendrimers confers monodispersity and susceptibility to functional modifications. Dendrimers comprise multiple hydrophobic pockets that allows for the entrapment of an array of biomaterials,

Table 7.3 Nanoemulsion applications for the treatment of neurological diseases

Targeting moiety or Target tissue	Biological or Drug molecule	Nanoemulsion attributes	References
Brain	Risperidone	Risperidone nanoemulsions demonstrated superior brain bioavailability, decreased liver distribution and an increased brain-drug delivery following an intraperitoneal administration	Đorđević et al. (2017)
Brain	Zolmitriptan	The chitosan mucoadhesive nanoemulsion employed to deliver, the anti-migraine drug, zolmitriptan demonstrated an increased $AUC_{0-\infty}$ with a concurrent decrease in the T_{max} within the brain as evaluated in comparison to an intravenously or intranasally administered drug solution	Abdou et al. (2017)
Brain	Valproic acid	Valproic acid-based nanoemulsion demonstrated superior brain bioavailability and decreased incidence of metabolite degradation related hepatoxicity	Tan et al. (2017)
Brain	Quetiapine fumarate	The formulation of a quetiapine fumarate-based nanoemulsion demonstrated an increased drug transport efficacy and direct nose-brain transport was accomplished following intranasal administration. This nanoemulsion based formulation exhibits a decreased T_{max} as compared to conventical administration of quetiapine fumarate	Boche and Pokharkar (2017)

these pockets are responsible for the sustained and controlled drug release nature of dendrimers (Soni et al. 2017; Duncan and Izzo 2005). The architecture of dendrimers consists of three distinct regions, the central core, branches, and the terminal functional groups. The central core contains a single atom or molecule that exists with a minimum of two identical functional groups. The branches radiate from the central core and exist with repeating units that are bridged together by at least one branched junction. The hallmark concentric layer of dendrimers (generations) is attributed from interconnected nature of the branches. The terminal for surface functional group of the dendrimer is responsible for the polymeric properties of the nanocarrier (Vogtle et al. 2009). Swami and co-workers (2015) formulated a docetaxel-loaded p-hydroxyl benzoic acid (pHBA) conjugated nanodendrimers conferred superior drug brain uptake as compared to unconjugated nanodendrimer or the available conventional commercial drug product (Swami et al. 2015). More examples of dendrimers applications for the treatment of neurological diseases are shown in Table 7.4 (Swami et al. 2015; Katare et al. 2015; Patel et al. 2016).

7.4 Polymeric Nanoparticles for Neurological Drug Delivery

The attractive multifaceted characteristics of natural or synthetic polymers make it ideal candidates as drug delivery carriers for nanoparticles. Polymer-based carriers for nanoparticles provides an efficacious and versatile platform that allows for the modifications of nanoparticles to meet specific requirements. These properties include increased drug entrapment efficacy, surface modifications, easy preparation, and protection against degradative enzymes and chemicals. There are two broad classes of polymers that can be employed for nano-neurological drug delivery, synthetic and natural. One of the most notable properties of polymeric nanocarriers is their ability to evade **reticuloendothelial system** (RES) accumulation and increase circulation and residence time. Polymeric nanodevices are susceptible to RES accumulation, therefore, hepatic metabolism, decreasing the drug half-life and therapeutic efficacy, which can be circumvented upon the conjugation of antibodies, proteins, peptides, and receptor-specific ligands (Kanazawa et al. 2017; Jain et al. 2015; Owens and Peppas 2006; Alexis et al. 2008). Table 7.5 illustrates the applications and research surrounding the use of polymeric nanoparticles for neurological drug delivery (Christoper et al. 2014; Jafarieh et al. 2015; Fornaguera et al. 2015).

Table 7.4 Dendrimers applications for the treatment of neurological diseases

Targeting moiety or Target tissue	Biological or Drug molecule	Dendrimer attributes	References
p-hydroxyl benzoic acid (pHBA)	Docetaxel	Docetaxel-loaded pHBA conjugated nanodendrimers conferred superior drug brain uptake as compared to unconjugated nanodendrimer or the available conventional commercial drug product	Swami et al. (2015)
Polyamidoamine dendrimer (PAMAM)	Haloperidol	The formulation of a PAMAM dendrimer with the presence of surface amine groups enabled the targeted drug delivery of haloperidol to the brain following an intranasal or intraperitoneal administration, demonstrated superior drug brain uptake as compared to the formulation drug control	Katare et al. (2015)
Glucosamine, sialic acid and concanavalin A	Paclitaxel	The formulation of a poly(propyleneimine) (PPI) nanodendrimer, surfaced modified with glucosamine, sialic acid, and concanavalin A ligands contributed to the enhanced biodistribution and increased accumulation of paclitaxel in the brain as compared to free paclitaxel	Patel et al. (2016)

Table 7.5 Polymeric Nanoparticle applications for the treatment of neurological diseases

Targeting moiety or Target tissue	Biological or Drug molecule	Polymeric nanoparticle attributes	References
Tween-80 surface coated PLGA nanoparticles	Zidovudine	Zidovudine-loaded PLGA nanoparticles coated with Tween-80 demonstrated higher brain-drug accumulation as compared to uncoated nanoparticles and conventional commercial drug product. The higher brain-drug accumulation can be attributed to the ability of the surface coating Tween-80 to adsorb apolipoprotein B and E intravascularly, which is responsible for inducing endocytosis medicated drug delivery	Christoper et al. (2014)
Chitosan-based nanoparticles	Ropinirole	The design of ropinirole-loaded chitosan mucoadhesive nanoparticles demonstrated an increased brain-drug accumulation as compared to the free drug solution. The mucoadhesive nature of chitosan enabled the drug delivery systems to have greater retention at the site of administration and allow for controlled drug release through mucosal membranes as compared to the free drug solution	Jafarieh et al. (2015)
8D3 antibodies	Loperamide	This study demonstrated the incorporation of a 8D3 monoclonal anti-body onto the surface of a PLGA nanoparticle. The 8D3 monoclonal anti-body, is an anti-body against the transferrin receptor, which is over-expressed on the blood–brain barrier. The incorporation of the monoclonal anti-body as a targeting ligand increases the permeability of the nanoparticle across the blood–brain barrier	Fornaguera et al. (2015)

7.4.1 Natural Polymers

7.4.1.1 Chitosan

Chitosan is a β 1–4 glucosamine oligomer which is derived from the deacetylation of chitin (Fig. 7.6), which primarily exists as a non-toxic and abundantly occurring polymer in nature (Du et al. 2019; Ai et al. 2012; Qi et al. 2004; Kendra and Hadwiger 1984). Chitosan and its derivatives demonstrate an array of advantages that include **biodegradability**, excellent biocompatibility, and extensive therapeutics applications such as but not limited to, anti-bacterial, anti-fungicidal, anti-viral, the potential to decrease blood glucose concentration and sterilization (Du et al. 2019; Ai et al. 2012). Chitosan is an attractive pharmaceutical biomaterial for drug delivery that can be exploited for its ability to confer control release properties, improved drug solubility, stability, and bioavailability while concurrently decreasing drug toxicity (Vivek et al. 2014; Nagpal et al. 2013).

Another attractive property of chitosan is its cationic polyelectrolyte nature which confers a strong electrostatic interaction with mucous or a negatively charged mucosal surface, this increased adhesive properties enhance drug internalization into target cells (He et al. 1998; Aspden et al. 1996). The mucoadhesive nature of chitosan was studied where the negatively charged sialic groups of mucin demonstrated the ability to form ionic interactions with positively charged outer surface of chitosan nanoparticles (Bhatta et al. 2012). This cationic property can also be exploited for the delivery of small interfering RNA (siRNA) for gene therapy. The polyanionic nature and large molecular weight of siRNA confers the inability of siRNA to freely cross the plasma membrane. The ability to complex nucleic acid with cationic chitosan nanoparticles can provide as a solution to circumvent gene delivery (Rudzinski and Aminabhavi 2010; Zhang et al. 2019).

The inherent chemical attributes of chitosan restrict its solubilization to an acidic environment only, whereas chitosan in a medium of neutral pH will not undergo solubilization. This property can be exploited for stimuli-responsive drug release. A recent study demonstrated the use of a transferrin receptor-targeted, bio-adhesive d-α- tocopheryl glycol succinate 1000 (TPGS)-chitosan micelles for drug delivery of docetaxel (DTX) for brain cell cancer. This study demonstrated the ability to

Fig. 7.6 Deacetylation of chitin to chitosan for application in neurological drug delivery platforms. Reproduced from (Rudzinski and Aminabhavi 2010) with permission from Elsevier B.V

exploit the protonation of the amino groups of chitosan in an acidic medium, since the intracellular pH of mature endosomes of cancer cells is acidic (~pH of 5.5), therefore, at acidic pHs this chitosan-based micelle has the ability to undergo dissolution and allow for the release of drug to site-specific cancer cells (Agrawal et al. 2017).

While the chemical properties of chitosan limit its dissolution in an acidic medium, the abundance of amino and hydroxyl groups of chitosan serves as target moieties for an array of chemical modifications that can improve aqueous solubility (Zhang et al. 2019). Quaternized chitosan's have been developed to enhance the water absorption capacity, bio adhesiveness, and permeation at physiological pH mediums, such applications include the development of trimethylated chitosan-graft-poly(ε-caprolactone) nanoparticle. This drug delivery vehicle demonstrated stability at physiological pH and enhanced gene vector ability as compared to native chitosan (Tang et al. 2014; Gruškienė et al. 2013).

Another study reported the advantages of chitosan as an effective drug delivery vehicle. Nagpal et al. (2013) demonstrated the formulation of a Tween-80® rivastigmine-loaded chitosan-based nanoparticle for the treatment of Alzheimer's Disease. The study reported that an increase in the concentration of chitosan resulted in an increase in the formulation's zeta potential, increasing the energy required for the coalescence of two or more particles, conferring greater physical stability. This also reported that an increase in the surfactant concentration of Tween-80®, there was a corresponding decrease in particle size, which creates the potential for effective formulation transport across the highly restrictive blood–brain barrier. Tween-80® also confers targeted brain delivery by binding to Apo-E proteins in the blood plasma. This Apo-E-nanoparticle conjugate binds to endothelial cells of the BBB and undergoes transcytosis across the endothelium. The conjugation of the chitosan-based nanoparticle with Tween-80®, also confers the ability of the system to evade the phagocytic action of the Sertoli cells, allows for increased drug circulation, retention, and controlled drug release (Nagpal et al. 2013).

7.4.1.2 Cellulose

Cellulose exists in nature as an abundant and an inexhaustible polysaccharide that consists of two repeating anhydroglucose monomers, β-glucopyranose, which chemically bonded through a 1-4 glucosidic bond (García-González et al. 2011). The attractive polymeric nature of cellulose can be exploited for an array of biomedical applications such as drug delivery, wound healing, and tissue engineering. Favorable chemical and physical properties of cellulose include good biodegradability and good biocompatibility. Cellulose was traditionally extracted from high fiber containing plants. It was responsible for plant growth, structural organization and the maintenance of tensile strength (Qazanfarzadeh and Kadivar 2016). Owing to the unique and exciting properties of cellulose, it has become an attractive polymer for the development of an environmentally friendly, biocompatible, and an effective matrix for the encapsulation of nanoparticles. Cellulose is a versatile polymer that

can be chemically tailored to meet the requirements of advanced delivery systems (Biliuta and Coseri 2019).

To enhance the ability to employ cellulose as a nanofiller, cellulose is subjected to hydrolysis and pretreated with a strong acid such as hydrochloric acid or sulfuric acid. The process of hydrolysis involves the esterification of the surface hydroxyl groups and the introduction of sulfur groups to cellulose. The resultant effect is the digestion of the amorphous domains of cellulose. This process is useful in the production of crystalline cellulose nanofibers or nanocrystals. While the hydrolysis process confers good crystallinity, the resultant material demonstrates diminished thermostability (Martínez-Sanz et al. 2011b). Neutralization of the acid sulfur groups of the cellulosic material with NaOH improves the material's thermostability (Martínez-Sanz et al. 2011a).

7.4.1.3 Sodium Alginate

Sodium alginate exists in nature as an anionic, water soluble, and linear polysaccharide which is primarily sourced from brown algae. This linear polysaccharide is comprised of two monomeric units, α-(1-4)-l-guluronic acid (G) and β-(1-4)-d-mannuronic acid (M). Alginate demonstrates the excellent ability to undergo **ionotropic gelation** with divalent cations (Ca^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+}, and Ba^{2+}) to produce stable hydrogels under safe and mild conditions (Wang et al. 2011; Donati et al. 2005).

Sodium alginate is commonly employed as a drug carrier owing to its biocompatibility, biodegradability, non-immunogenicity, and drug encapsulating efficacy (Li et al. 2011). The encapsulation of hydrophobic molecules is impaired by the presence of hydroxyl and carboxyl groups present on the chemical backbone of alginate, which decreases the affinity of hydrophobic molecules (Yang et al. 2011). A recent study reported the use of bacterial cellulose nanocrystals as a Pickering emulsifier to improve the drug loading capacity and stability of alginate as a delivery vehicle (Yan et al. 2019). Another study reported the ability of alginate hybridized with chitosan nanoparticles to encapsulate and deliver extremely hydrophobic drug molecules while increasing the **mucoadhesiveness**, bioavailability, and controlled and site-specific release (Niaz et al. 2016). Another study exploited the advantages of alginate and its gelling properties and the techniques of microfluidics and reported the formulation and design of alginate nanoparticles, with particle size diameters ranging between 10 and 300 nm (Rondeau and Cooper-White 2008).

7.4.2 Synthetic Polymers

7.4.2.1 Poly(ethylene) Glycol

Poly (ethylene) glycol (PEG) exists as a linear or branched neutral polyether that demonstrates solubility in water and most organic solvents and is available in a variety of molecular weights (Fig. 7.7). PEG is an attractive polymer in the biotechnical and biomedical arena owing to its interesting properties. PEG is unusually effective in the exclusion of other polymers in an aqueous environment, which can be exploited for protein rejection and formation of a two-phase system with other polymers. The remarkable non-toxic nature of this polymer prevents undesirable interactions with proteins and cellular components, while interacting with cellular membranes (Zhu 2010; Peppas et al. 1999; Harris 1992). The favorable properties of PEG, such as poor toxicity and immunogenicity, have conferred an FDA approval as such this polymer is used in an array of pharmaceutical and cosmetic applications (Harris 1992).

PEG polymers are commonly employed as "stealth" polymers in drug delivery. The hydrophilic and flexible nature of PEG polymers allows for the prevention of absorption of serum proteins onto surfaces, this is accomplished by the formation of a thick and dynamic hydration shell encapsulating the surface of the nanoparticle. Consequently, the PEG-grafted nanoparticles demonstrate the ability to evade the mononuclear phagocytic system thereby increasing blood circulation time (Lin

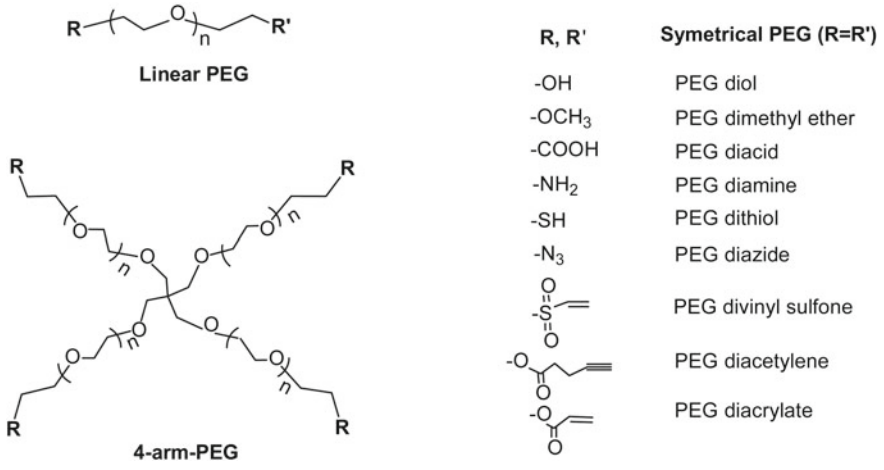

Fig. 7.7 PEG exists as a linear or branched (4-arm) polyether. The basic chemical of structure of the polymer contains a PEG diol, with two hydroxyl functional group ends. The functional group ends of the PEG structure can be converted to methyloxyl, carboxyl, and acrylate group ending. The functional groups confer an array of properties that are useful for different cargo materials. Reproduced from (Zhu 2010) with permission from Elsevier B.V

and Anseth 2009). In a previous study, it was demonstrated that PEGylated nanoliposomes as compared to standard nanoliposomes, that PEGylation increases blood circulation time up to 5 h (Klibanov et al. 1990).

While liposomes have been extensively utilized and adopted as a drug delivery vehicle, liposomes exhibit some challenges such as instability and non-uniform size distribution. The non-uniform size distribution inhibits larger liposomes from exploited the enhanced permeability and retention (EPR) effect. The instability of liposomes can attribute to "burst" drug release during extended blood circulation, contributing to undesirable side-effects (Barenholz 2012; Olson et al. 1979). Shen and co-workers (2016) demonstrated the formulation of a self-assembled core-polyethylene glycol-lipid shell (CPLS), that demonstrated superior stability and size uniformity. The self-assembly process involves the bonding of the free ends of the PEG chains to the anchored lipids, forming a complete lipid bilayer encapsulating the nanoparticle. The nanoparticle core and the PEG polymer chain are connected conferring stability to the drug delivery vehicle (Shen et al. 2017).

7.4.2.2 Poly Lactic-co-Glycolic Acid

Poly lactic-co-glycolic acid (PLGA) is an exciting and promising biomaterial that demonstrates great potential as a drug carrier and as scaffolds in tissue engineering. PLGA is a synthetic polyester co-polymer synthesized from glycolic acid and lactic acid monomers. PLGA advantages include excellent biodegradability, biocompatibility, mechanical strength, modifiable erosion times and is an FDA approved polymer (Muthu 2014). The degradation of PLGA can be exploited for controlled drug release. PLGA demonstrates the possibility to modify the physical attributes of the drug-polymer matrix to obtain sustained drug release by tailoring the appropriate parameters such as lactide and glycolide ratio, polymeric molecular weight, and drug concentration (Allison 2008). PLGA co-polymer can be synthesized from an array of chemical reactions, however, polycondensation and ring-opening polymerization reactions are common. Reaction conditions and process parameters significantly contribute to observable physicochemical properties exhibited from the formulated polymer (Mir et al. 2017). The molar ratio of primary monomers (glycolic acid and lactic acid) significantly influences the physicochemical properties of the polymer such as **glass transition temperature**, degree of crystallinity, mechanical strength, and the ability to hydrolyze. PLGA comprising of a 50:50 molar ratio of elementary monomers exhibits a faster degradation rate as compared to PLGA comprising of a higher quantity of either of the monomers (Ding and Zhu 2018; Makadia and Siegel 2011).

PLGA can be formulated with co-polymers to further enhance the polymeric properties for drug delivery. In a recent study, a PLGA-chitosan nanoparticulate drug delivery system was formulated for the treatment of depression. This system demonstrated mucoadhesive behavior which improved drug transport across biological membranes in addition to the nanoparticulate system improving drug delivery (Tong et al. 2017). In another study, the controlled release property of PLGA was

exploited, where a thermal responsive in situ gel loaded with risperidone PLGA nanoparticles. This system demonstrated prolonged anti-psychotic activity with a concurrent decrease in extra-pyramidal adverse effects (Muthu 2014).

7.5 Clinical Considerations and Regulatory Requirements

Nanosystems demonstrates an array of advantages attributed from their unique physicochemical properties and phenomena exhibited at their smaller particle size (Fig. 7.8). Nanosystems can be tailored for the targeted and site-specific delivery of drugs and biomaterials, thereby reducing side-effects and enhancing overall clinical outcomes to contributing to the advancements in medical devices and instrumentation for surgical interventions thereby preventing invasiveness and reducing the chance of post-operative infections. Research in nanosystems is directed at the development of targeted and site-specific delivery technology (example: nanoliposomes and nanomicelles) to the development of nanoparticles to enhance diagnostic efficacy (examples: carbon nanotubes). However, the need for research to be directed and focused on the fate and distribution of nanoparticles in biological systems is imperative. The risk and safety profiles should be clearly outlined and support the overall application of nanoparticles in biomedicine (Sharma et al. 2018; Kumar et al. 2018; Sanvicens and Marco 2008).

The responsibility of regulatory agencies, such as the US Food and Drug Administration (FDA), European Medicines Agency (EMA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), and the South African Health Products Regulatory Authority (SAPHRA),

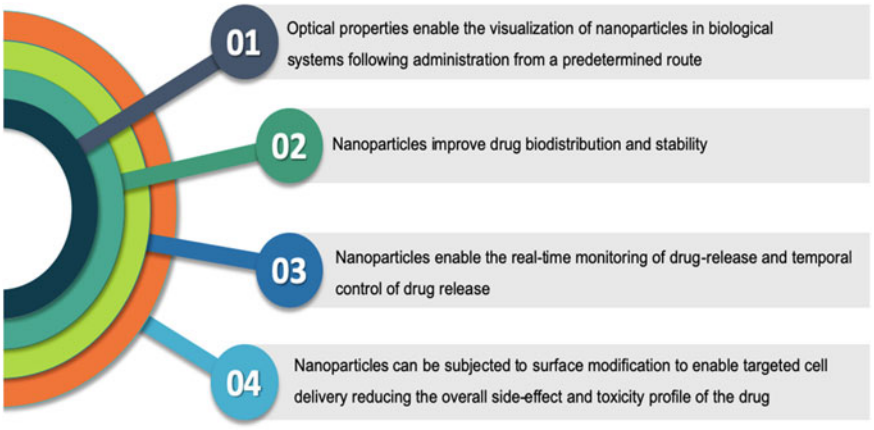

Fig. 7.8 Diagrammatic illustration demonstrating the array of advantages associated with nanoparticles as drug delivery vehicles

to collaborate to ensure that challenges are circumvented effectively and immediately. Public health care professionals also serve as a critical and central role, as they are able to collect, analyze, and publish epidemiolocal and clinical data for the development of sound policies and dossiers for the implementation and application of nanoparticles in drug delivery and biomedicine (Pautler and Brenner 2010).

While the developmental process of nanosystems demonstrates an array of difficulties and challenges with concurrent competition from conventional drug therapies, there are nanosystems that have been approved for clinical application and market entry, by the FDA, EMA, and other regulatory agencies. The challenges responsible for impeding the market entry of nanosystems include the elevated development cost associated with its clinical and biological development and regulatory approval, which is not counterweighed from the inadequate sale of nanomedicine (Tekade et al. 2017; Wang et al. 2015).

Another critical parameter that impedes the development and market entry of nanosystems is the limited availability of protocols for pre-clinical development and characterization of nanosystems. As an alternative, the regulatory requirements for conventional drug therapy have often been employed to assess the safety, toxicity, and biocompatibility of nanosystems. The active pharmaceutical ingredient of the nanoparticle dictates and outlines the context regulatory requirements that need to be meet (Sainz et al. 2015; Dobrovolskaia and McNeil 2013; Muthu et al. 2014).

The physicochemical properties and surface modifications of nanosystems are primarily responsible for its clinical application. Currently, only methods to determine and evaluate the physicochemical properties, such as particle size, size variability, and charge are available. However, regulatory agencies need to formulate a framework that can be employed to assess the effect of these physicochemical properties on the performance of nanosystems, such as protein binding, drug release, and the ability to demonstrate targeted drug delivery (Desai 2012).

The development of a concise framework by regulatory authorities to assess preclinical immunotoxicity is vastly needed. Since nanoparticles demonstrate the ability to interact and undergo absorption by immune cells and nanoparticles such as carbon nanotubes and dendrimers need to be subjected to specific surface modifications to eliminate the inherent toxic nature of the nanoparticle (Desai 2012).

7.6 Other Challenges and Considerations for the Regulatory Requirement

Nanosystems offers an array of advantages for the treatment and diagnosis of neurological disorders, there are multiple hurdles that are associated with neurological nanosystems such as the transition of nanosystems from laboratories to a patient's bedside. The physicochemical characterization of nanoparticles for nanosystems are critical parameters that are required for adequate regulation of the safety and activity of the produced nanoparticle-based formulation within acceptable pharmaceutical

tolerances. The therapeutic index of nanoparticle-based nanosystems is another critical parameter that requires significant attention, as it is dependent on the specific physicochemical characteristics of the formulation and equipment dependent confers difficulty in the commercialization of these nanoparticles-based nanosystems formulations. Nanoparticles fabricated and synthesized in benchtop laboratories are not often characterized and analyzed for clinical application and evaluation. The fabrication of optimized nanoparticles for nanosystems would require the analysis of multiple parameters concurrently (examples: surface charge, length, particle diameter, shape, stability and targeting moiety), because each of these parameters confers a specific property, and it would be imperative to determine the interaction and relationship and quantify each of these parameters as a single functional unit, as opposed to the conventional analysis of an individual parameter (Ajetunmobi et al. 2014). The clinical use of nanoparticles which are complex and sophisticated drug delivery technology requires a compressive and extensive understanding of these properties as slight adjustments to the manufacturing process or raw materials employed can result in structural changes which may critically alter the biological and biodistribution profile of the nanoparticle (Duncan and Gaspar 2011).

Therefore, the need for regulatory bodies to develop specific guidelines and requirements for analytical characterization would significantly enable the smooth shift from benchtop laboratories to a patient's bedside, not only to establish physicochemical properties (example: size and size variability), but also to determine drug release, biodistribution, drug metabolism, and protein binding (Tinkle et al. 2014).

With the emergence of nanosystems, the concurrent field of nanotoxicology has gained significant attention over the past decade. Adverse effects of nanoparticle-based nanosystems need to be clearly addressed and determined and have been previously reported to include deaths resulting from cardiovascular to respiratory depression (Brook et al. 2004). Comparatively, an array of other studies has demonstrated the blood-related adverse effects of nanoparticles such as thrombosis and platelet aggregation, examples include cationic (polystyrene or gold) nanoparticles illicit the ability to induce hemolysis (Jong and Borm 2008). The physicochemical properties of nanosystems are responsible for its ability to adsorb onto serum proteins and interact with immune cells, direct the need to tailor pre-clinical studies to evaluate immunotoxicology and biocompatibility (Dobrovolskaia and McNeil 2013).

Another challenge that limits the clinical application of nanoparticle-based nanosystems is the inadequate understanding of the relationship between its physicochemical properties and the demonstrated clinical pharmacokinetic profile observed, which is further limited by the use of conventional animal models, as these models provide insufficient data regarding the toxicity and biodistribution of the nanoparticle. Therefore, it is imperative that regulatory bodies develop a concise and inclusive list of tests that are a mandatory requirement for clinical application of nanoparticles (Ajetunmobi et al. 2014).

The nature and extent of data to be provided before and during the product life cycle is another hurdle that needs to be circumvented for the regulation of nanosystems. The European marketing authorization applications allow for scientific counseling between applicants and the regulatory body, which is provided from the

onset of research and development. This significantly contributes to the efficient harmonization of nanosystems development (Gaspar 2010).

Despite the lack of concise guidelines for the development of nanosystems, there have been significant efforts from regulators across the United States, Europe, and Japan as well as industry for the fabrication of comprehensive and inclusive regulatory frameworks through the ICH (Wagner et al. 2006). Pharmaceutical industry has demonstrated substantial interest in "proof of concept" and clinical development for nanosystems. Altogether, this joint collaboration creates the platform for the promotion of concise guidelines to determine the safety and toxicity of nanosystems (Sainz et al. 2015).

Prior to the commercialization and marketing of nanosystems, a pharmacoeconomic analysis will provide critical insight into the socioeconomic ramifications of nanosystems as compared to conventional treatment. Critical parameters and indicators such as increment in QALYs (quality-adjusted life expectancy years) and financial implications associated with consecutive hospitalizations should be considered for the fabrication and clinical application of nanosystems (Gaspar et al. 2014).

7.7 Summary and Outlooks

The development of novel and advanced drug delivery technology is urgently required for the successful treatment of neurological diseases. Nanosystems serve as an attractive platform that has the ability to circumvent an array of challenges associated with the conventional treatment of neurological diseases such as nanoliposomes, nanoemulsions, carbon nanotubes, and dendrimers. While significant advances have been made over the past decade in the neuro-nanomedical arena, the medical fraternity remains crippled by the inadequate movement of nanosystems from benchtop laboratories to a clinical trails and applications. Regulatory authorities in conjugation with industry urgently need to address the lack of legislative requirements for the up-scale manufacturing and clinical application of nanosystems. The development of a concise and inclusive framework for nanosystems need to focus on the fate of nanosystems, toxicity, long-term exposure, and possible environmental burden. Apart from the need for concise safety data is a critical parameter that will propel the clinical application of nanosystems,a pharmacoeconomic analysis will provide critical insight into the socioeconomic ramifications of nanomedicines as compared to conventional treatment. QALYs (quality-adjusted life expectancy years) and financial implications associated with consecutive hospitalizations will provide cognizance for the discrepancy in risk–benefit ratio between developed and developing countries.

Important Notes

- While nanotechnology employed for the delivery of drug and bioactive molecules for the treatment of neurological conditions remains in its infancy,

there has been a vast array of advances and successful applications in the field that serves as a platform to propel the emergence of clinically appropriate formulations to reach the patient's bedside.

- Nanotechnology employs nanometer-scale devices, materials, and systems that confer superior physicochemical properties for drug and bioactive molecule delivery. Decreased particle size allows for drug delivery vehicles to offer an enhanced intercellular uptake, overall formulation stability, and superior biodistribution as opposed to conventional drug delivery techniques.
- Over the past few years, nanotechnology has been significantly exploited for its ability to be formulated for site-specific delivery of an entrapped drug or bioactive molecule. Site-specific drug delivery is associated with a decreased administration of drug for the required therapeutic outcome and limited adverse drug reactions.
- In the past decade, there has been significant advances in the development and utilization of the versatility of nanomedicines. Nanoparticle-based nanomedicine can be divided into two broad categories based on their formulation technique, i.e., organic or inorganic nanoparticles. Organic nanoparticles include nanomicilles, nanoliposomes, nanodendrimers, and polymeric nanoparticles, while inorganic nanoparticles include gold or silica-based nanoparticles, fullerenes, and quantum dots. The versatility of available formulations allows for the diversity in clinical applications.
- Nanomedicine has demonstrated to overcome the limitations of neurological drug delivery by overcoming the hindrance of the blood–brain barrier and reaching the targeted site.

Questions for Future Research

- The clinical application of nanomedicine for the delivery of drugs and bioactive molecules needs to be accompanied by concise and specific scientific data regarding the biodistribution, safety, toxicity, shelf-life, and possible adverse reactions directly associated with nanomedicine.
- The clinical application and market entry of nanomedicine for the delivery of drugs and bioactive molecules need to be accompanied by environmental safety data that concisely and scientifically outlines the environmental burden that nanomedicines could contribute to, should there be environmental contamination.
- The development of a concise, scientific, and structured framework by regulatory agencies is of paramount importance, as the specifications for the

acceptable market entry of nanomedicines, such that biodistribution, toxicity, drug release, metabolism, and formulation stability need to be clearly defined.

- The development of a concise, scientific, and structured framework by regulatory agencies is of paramount importance for the pre-clinical and clinical studies for nanomedicine. The lack thereof is the critical inhibitor of nanomedicine reaching the patient's bedside.
- Prior to the market entry of nanomedicine, the need for concise and scientific pharmacoeconomic data is needed. The need to assess the relationship between the quality-adjusted life expectancy years and the financial implications with associated hospitalization for the clinical application of nanomedicine as compared to conventional treatment is of critical importance.

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Glossary

Bioconjugation Adsorption or covalent attachment of a biomacromolecule on the outer surface of another chemical entity.

Biodegradability He capacity of a material to undergo degradation in a biological environment.

Glass transition temperature The temperature at which a polymer undergoes transition from a rubbery state to a glassy state.

Graphene A two-dimensional carbon material possessing a honeycomb lattice and Dirac-like low-energy excitations.

Ionotropic gelation A gelation process mediated by the crosslinking of polyelectrolyte molecules in the presence of multivalent counter ions.

Mucoadhesiveness The ability of a material to adhere to mucosal tissues upon administration to a biological body.

Protein corona A dynamic protein layer on the surface of a nanocarrier. Its composition changes continuously due to ongoing protein desorption and absorption.

Quantum dots Fluorescent semiconductor nanocrystals that find use in imaging applications.

Reticuloendothelial system A major component of the host defense system contributing to the clearance of particulate materials and bacteria from the bloodstream.

Risperidone A benzisoxazole derivative that has been exploited as an anti-psychotic agent.

Surface functionalization Modification of surface properties for specific purposes.

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Part III
Systemic Delivery Techniques Based on Biological Materials

Chapter 8
Lipid-Based Nano-delivery of Phytobioactive Compounds in Anti-aging Medicine

Oleh Lushchak, Roman Karpenko, Alina Zayahckivska, Alexander Koliada, and Alexander Vaiserman

Abstract Aging population presents a major public health challenge across developed societies. Since phytobioactive compounds (PBCs) including resveratrol, quercetin, curcumin, catechin, and epigallocatechin-3-gallate have been repeatedly reported to demonstrate anti-aging properties, they are increasingly investigated for their anti-aging potential now. The therapeutic efficiency of orally administered PBCs is, however, largely limited by their poor stability, solubility in the gastrointestinal tract, and, subsequently, bioavailability. Apart from the use of polymeric nanoparticles in therapeutics delivery as depicted in Section II, biomaterials have been widely used as drug carriers. One of these biomaterials is lipids, which are a large and diverse group of naturally occurring organic compounds important to cell physiology. It has been reported that PBC-loaded lipid nanocomposites provide many benefits over their conventional formulations, including improved solubility and stability, prolonged half-life, enhanced epithelium permeability and bioavailability, and also improved tissue targeting and minimized side effects. This chapter will summarize recent advances in this research area.

Keywords Lipid · Anti-aging · Nanomaterials · Phytobioactive compounds · Healthspan

8.1 Introduction

The general trend of extended average life expectancy is observed in most developed countries. According to demographic projections made by WHO, the number of people older than 65 years will reach about 1.5 billion in 2050 that is almost

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three-fold more than in 2010 (World Health Organization (2012)). However, the general extension of the lifespan is not necessarily accompanied with traits related to **healthspan** (Hansen and Kennedy 2016). It means that we also have to pay attention to the quality of life together with its extension. Notably, some health benefits were already described for drugs which are able to extend the lifespan (Piskovatska et al. 2019a). Since aging is already defined as a major risk factor for many pathological conditions, fast gown fraction of elderly people is an important challenge for most modern societies. Aging affects the progression of cardiovascular and neurodegenerative diseases, osteoporosis, T2D, and varied cancer types to be faced as the significant problems for healthcare system (Beard and Bloom 2015). Every year, increasing amount of people define aging as diseases and thus it can be treated. However, successful treatment requires the discovery of anti-aging drugs with fewer side effects (Vaiserman et al. 2016), development of delivery systems to increase drug efficiency, and personalized multidrug treatments. Moreover, all the treatments are affected by factors contributing to the determination of life expectancy and development of pathologies. Parental programing of offspring traits (Vaiserman and Lushchak 2019), dietary interventions (Costa et al. 2019), nutrition (Lushchak et al. 2019), microbiome (Vaiserman et al. 2017), and lifestyle are among main factors affecting tightly linked aging and metabolism.

Many compounds or drugs were described to extend the lifespan and healthspan of model organisms (Piskovatska et al. 2019a). However, only resveratrol, rapamycin, aspirin, and metformin have extended the lifespan in worms, fruit fly, mice, and rats. Being effective in model organisms, metformin showed significant health outcomes in human being (Piskovatska et al. 2019b). Phytochemicals, a plant-derived natural compounds, are promising molecules for the development of novel drugs and dietary supplements for treating age-related pathologies. They are mostly secondary metabolites that protect plants from environmental stresses such as rush conditions, pollutants, and infections. Dietary supplementation with **phytobioactive compounds** (PBCs) such as resveratrol, quercetin, curcumin, epigallocatechin gallate, catechin, and sulforaphane have been extensively reported to cause beneficial effects with prolongevity properties (Corrêa et al. 2018; Santín Márquez et al. 2019). They have been shown to act as antioxidants (Franco et al. 2019), have anti-inflammation (Zhu et al. 2018), anti-tumor (Chikara et al. 2018), cardioprotective (Shah et al. 2019) and neuroprotective (Sarker and Franks 2018) properties, and other anti-aging activities in animal and human studies.

Being effective, anti-aging agents PBCs possess the properties that significantly limit their **bioavailability** due to low solubility, chemical instability, intrinsic dissolution rate, low absorption, scarce distribution, and poor accumulation in the human body (Khadka et al. 2014). This significantly limits their use via oral administration. However, specific formulations-based development of certain types of PBC-loaded nanoparticles (NPs) suitable for oral administration may increase the stability and solubility, prolong half-life, and improve cell and tissue permeability and bioavailability of compounds. Moreover, so-called nanoformulations of PBS may enhance tissue-specific delivery and decrease possible side effects (Date et al. 2016; Lin et al. 2017). In recent decade, these nanotechnological formulations have been increasingly

applied in treating chronic age-related pathologies such as cardiovascular diseases (Li et al. 2017), type 2 diabetes (Jeevanandam et al. 2015), obesity (Zhang et al. 2018), neurodegeneration (Silva Adaya et al. 2017), and cancer (Qiao et al. 2019). In this chapter, we will focus on the lipid-based nanoformulations, their applications with specific insight into NPs loaded with resveratrol, quercetin, curcumin, genistein, and epigallocatechin gallate.

8.2 Main Types of Lipid-Based Nano-delivery Systems and Possible Applications

The problems related to low bioavailability of PBCs can be partially solved by the use of lipid-based **nanocarriers** for the oral administration. Bioavailability might be further improved by surfactants and emulsifiers that are important counterparts of lipid NPs (Chakraborty et al. 2009). Most useful lipid-based **nano-delivery** systems are nanoemulsions, solid lipid NPs (SLNs), self-emulsifying drug delivery systems (SEDDSs), and nanostructured lipid carriers (NLCs) (Fig. 8.1) (Hsu et al. 2019). They carry bioactive compounds to increase their solubility and bioavailability. Lipid NPs have good solubility in **lipids**, and thus encapsulated active compounds possess improved pharmacokinetic properties, biocompatibility, and reduced toxicity (Dumont et al. 2018). These formulations are also easily absorbed

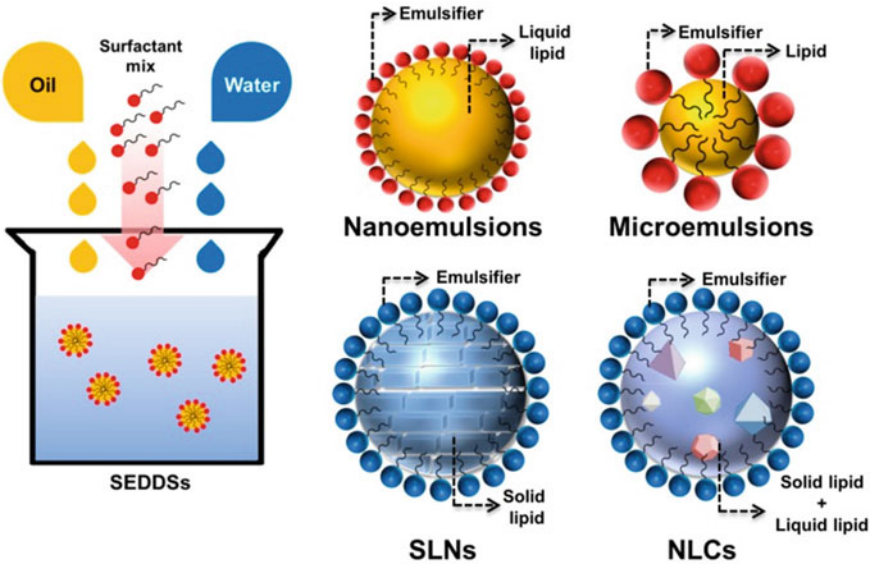

Fig. 8.1 Structures of lipid-based NPs: self-emulsifying drug delivery systems (SEDDSs), nanoemulsions, microemulsions, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). Reproduced from (Hsu et al. 2019) with permission from MDPI

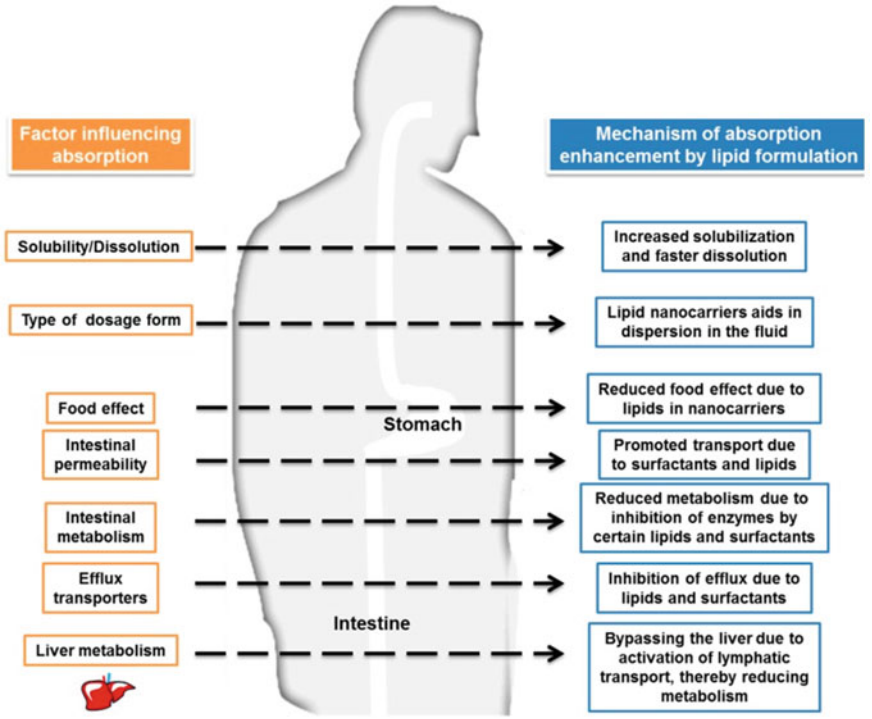

Fig. 8.2 Possible mechanisms for enhancement of PBC bioavailability using lipid-based delivery systems. Reproduced from (Hsu et al. 2019) with permission from MDPI

by human body (Fig. 8.2). In addition, they can be digested to produce emulsions with high surface area that further may be enzymatically hydrolyzed to release active compound in easily absorbed form (Joyce et al. 2016), see also Fig. 8.3 for schematic representation.

8.2.1 Nanoemulsions

Nanoemulsions (NE) are heterogeneous mixtures of oil droplets in aqueous solution that are further stabilized by an emulsifier (McClements 2011). They are generally produced by directed assembly of the compounds. Drug or active compound can be loaded into the oil cores of NE to improve oral administration (Kumar and Sarkar 2018). The mixture is kinetically stable with no flocculation or coalescence during long-term storage. Nanoemulsions are prepared of natural components or GRAS. Surfactants and co-surfactants are used in NE to reduce toxicity and increase stability of the compound. The most used particles of the nanoemulsions are peptides, proteins,

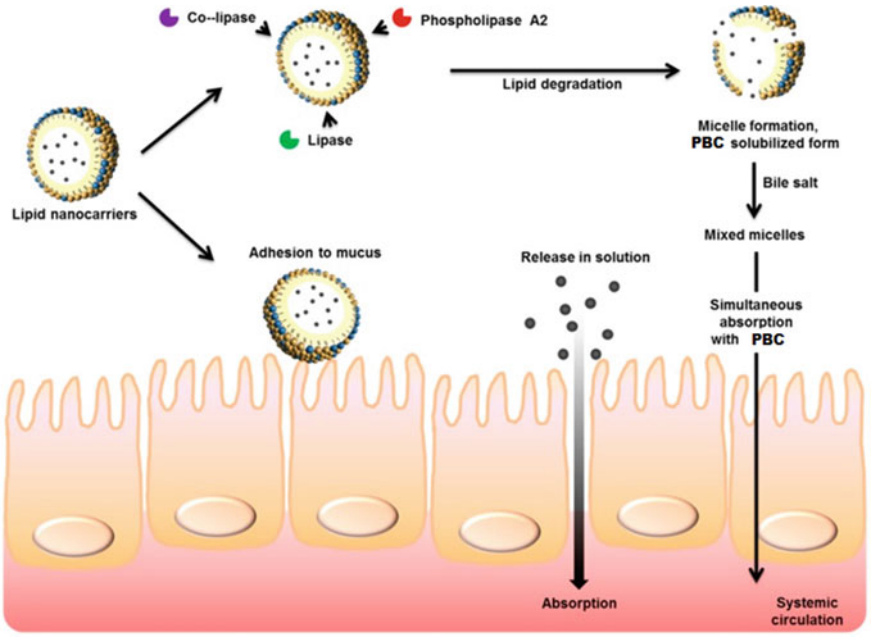

Fig. 8.3 Possible pathways of gastrointestinal absorption of orally administered lipid NPs. Reproduced from (Hsu et al. 2019) with permission from MDPI

polysaccharides, phospholipids, and small molecule nonionic surfactants (Tagami and Ozeki 2017).

Three methods are mostly used to prepare NE which include phase inversion temperature synthesis, high-pressure homogenization, and **microfluidization** (Goh et al. 2015). The use of rapidly diffusing and electrostatically stabilized emulsifiers with low molecular weight can provide complete dispersity. The use of proper synthesis method in combination with surfactant gives the possibility to create tiny droplet size with large surface area. Thus, they can easily be absorbed in the gastrointestinal tract with significantly increased availability of bioactive compounds compared with conventional emulsions. Nanoemulsions increase bioavailability of PBCs due to increased solubilization, prolonged **gastric residence time**, stimulated lymphatic absorption, reduce the effects of efflux transporters, and inhibit metabolism (Salvia Trujillo et al. 2017).

An average size of NE particles varies from less than a nanometer to more than 400 nm and is strongly dependent on the compounds used. For example, curcumin-loaded NPs studied by Chen and colleagues were nanoemulsion prepared from ethanol, isopropyl myristate, tween 80, and tween 20 and had an average size of about 100 nm (Chen et al. 2013). Nanoemulsions of curcumin for cancer chemotherapy were prepared with soybean oil and hydrogenated L-α-phosphatidylcholine and has an average particle size of 55 nm (Anuchapreeda et al. 2012).

NE may carry out different types of compounds with different targets. Fat-soluble vitamins A, E, and D can be loaded within the oil core of liquid droplets and thus be protected from chemical and enzymatic degradation and released after ingestion. It has been shown that NE loaded with b-carotene was twice more effective. Nanoemulsions were tested to increase delivery of varied active compounds when applied orally, transdermal or intranasal administration. Lipid nanoformulation of antimalarial agent primaquin gave possibility to decrease amount of applied drug by 25% with respect to increase of liver drug concentration by 45% (Singh and Vingkar 2008). Oil-in-water nanoemulsions of anti-HIV protease inhibitor Saquinavir increased drug oral bioavailability and its brain disposition (Vyas et al. 2008). Oral administration of NE loaded with paclitaxel significantly increased amount of drug in the circulation and absorbed by kidneys, liver, and lungs (Tiwari and Amiji 2006). Intranasal administration of NE formulations of olanzapine and risperidone showed the ability of direct nose-to-brain transport that bypasses the blood–brain barrier with about four-fold increased efficacy (Kumar et al. 2008a, b). Finally, dermally applied celecoxib-loaded NE showed a significant decrease of inflammation (Baboota et al. 2007).

8.2.2 Self-emulsifying Drug Delivery Systems

Mixtures of oils, surfactants, solvents, and compound spontaneously form nanoemulsions or microemulsions upon dilution with water under soft agitation (Chintalapudi et al. 2015; Knaub et al. 2019). These self-emulsifying drug delivery systems (SEDDS) can include particles with diameters from 20 to 300 nm. An exciting property of SEDDS is the possible formation of nanosized oils during self-assembly in the fluid of gastrointestinal tract (Dokania and Joshi 2015). Two main types of SEDDSs are self-nanoemulsifying (SNEDDSs) and self-microemulsifying drug delivery systems (SMEDDSs). SNEDDSs with the droplet size under 100 nm are generally opaque or translucent formulations. SMEDDSs are transparent microemulsions that become thermodynamically stable after ingestion. Droplet diameter, composition, lipid digestibility, and lipophilicity of the active compound affect its bioavailability when released from particles (Date et al. 2010). This type of nanocarriers are generally formulated by using lipids and surfactants, sometimes co-surfactants, that are generally recognized as safe according to FDA. Technically, SEDDSs can be produced by phase inversion composition, low-energy emulsification, solvent displacement, or phase inversion temperature methods (Date et al. 2010).

Significant volumes of typical emulsions have to be consumed to provide the therapeutically tractable amounts of bioactive compound. Emulsions include water that is required for their productions. Comparably large amount of water included may enhance hydrolysis and/or precipitation at long-term storage and in this way reduce stability and absorption after oral ingestion. Thus, oral administration SEDDS is an application of emulsion concentrate that does not require water for the production.

SEDDSs possess varied advantages for oral administration. These formulations improve physicochemical stability of delivered drug with possibility to be encapsulated with the vehicle to increase acceptability by recipient (Khan et al. 2012). Agitation required for the formation of nanoemulsions may be simply provided by digestive motility of GI tract. The spontaneous formation of an interface between the oil droplets and external phase is caused due to swelling of liquid crystalline phase that is formed between water and oil phases (Khan et al. 2012). An increased production of emulsion droplets may also be induced by excess amounts of the lipids in GI tract derived from SEDDSs. They trigger the secretion of bile acids into the lumen. Together with higher quantities of cholesterol and phospholipids in the presence of lipids and emulsifiers, the lipid-rich environment provides intensive formation of droplets. Thus, drugs or bioactive compounds with low solubility initially loaded into SEDDS will be incorporated in micelles that are characterized by high absorption rate.

Ingested SEDDSs provide increased bioavailability by increasing the stability of bioactive compound in the gastro-intestinal environment via minimizing the first-pass effects (Chatterjee et al. 2016). Also, formulations inhibit efflux mediated by P-glycoprotein (Negi et al. 2013). Finally, there is methodologically simple production of SEDDSs and simple delivery by the oral consumption with low intersubject variability and effects caused by food ingestion.

SEDDSs have to be encapsulated into gelatin capsules for oral administration. However, the material of the shell might be incompatible with the formulation. This fact may cause the precipitation of the active ingredients, the requirements of storage at lower temperature, or changes of the preparation protocols (Dokania and Joshi 2015). These problems can be partially solved if liquid SEDDSs are converted into solid state. Thus, more stable and convenient forms for easier handling and delivery may be created by methods such as granulation, **freeze drying**, spray drying, or be achieved by adsorption to carriers.

SEDDS loaded with varied bioactive compounds or drugs with anti-fungal, anti-seizure, anticoagulant or anthelmetic properties were tested to treat inflammation, cancer, hypercholesterolemia, liver cirrhosis and bleeding in the brain. For example, SEDDs-based formulations of anti-inflammation low water-soluble drugs ketoprofen and celecoxib showed three- and four-fold higher effectivity at oral administration, respectively (Patil et al. 2004; Song et al. 2014). The paclitaxel SEDDS formulation showed about five-fold higher oral bioavailability of the drug compared with that of the orally dosed Taxol or SEDDS formulation without HPMC (Gao et al. 2003). In vivo treatment with nanocarriers-loaded atorvastatin significantly reduced serum lipid levels in a triton-induced hypercholesterolemia model in male Albino Wistar rats as compared with calcium salt (Kadu et al. 2011). Moreover, the in vivo study indicated ~ three-fold increased delivery when silybin-S-SEDDS produced in the presence of HPMC were used (Wei et al. 2012). A two-fold increased bioavailability of lipophilic CoQ10 was observed for the SEDDS compared to a powder formulation (Kommuru et al. 2001).

8.2.3 Solid Lipid NPs

SLNs or solid lipid NPs represent another formulation and drug delivery system. This type of nanocarriers consists of solid lipid core matrix stabilized by surfactants or emulsifiers and thus may solubilize molecules with lipophilic properties (Muchow et al. 2008). The use of biodegradable and biocompatible solid lipids in the formulations of SLNs strongly increases their applicability and safety. Triglycerides, wax, fatty acids, cholesterol, and glycerol derivatives such as monostearate, palmitostearate, or behenate are combined with either natural or synthetic solid lipids to form stable SLNs nanosystems (Santo et al. 2013). SLNs can be produced by melting microemulsification, melting or cold homogenization. Solid lipids must be melted by hot homogenization before combining with other components (Weiss et al. 2008). However, if drug or active compound of formulation is sensitive to temperature, then cold homogenization may be used. SLNs are mostly spherical particles with average diameter 10–1000 nm.

Lipases in the stomach start to digestion orally applied SLNs. Simple mechanical mixing of particles with gastric fluids results in the formation of crude emulsion that can be further digested by intestinal fluids (Lin et al. 2017). Small size and properties of SLNs allow them to attach to gastrointestinal mucus and diffuse into intervillar space. Absorption of particles may be further increased by using emulsifiers that reduce membrane fluidity and coat the surface of SLNs. Moreover, chemically labile bioactive compounds can be partially protected by solid state of the lipid. Also, slower digestion of SLNs may prolong release of the carried active compound (Dolatabadi et al. 2015).

Since there are obvious benefits of SLNs use for oral delivery of bioactive compounds, several disadvantages limit their broader application. Firstly, the loading space of active compound can be thereby reduced because solid lipids are densely packed into matrix (Dolatabadi et al. 2015). Secondly, partial loss of active compound may occur due to aggregation and gelation of particles during storage. Thirdly, the liquid lipid incorporation into the crystalline matrix can partially change the properties of the core due to “lattice defects” (Garcês et al. 2018). Finally, so called “burst escape” of the drug from the NPs may cause toxic effects. Thus, the SLN formulations have to be significantly improved to decrease the drawbacks of SLNs.

Solid lipid NPs of different formulations were successfully loaded with antibacterial, antifungal, antischistosomal, anti-inflammation, and antirheumatic drugs and compounds. SLN formulation of carvacrol showed significant benefits in rat with lung damage by partial prevention of oxidative stress and histological damages caused by smoke inhalation (Carvalho et al. 2019). An increased trans-resveratrol penetration through the skin for up to 45% was shown for NPs loaded with ionic liquid-melinjo seed extract (Trinovita et al. 2019). SLN formulations of antirheumatic drugs methotrexate and doxycycline with yield of 65–80% were characterized by sustained release of both drugs for about two days without any significant interaction suggesting their use to treat chronic inflammation (Vijaya and Ram Kishan 2018).

Interestingly, praziquantel-containing SLN formulation shows enhanced bioavailability and antischistosomal efficacy against murine *S. mansoni* infection. SLN-PZQ use is able to decrease applicable drug concentration by five-fold with a significantly higher reduction in hepatic and intestinal tissue egg amounts and strong disappearance of deposited immature eggs (Radwan et al. 2019). Treatment with SLN loaded with myricitrin abrogated diabetes-related changes by increasing the activities of antioxidant enzymes, and reducing levels of oxidative stress markers and apoptotic signatures (Ahangarpour et al. 2019).

8.2.4 Nanostructured Lipid Carriers

Nanostructured lipid carriers (NLCs) consist of partially crystallized lipid particles dispersed in an aqueous phase including emulsifier (Khan et al. 2012). NLC may have some advantages in comparison with other colloidal carriers, for instance, NLC can be loaded with high amount of drug with increased encapsulation efficiency and stability. Carriers may further increase stability of bioactive compounds and their bioavailability. NLCs as second-generation lipid-based NPs consisted of solid and liquid lipids with improved physical stability. Moreover, the release of bioactive agents from NLCs can be easily modulated by adjusting the ratio between the liquid and solid lipids. A number of preparation methods are suitable for NLC production such as solvent evaporation or injection, emulsion–solvent diffusion, membrane extrusion, multiple or microemulsions, sonication, phase inversion high-pressure homogenization (McClements 2011). The latter does not require a solvent and thus is preferred over other methods. Moreover, this method is already well established and extensively used in pharmacology.

A nature of NLCs makes them especially useful for enhanced absorption in gastro-intestinal tract by lymphatic uptake via M cells (Managuli et al. 2018). NLCs promote absorption by increasing carrier transport through intestinal bulk fluid and brush border of enterocytes. Moreover, P-glycoprotein efflux can be inhibited by the components of NLC shell surfactants (Negi et al. 2013). Nanostructured lipid carriers were successfully used for oral, skin, eye, or lung drug applications. NLC-based gels loaded with celecoxib and valdecoxib were showed to be effective at treating inflammation and allied conditions in rats (Joshi and Patravale 2006, 2008). These formulations were two- and four-fold more effective as compared to other ones. Ibuprofen-containing NLCs displayed controlled-release property with four fold increase of ocular drug delivery (Li et al. 2008). NLC loaded with simvastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor with beneficial effects on coronary diseases and mortality rate in patients with hypercholesterolemia, showed better release, pharmacokinetics, and increased bioavailability for about five-fold (Tiwari and Pathak 2011).

8.3 Nanoformulations of Anti-aging Drugs

Many lipid nano-delivery systems loaded with plant-based bioactive compounds such as resveratrol, genistein, quercetin, curcumin, and epigallocatechin-3-gallate (EGCG) (for chemical formulas, see Fig. 8.4) have been demonstrated to be efficacious in modulating oxidative stress and related chronic inflammation mediating most aging-associated disorders. Results from studies reporting antioxidant and anti-inflammation effects of these nano-delivery systems are discussed in subsections below.

8.3.1 Nano-curcumin

Curcumin is a naturally occurring polyphenolic compound with a wide range of beneficial biological functions, including antioxidant, anti-inflammation, and anticancer activities (Sarker and Franks 2018). Its therapeutic potentials in combating aging-associated conditions, including chronic inflammation, hypertension, type 2 diabetes, atherosclerosis, cardiovascular and neurodegenerative diseases, osteoporosis, and also chronic kidney and ocular diseases are well documented (Kumar et al. 2010). Presently, the healthspan-promoting capacity of curcumin is increasingly investigated in clinical trials (Salehi et al. 2019). The clinical use of this compound is, however, limited because of its susceptible nature to high temperature, alkaline pH,

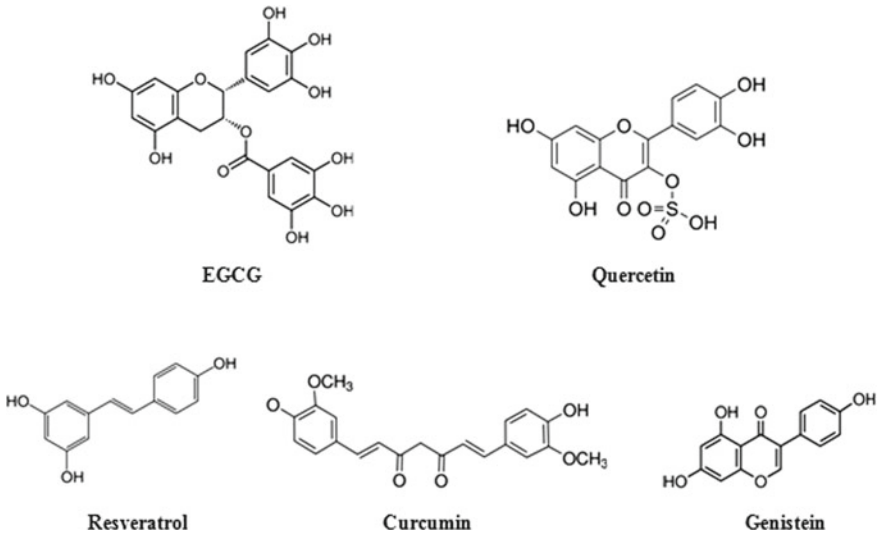

Fig. 8.4 Chemical formulas of PBCs most commonly used to integrate in lipid nano-delivery systems

as well as presence of oxygen and light. Therefore, it is extremely difficult to maintain its bioactivity during processing, storage, and consumption (Nayak et al. 2016). Its therapeutic potential is, however, substantially limited through low aquatic solubility and gastrointestinal stability, leading to poor bioavailability (Kumar et al. 2010). Therefore, developing nano-delivery systems aimed at improving bioavailability of curcumin is considered as a promising therapeutic option now (Flora et al. 2013; Ahmad et al. 2016).

Recent advancements in the nanotechnology-based applications offer an opportunity to enhance the stability and bioactivity of curcumin to overcome its pharmacokinetic mismatch. Among various nano-delivery systems, lipid-based ones are the most well-studied delivery systems aimed at enhancing its stability and pharmacokinetic profile both for pharma and food applications (Nayak et al. 2016). Greater therapeutic potential of curcumin-loaded lipid nanoformulations in comparison to that of native curcumin was repeatedly reported in vitro *and* in vivo. Both its solubility and bioavailability were demonstrated to be significantly improved by encapsulating in nano-delivery systems, including the lipid ones, and it caused enhancement of its pharmacological activity (Shome et al. 2016). In particular, loading of curcumin in N-trimethyl chitosan surface-modified solid lipid NPs resulted in a substantial improvement of its oral bioavailability and brain distribution compared to those of free curcumin (Ramalingam and Ko 2015). In an induced cerebral ischemia rat model, the bioavailability of curcumin in the brain was shown to be 16 times greater if it was loaded in solid lipid NPs compared to that of the native curcumin (Kakkar et al. 2013). Therefore, the nano-delivery of curcumin is regarded now as an efficient approach to enhance its bioavailability in a target-specific manner and to improve its therapeutic potential in combating aging-related disorders. Some studies have indeed demonstrated the enhanced oral bioavailability of curcumin-loaded solid lipid NPs to the brain, highlighting its therapeutic potential to treat neurodegenerative disorders (Ramalingam and Ko 2015,2016; Sadegh Malvajerd et al. 2019). For example, in an aluminum-induced model of Alzheimer's disease, orally administered curcumin-loaded solid lipid NPs resulted in 32–155 times enhanced bioavailability of curcumin relative to the control mouse group, and also in abolishing of adverse behavioral changes and biochemical and histopathological alterations in the brain induced by exposure to aluminum (Kakkar and Kaur 2011). The protective potential of curcumin-loaded solid lipid NPs in ameliorating the complete Freund's adjuvant (CFA)-induced arthritis, supposedly due to attenuation of the antioxidative and anti-inflammation responses, has been also demonstrated (Arora et al. 2015). Recently, in a double-blind randomized placebo-controlled clinical trial, nano-curcumin improved glucose indices, lipid profiles, and ameliorated inflammation in overweight and obese patients with non-alcoholic fatty liver disease (Jazayeri Tehrani et al. 2019). In the study conducted in the breast cancer cell line, an evidence has also been obtained that loading curcumin into nanostructured lipid carriers can enhance its cell penetration and cytotoxic anticancer properties (Kamel et al. 2019).

8.3.2 Nano-auercetin

Quercetin is a flavonoid widely presented in a number of grains, fruits, and vegetables. Antioxidant, anti-inflammation, antihypertensive, anti-obesity, antidiabetic, anti-atherosclerotic, and anti-hypercholesterolemic properties of this compound were repeatedly reported (Anand David et al. 2016). However, its health benefits are substantially limited because of very low bioavailability (less than 2% after a single oral dose) that is attributed to its low absorption, and also extensive metabolism and fast elimination from the body (Kawabata et al. 2015; Ganesan et al. 2017). The estimated absorption of quercetin glucoside (the naturally occurring form of quercetin), ranges from 3 to 17% only of quercetin ingested in healthy persons (Li et al. 2016). The innovative nano-delivery approaches are developed now to overcome these challenges. For example, substantially enhanced bioavailability of quercetin-loaded solid lipid NPs compared to a pure quercetin powder was reported in Caco-2 cell study (Vijayakumar et al. 2017). The improved topical delivery of quercetin by nanostructured lipid carrier systems was also demonstrated (Bose and Michniak Kohn 2013). Some studies demonstrated a therapeutic potential of such NPs in combating aging-associated disorders such as the Alzheimer's disease. In a rat model of Alzheimer's disease, substantially better memory retention vis-à-vis was observed in animals intravenously administered with quercetin-loaded solid lipid NPs compared to that in animals administered with pure quercetin (Dhawan et al. 2011). Moreover, a higher delivery of quercetin to the brain along with the enhanced antioxidant effect in the brain cells was found in these NPs. In addition, a formulation of quercetin-based solid lipid NPs was shown to be more efficient than free quercetin in an ovariectomized rat model in restoring bone mineral density in osteopenic animals (Ahmad et al. 2016).

8.3.3 Nano-resveratrol

Resveratrol is a well-known polyphenolic compound with many anti-aging activities. Its anti-aging effects are believed to be attributed to the capacity to activate the mammalian silent information regulator 1 (SIRT1), and also to modulate the activity of proteins playing important regulatory roles in aging processes, including the Akt (protein kinase B), peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), NFκβ, and the FOXO family members (Camins et al. 2009). Many of these activities are similar to those observed in **calorie restriction** (CR) treatments (Li et al. 2017). The effectivity and safety of resveratrol have been documented to date in 244 clinical trials, where the therapeutic potential of this compound in treating aging-associated conditions such as hypertension, obesity, metabolic syndrome, type 2 diabetes, cardiovascular disorders, stroke, chronic inflammation and kidney diseases, Alzheimer's disease, and cancer was shown (Berman et al. 2017; Singh et al. 2019).

The therapeutic applicability of resveratrol is, however, substantially limited because of its fast metabolism and poor bioavailability (Smoliga and Blanchard

2014), and also very low water solubility causing its poor absorption by oral administration (Chauhan 2015). To overcome these limitations, nanosized resveratrol-loaded formulations have been recently developed and investigated. For example, the bioavailability of *resveratrol loaded in* N-trimethyl chitosan-g-palmitic acid surface-modified solid lipid NPs was shown to be 3.8-fold higher than that from resveratrol suspension (Ramalingam et al. 2016). In male Wistar rats, two times higher bioavailability of the trans-resveratrol in the brain, kidney, and liver was observed through oral delivery with trans-resveratrol-loaded lipid core nanocapsules compared to the free trans-resveratrol (Frozza et al. 2010). Gastrointestinal safety has also been found to be significantly improved in the same animal model compared to the free trans-resveratrol. Since resveratrol is considered to be a promising candidate for the treatment of Alzheimer's disease due to its neuroprotective properties, the resveratrol-loaded solid lipid NPs functionalized with monoclonal antibodies against the transferrin receptor have been developed to enhance its bioavailability to the brain (Loureiro et al. 2017). These NP–antibody conjugates demonstrated increased cellular intake; therefore, they were proposed as promising nanocarriers to transport resveratrol to the brain tissues in efforts to treat Alzheimer's disease. Recently, solid lipid NPs loaded with resveratrol were shown to have therapeutic effect for protecting the myocardium and reducing the doxorubicin-induced cardiotoxicity in mice (Zhang et al. 2019). Oral administration of resveratrol-loaded solid lipid NPs also was found to improve insulin resistance through targeting expression of SNARE proteins in adipose and muscle tissues in rats with type 2 diabetes (Mohseni et al. 2019).

8.3.4 Nano-genistein

Genistein is a phytoestrogenic isoflavone found in soy. It is known to be able to combat aging-associated pathological conditions such as oxidative stress, inflammation, osteoporosis, obesity, type 2 diabetes, neurodegenerative diseases, and several cancers (Saha et al. 2014). Its bioactivity is, however, substantially decreased due to a very low solubility and poor bioavailability. Moreover, since genistein is an estrogen-like substance, its high doses may cause toxicity and endocrine-disrupting effects (Patisaul 2017). Recently, innovative nanoscale materials, including the lipid ones, were developed with aim to improve the oral delivery and to overcome the potential toxic effects of this substance (Rassu et al. 2018). For instance, the oral bioavailability of genistein loaded in the solid lipid NPs was found to be significantly increased in rats compared to that of its suspension or bulk powders (Kim et al. 2017).

8.3.5 Nano-epigallocatechin-3-Gallate

Epigallocatechin-3-gallate (EGCG) is polyphenol compound (catechin) contained in green tea. This compound was shown to exhibit a lot of anti-aging and healthspan-promoting activities including antioxidant, anti-atherogenic, anti-inflammation, and anti-tumor ones (Khan and Mukhtar 2018). The epidemiological findings on these properties are, however, inconsistent and frequently conflicting with results of in vitro investigations. This contradiction is believed to be due to poor stability and low bioavailability of this compound (Krupkova et al. 2016; Chu et al. 2017). In order to enhance its bioavailability, innovative solid lipid NPs and other nanocarrier-based delivery systems have been recently developed (Granja et al. 2017; Dai et al. 2019). Encapsulating EGCG in lipid NPs is regarded as an appropriate approach to avoid the oxidation and **epimerization** of drugs, which are known to be common processes that result in reducing their bioavailability and, thereby, to limiting their therapeutic effectivity (Fangueiro et al. 2014). In a study by (Frias et al. 2016), EGCG loaded into solid lipid NPs (particle size ~300–400 nm) demonstrated higher stability and larger potential for oral delivery compared to those of non-processed EGCG. EGCG-loaded folic acid functionalized NPs were demonstrated to be biocompatible with epithelial Caco-2 cells, and EGCG transport across the intestinal barrier was estimated to be 1.8-fold higher than that of native EGCG (Granja et al. 2019). Two-fold larger oral bioavailability over free EGCG and improved ability to treat the Alzheimer's disease was also found in EGCG-loaded nanolipid particles (Smith et al. 2010). The EGCG-loaded lipid NPs (<300 nm) have also been shown to have a potential for the treatment of aging-related ocular diseases, such as dry eye, age-related macular degeneration, glaucoma, diabetic retinopathy, and macular oedema (Fangueiro et al. 2014).

8.3.6 Other Phytocompound-Based Lipid Nanocomposites

Enhancement of therapeutic potential was also found in several other phytocompound-based lipid nanocomposites compared to their pure constituents. Such effect was found, e.g., for puerarin which is the major bioactive constituent in kudzu roots widely used in China for the treatment of various cardiovascular disorders. Solid lipid NPs loaded with puerarin demonstrated three times higher bioavailability following oral administration in heart and brain tissues compared to the free puerarin (Luo et al. 2011). The same effect was observed for the alkaloid piperine which is the main active ingredient of black pepper. In a rat model of the complete Freund's adjuvant (CFA)-induced arthritis, either topical or oral administration of piperine-loaded solid lipid NPs caused significant reduction in TNFα protein levels, assuming that treatment with such NPs has antirheumatic therapeutic potential (Bhalekar et al. 2017). Improved therapeutic potential against myocardial ischemia–reperfusion injury has also been shown in rats treated with solid lipid NPs

loaded with the total flavonoid extract derived from *Dracocephalum moldavica L.* compared to that of the non-modified extract (Tan et al. 2017).

8.4 Summary and Outlooks

There is convincing evidence that PBCs have a substantial potential in preventing and treating various aging-associated chronic disorders. However, poor stability, solubility in the gastrointestinal tract, and bioavailability largely limit their clinical applications. Currently, many nanocarrier-based systems intended to delivery of PBCs to target organs are developed to protect them from premature enzymatic degradation and metabolism, enhance the solubility and stability, increase the absorption in the gastrointestinal tract, and also to prolong their circulation time, thereby limiting side effects of these compounds (Conte et al. 2017; Lai et al. 2020; Martínez Ballesta et al. 2018; Lai 2013; Jampilek et al. 2019). Phytonanotherapy represents a promising innovative approach that may provide an opportunity to overcome a lot of drawbacks intrinsic to conventional therapeutic strategies. PBC-loaded nanoformulations are believed to provide synergistic health benefits because such a therapeutic modality can be clinically equivalent to a standard treatment mode with conventional drugs, but with minimum side effects (Anand David et al. 2016). Thus, this approach may provide an approach alternative to therapeutic modalities commonly used for management of aging-associated pathological conditions and give an opportunity to overcome disadvantages related to the use of conventional medications. Among nanosystems aimed at delivering PBCs to target organs and tissues, lipid-based nano-delivery systems are the most well-studied systems aimed at enhancing the stability and pharmacokinetic profiles of PBCs in various pharmacological and food applications (Nayak et al. 2016). One beneficial feature of such delivery systems is that they can help in delivering PBCs to vital body organs, particularly the brain, following the oral delivery (for schematic representation, see Fig. 8.5). The available research evidence indeed suggests that bioavailability of PBCs loaded in nanocarriers can be up to 5–10 times higher than that of their bulk counterparts (Ganesan et al. 2017). In conclusion, it can be stated that substantial steps have been already taken to bringing a nonotechnology-based approach closer to application in clinical practice. In light of the unresolved questions, however, additional research is certainly needed to further improving the long-term safety, efficiency, and cost-effectiveness of PBC-loaded lipid nano-delivery systems.

Important Notes

- The development of efficient means for the human healthspan extension is a priority task for researchers worldwide.

- Phytobioactive compounds (PBCs) demonstrate anti-aging properties such as antioxidant, anti-inflammation, cardioprotective, and anti-tumor activities.
- The therapeutic efficiency of orally administered PBCs is, however, largely limited by their poor stability, solubility in the gastrointestinal tract, and bioavailability.
- Lipid-based nano-delivery systems are increasingly used now to enhance the bioactivity of PBCs and improve their potential in delaying and/or preventing aging-associated pathological conditions.
- PBC-loaded lipid nanocomposites provide many benefits over their conventional formulations, including improved solubility and stability, prolonged half-life, enhanced epithelium permeability, and bioavailability, and also improved tissue targeting and minimized side effects.

Questions for Future Research

- **How can nano-delivery designs be incorporated with optimized release profiles specific to physicochemical properties of the loaded PBCs?** For some PBC-loaded nano-delivery systems, the burst drug release may potentially cause cellular toxicity. The very slow drug release can, in turn, lead to inadequate therapeutic effect in treating the disease. Therefore, the development of innovative nano-delivery designs with optimized release profiles

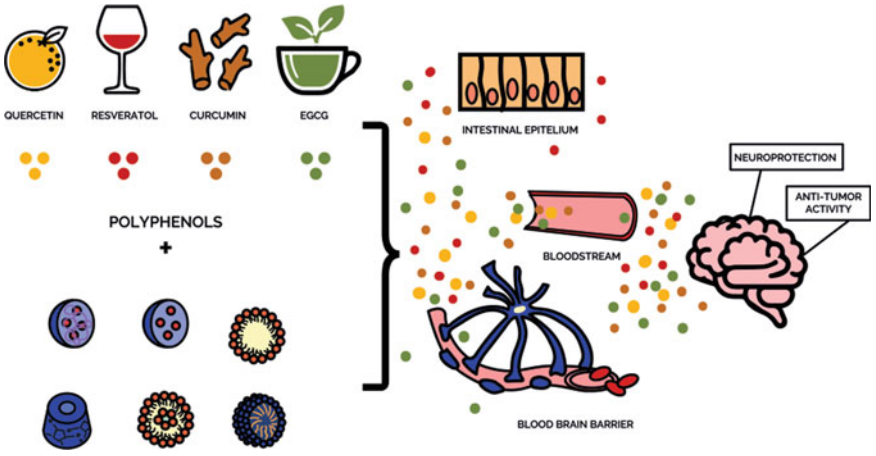

Fig. 8.5 Schematic representation of lipid nanotechnology based systems used for brain delivery of PBCs

specific to physicochemical properties of loaded PBCs presents an important research challenge.

- **How safe can nanomaterials be used in anti-aging medicine?** Advances in research in nanomaterials have streamlined the development of biogerontological interventions, but currently studies on the biological safety of those materials are lacking. Nanomaterials that are being used to encapsulate PBCs need to be further thoroughly investigated to determine if these carriers themselves have any harmful effects, especially if they will be used over a long period of time by patients.
- **What is the fate of the nanomaterials after administration?** Right now, our understanding of the physiological fate of a nanomaterial after administration is insufficient. It is unclear whether these nanocomposites can be metabolized into potentially harmful products. An important question to be addressed is whether the orally administered nanomaterials may be completely degraded and excreted after delivering their drug load. In addition, it is important to determine whether lipid nanomaterials can bioaccumulate in the human body. Finally, there is a concern that nanomaterials may constitute a biohazard when excreted in urine or feces and accumulated in the environment. These have to be verified before applications of nanomaterials in clinical practice for tackling aging.

Glossary

Bioavailability The fraction of absorbed drug or active compound reaching the systemic circulation.

Calorie restriction A reduction in calorie intake without malnutrition.

Epimerization A chemical process in which an epimer is converted to its chiral counterpart.

Freeze drying A method of removing water from a frozen material via sublimation of ice crystals.

Gastric residence time The length of time during which a material is kept in the stomach.

Healthspan The period of life spent in good health, free from the chronic diseases and disabilities of aging.

Lipids Organic molecules with hydrophobic or amphiphilic properties able to form structures such as vesicles, liposomes, or membranes in an aqueous environment.

Microfluidization A homogenization technique in which high pressure is applied to a fluid and is used to force the fluid to pass through microchannels. It is used extensively for generation of emulsions and nanoemulsions.

Nanocarriers Materials with particle size up to 100 nanometrers used to increase bioavailability of drugs with low solubility and absorption.

Nano-delivery Delivery of drugs or substances with nanocarriers.

Phytobioactive compounds Compounds of natural origin, being able to affect varied processes in biological systems.

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Chapter 9
Albumin-Based Carriers for Systemic Delivery to Tackle Cancer

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Abstract Proteins have been used to fabricate and design carriers for systemic drug delivery. One of the proteins that shows great potential for the applications in systemic delivery is albumin which is the most abundant plasma protein. This protein has been widely used in nanotechnology-based treatments of many diseases as a promising drug carrier. Taking cancer, an age-associated disease, as an example, the use of albumin nanoparticles as a drug carrier overcomes some of the limitations of chemotherapy such as severe side effects, nonspecific targeting, insolubility in aqueous solutions, short-term retention in bloodstream, and damage to normal cells. There are three main modes for delivering chemotherapeutic drugs with albumin: (1) covalent conjugation of the drug to albumin (prodrugs), (2) non-covalent coupling of the drug to albumin, and (3) encapsulation of the drug into albumin nanoparticles. In this chapter, we will illustrate the use of albumin-based carriers in anti-aging medicine, particularly in cancer treatments. In particular, we will present an overview of studies examining the anticancer effects of natural and synthetic drugs loaded to albumin (in all three modes) and related challenges.

Keywords Albumin · Protein · Drug carrier · Drug delivery · Prodrug · Chemotherapeutic drugs · Nanoparticle

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9.1 Introduction

Drugs that entered the blood stream will be circulated bodywide and retained in the body for several hours. In the treatment of disorders like cancer, chemotherapeutic agents attack cancer cell as well as healthy ones and cause severe side effects. In addition, other challenges associated with chemotherapy include short retention time, low cellular uptake, nonspecific targeting, cytotoxicity, and drug resistance. A drug delivery system is therefore essential for overcoming these limitations. The emergence of nanotechnology has been provided potential solutions to these difficulties. The superiorities of drug nanocarriers include specific targeting, excellent dispersibility, enhanced cellular penetrability, and enhanced cellular uptake. A variety of nanoparticles have been used as drug delivery systems such as metals, metal oxides, proteins, etc. (Nosrati et al. 2019a; Sharma et al. 2018). Protein nanoparticles have drawn particular interest due to their biodegradability, non-toxicity, biocompatibility, drug-binding capacity, and low costs. Recently, albumin nanoparticles have drawn particular research interest due to their biological functions including balancing plasma pH, stabilizing endothelium, transporting molecules intercellularly, and antioxidant activities in serum (Fasano et al. (2005); Bairagi et al. 2015). The application of albumin as a drug carrier was found to significantly ameliorate the low half-time and rapid degradation of a variety of chemotherapeutics.

There are three main ways for preparing albumin-based drug delivery systems: (1) covalent conjugation of the drug to albumin (prodrugs), (2) non-covalent coupling of the drug to albumin, and (3) encapsulation of the drug into albumin nanoparticles (Hoogenboezem and Duvall 2018). In the covalent binding mode, the drugs modified with maleimide groups can be conjugated to the cysteine 34 residues or tyrosine/lysine amino acid groups on albumin via chemical bonding to form a new compound. This strategy has been used for a number of drugs such as doxorubicin and methotrexate to enhance their therapeutic performance (Kratz and Elsadek 2012; Kratz 2008; Larsen et al. 2016).

9.2 Overview of Albumin

Albumin is a globular, water-soluble, and carbohydrate-free protein produced in the liver about 15 g/day. It is abundant in blood serum (at 3.5–5.0 g/dL; takes 60–65% of total serum proteins) and has a half-life of approximately 19 days. It helps in the growth and repairment of body tissues. Albumin is often obtained from human, bovine, and horse serum as well as egg white for commercial and research purposes (Peters and Brennan 1996).

9.2.1 Human Serum Albumin (HSA)

In the human body, HSA (approximately 66.5 kDa) serves the functions of transporting cellular information such as fatty acids, thyroid hormones, bile acids, folate, copper, zinc, and calcium. HSA also plays a role in inducing and maintaining oncotic pressure and in drug pharmacokinetics due to its affinity for a variety of drugs which allows it to transfer drugs to target sites (Liang et al. 2014; Ghuman et al. 2005; Yang et al. 2012). HSA proteins are encoded by ALB gene in chromosome 4 and consist of three domains (I, II, and III). Each domain has two subdomains (A and B). Subdomains A and B consist of 6 and 4 anti-parallel α-helices, respectively. Each HSA molecule contains 35 cysteine residues, of which 34 have disulfide bonds and one has a free sulfhydryl group (Yang et al. 2012; Wang et al. 2015; Peters 1995). HSA contains a large number of acidic and basic amino acids, which contributes to its high solubility and excellent binding ability to various molecules (Peters and Brennan 1996; Kragh-Hansen et al. 2013). The binding ability enhances the absorption of bound molecules by various cells (Varshney et al. 2010; Falé et al. 2011). HSA has been extensively used in the pharmaceutical industry due to its superiorities including biocompatibility, non-toxicity, biodegradability, abundance, and ease preparation.

9.2.2 Bovine Serum Albumin (BSA)

The mature form of BSA has a length of about 583 amino acids with a molecular weight of 66.5 kDa. It is also widely used in pharmaceuticals, biochemistry, biotechnology, and immunology because it is stable, available, ease isolate, and inexpensive (Majorek et al. 2012; Masuelli 2013).

9.2.3 Egg White Albumin (Ovalbumin)

Ovalbumin is the main protein found in egg whites. It consists of 385 amino acids at a molecular weight of 42.7 kDa (Nisbet et al. 1981; Stein et al. 1991). Ovalbumin is often used as drug vehicles, especially for the controlled release in target areas due to its abundance, heat and pH sensitivity, and low costs (Wongsasulak et al. 2010).

9.3 Albumin as a Drug Carrier

With the various advantages of albumin, it has been often used as a drug nanocarrier for the treatments of a range of diseases (Kratz 2008; Peters and Brennan 1996). Due to the exposed thiol, amino, and carboxyl groups on the surface of albumin, charged

molecules can easily attach to its surface (Irache et al. 2005). Therefore, albumin has often been used to deliver genes and chemotherapy agents in order to minimize the potential problems associated with free drugs and genes such as easy degradation, toxicity, poor solubility, and nonspecific targeting.

9.3.1 Albumin as Carrier of Platinum Drugs

Platins and platinum drugs (such as cisplatin, carboplatin, and oxaliplatin) are extensively used as chemotherapeutic drugs for relieving symptoms of metastatic cancers (chemical structures of some platinum drugs are shown in Fig. 9.1). For instance, cisplatin which is FDA approved is the first generation of platinum-based anticancer drugs. It is from the alkylating drug groups and also known as cisplatinum, neoplatin, and cis-diamminedichloridoplatinum (II) (CDDP) (Kelland 2007; Hato et al. 2014; Dilruba and Kalayda 2016; Hartmann and Lipp 2003; Ralhan and Kaur 2007). Cisplatin is often used to treat and control a variety of cancers such as ovary, uterus, and testis cancers. Carboplatin is used to treat ovarian and lung cancers, and oxaliplatin is used to treat pancreatic and colon cancers (Kelland 2007; Hato et al. 2014). The platinum drugs bind to the purine bases of the DNA molecules of the target cells and cause DNA damage and disruption of the DNA repairment process, then **apoptosis**. Overall, the alkyl groups of **alkylating agents** bind to many electronegative groups in cells. They inhibit tumor growth by directly attacking DNA molecules and cross-linking the guanine bases in the double-stranded DNA chain. This makes the DNA chains unable to detach and eventually stops cells proliferation. In addition, these drugs disrupt DNA base pairing by adding methyl or other alkyl groups to DNA molecules, which disrupts the DNA replication process (Hato et al. 2014; Dilruba and Kalayda 2016; Ralhan and Kaur 2007; Oun et al. 2018; Galluzzi et al. 2012; Dasari and Tchounwou 2014). However, there are challenges associated with these drugs, including drug resistance and severe side effects (such as neurotoxicity which is manifested by peripheral neuropathy) (Dilruba and Kalayda 2016; Oun et al. 2018).

The second generation of platinum-based anticancer drugs is carboplatin. This drug was developed to reduce the dose-dependent toxicity of cisplatin. The mechanism of action of carboplatin is similar to that of cisplatin. Similar to cisplatin, drug

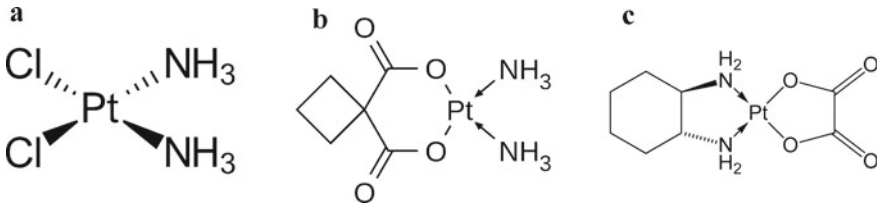

Fig. 9.1 Chemical structures of (a) cisplatin, (b) carboplatin, and (c) oxaliplatin

resistance to carboplatin can occur, which is a major challenge in the use of this drug in clinics (Calvert et al. 1982; Stewart 2007). Oxaliplatin (eloxatin) is the third generation of platinum-based drugs which was developed to overcome the drug resistance of cisplatin and carboplatin. In the treatment of metastatic colorectal cancer, it is prescribed in combination with 5-fluorouracil and folinic acid. Like cisplatin and carboplatin, the mechanism of action of oxaliplatin is to prevent the replication of DNA molecules. (Dilruba and Kalayda 2016; Wheate et al. 2010).

Platinum (IV) prodrugs are a group of platinum-based anticancer drugs that can easily be functionalized with biological ligands and overcome drug resistance. These drugs also allow chemical modifications and adjustments due to the presence of more ligands. These drugs are stable in the bloodstream due to a low level of interactions with plasma proteins. This class of drugs has the following advantages: 1—since these drugs are stable, they can be administrated orally; 2—they have less side effects; and 3—by modifying them, their pharmacological properties can also be modified, for example for specific targeting tumor sites. Generally, given the dose-dependent toxicity, tumor recurrence, drug resistance, low drug accumulation at tumor sites, and consequently low antitumor efficacy, delivery strategies are needed to improve the efficacy of platinum drugs in cancer treatment (Oberoi et al. 2013). By developing carriers for specific targeting delivery, the efficacy of these drugs can be improved.

The application of nanocarriers (such as gold nanoparticles, protein nanoparticles, peptides, lipid nanoparticles, carbon nanoparticles, and other nanomaterials) has recently been expanded (Johnstone et al. 2016). The design and development of targeting platinum (IV) prodrugs can increase the specificity of platinum drugs in targeting tumor sites and reduce the associated side effects. Accordingly, Mayr et al. synthesized a functionalized cisplatin and oxaliplatin prodrugs that contain maleimide groups (for specific binding to albumin) (Mayr et al. 2017). In fact, the presence of the maleimide groups allows selective binding to serum albumin in the bloodstream, which leads to an accumulation of the drug in the target tumor site by EPR effect and also prolong of drug retention by preventing renal excretion. In vivo experiments also confirmed the long-term accumulation of the prodrugs containing maleimide groups in tumor sites. Evaluation studies of the effects of the prodrugs on mice bearing CT26 tumor have also shown higher antitumor activities of the oxaliplatin prodrug as compared to cisplatin (Mayr et al. 2017).

Another limitation of cisplatin is its poor solubility in water. Although the solubility of cisplatin in DMSO is very high, dissolving in DMSO decreases its toxicity and consequently reduces its therapeutic effects against cancer cells. In a study, dry powder of cisplatin–albumin mesospheres were post-loaded with cisplatin in a DMSO solution. It was observed that cisplatin efficiently was absorbed onto albumin mesospheres and maintained its toxicity at 100%. Albumin microspheres have the potential to be an effective carrier for cisplatin drugs due to their functional groups such as carboxyl groups. The established formulation was designed for intratumoral injection or inhalation because of the excellent stability of this formulation in air and in solutions. A drug release assay also showed a rapid release of cisplatin over 24 h. Finally, the anticancer effects of the formulation on different cancer cells were investigated, and the efficacy of the formulation in deteriorating the cancer cells

was demonstrated (Lee et al. 2015). In 2015, Shi et al. synthesized nanoparticles containing HSA and calcium phosphate (CaP) (which were sensitive to pH and redox) to deliver cisplatin prodrugs. They investigated the antitumor effects of the system against tumor cells (Shi et al. 2015). Also, Catanzaro et al. designed and synthesized glutathione-sensitive albumin nanoparticles to carry cisplatin and investigated the toxicity and anticancer properties of the system (Catanzaro et al. 2017).

9.3.2 Albumin as Carrier of Curcumin

Turmeric, one of the most popular spices used in the food industry (Aggarwal and Sung 2009). Turmeric contains many components, including turmerone and coloring agents called curcuminoids. Curcuminoids include demethoxycurcumin, 5′-methoxycurcumin, dihydrocurcumin, and cyclocurcumin (Prasad and Aggarwal 2011; Kiuchi et al. 1993).**Curcumin** ($C_{21}H_{20}O_6$; also known as diferuloylmethane) is the most important component of turmeric which accounts for up to 8% of its dry weight (Fig. 9.2). Many properties of turmeric as well as its yellow color are related to curcumin (Modaresi et al. 2017; Aggarwal et al. 2007; Chattopadhyay et al. 2004). This compound was first extracted and purified from turmeric in 1815 (Vogel and Pelletier 1815). Curcumin has many properties including lowering cholesterol, inhibiting lipoxygenase and cyclooxygenase, inhibiting the proliferation of tumor cell, and inhibiting proteases. It also exhibits antioxidant, antibacterial, and anti-inflammatory activities (Modaresi et al. 2017). High doses of this substance prevent the proliferation of cancer cells without harming healthy ones. Curcumin exerts its therapeutic effects through the induction of apoptosis and cell death and also inhibition of cellular signaling pathways that disrupts the proliferation of cancer cells (Kunnumakkara et al. 2017). The anticancer activities of curcumin have been shown against various cancers including breast, prostate, and lung cancers (Kunnumakkara et al. 2017). This golden substance has some limitations, including low solubility in water which causes poor absorption. Curcumin, due to its hydrophobicity, binds to fatty acyl chains upon penetration into cell membranes leading to a poor absorption by the cells (Nagahama et al. 2016; Barry et al. 2009; Tsukamoto et al. 2014). One way to overcome these limitations is to develop new delivery strategies to transfer the

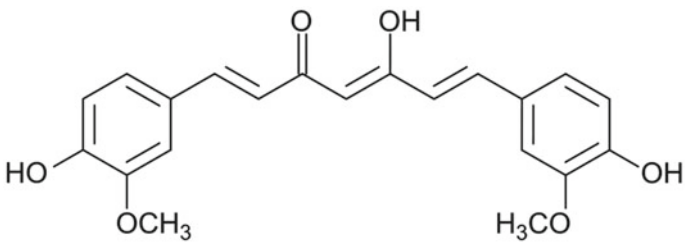

Fig. 9.2 Chemical structure of curcumin

compound to the target site and thereby increase its therapeutic efficiency. The use of nanotechnology-based delivery vehicles can increase the absorption and specific targeting (Sun et al. 2012).

One of the carriers used to deliver curcumin is albumin protein nanoparticles. Albumin nanoparticles exhibit advantages such as better targeting and controlled release. These nanoparticles can also encapsulate a variety of drugs due to their multiple specific and nonspecific binding sites (Sun et al. 2012). Binding of curcumin to albumin results in an increase in solubility of curcumin in water (Thomas et al. 2014). A study was conducted to investigate the in vivo and in vitro therapeutic effects of curcumin conjugated with BSA (CUR–BSA prodrug) (Aravind and Krishnan 2016). The in vitro therapeutic effects of the formulation were investigated on DLA murine lymphoid cell lines (Dalton lymphoma ascites). It was found that CUR–BSA decreased the viability of DLA cells in a dose-dependent manner. In the in vivo assay, it was also observed that the injection of the compound did not lead to a weight loss in healthy animals and did not show any toxic effects. Administration of CUR–BSA nanoparticles on mice bearing DLA tumors have shown a reduction in tumor sizes. It was found that upon intraperitoneal injection of the compound into the animal models seven days after tumor development, the size and number of tumor cells were significantly reduced and the longevity of the animals was increased. Also, injection of the compound immediately after tumor formation showed excellent antitumor effects (Aravind and Krishnan 2016).

In another study, the in vitro and in vivo antitumor effects of BSA–CUR were investigated against murine melanoma cells and a murine melanoma model. The BSA–CUR formulation was synthesized using the **desolvation** method into negatively charged spheres at a size of 150 nm. The encapsulation rate of curcumin into albumin was about 45%. It was observed that the release of curcumin from the albumin nanoparticles was slow and diffusion dependent. The anticancer effects of BSA–CUR and free curcumin were evaluated in an animal model and cells. It was found that BSA–CUR showed more antitumor effects as compared to free curcumin. BSA–CUR and curcumin reduced cell viability in vitro by 45% and 55%, respectively. Therefore, it can be concluded that albumin can be a suitable carrier for curcumin and may be applied in the treatment of melanoma in clinics (Camargo et al. 2018).

Use of nanoparticles made from albumin to transport hydrophobic drugs to tumor sites is a promising strategy. Kim et al. investigated the simultaneous delivery of paclitaxel and curcumin by albumin nanoparticles to cancer cells and their anticancer effects. The nanoparticles had a spherical shape at a size of approximately 250 nm. It was also seen that the release rate of paclitaxel and curcumin was approximately 97% and 76%, respectively, within 24 h. They observed that the prepared nanoparticles efficiently entered the target cells (Mia Paca-2 cells) (Kim et al. 2016). In another attempt, the antitumor effects of curcumin conjugated to HSA nanoparticles were also investigated. CUR–HSA nanoparticles were synthesized with a size of 130–150 nm. It was found that the solubility of these nanoparticles in water was over 300 times higher than that of free curcumin. In vivo experiments on drug distribution and transport in vascular endothelial cells have also illustrated the superiorities of

CUR–HSA nanoparticles over free curcumin. It was also observed that the rate of drug accumulation in the tumor site after injection of the nanoparticles (CUR–HSA) was about 14 times higher than free curcumin. Antitumor effects study of these nanoparticles have also shown a suppression of tumor growth by up to 66% (Kim et al. 2011).

Interestingly, BSA was used as a sulfur origin in the Bi_2S_3 preparation process (Nosrati et al. 2019b; Wang et al. 2016). In this case, BSA was used not only as a sulfur origin but also as a coating agent. In addition, because of the high drug capacity of BSA, the prepared nanoparticles were used as a drug carrier (Nosrati et al. 2019b). Nosrati et al. used BSA-coated Bi_2S_3 nanoparticles as a **radiosensitizer** and a carrier for CUR (Fig. 9.3) (Nosrati et al. 2019b). The main goal of this study was to increase the dose of radiation at the tumor site while decrease it in the surrounding healthy tissues (Sridhar and Symonds 2009; Sridharan et al. 2015; Li et al. 2018; Zang et al. 2019; Huang et al. 2019). In radiotherapy, researchers confronted the adverse effects on healthy tissues and challenges related to hypoxic cells. Therefore, the development of nanomaterials as both radiosensitizer and carrier of drugs have gained significant attention for successful therapies (Nosrati et al. 2019b). They also reduced the needed therapeutic doses and side effects and ultimately improved the therapeutic efficiency. Also, CUR as a natural **radioprotector** was used in this work. Therefore, using a radiation-protecting compound can protect healthy cells. Indeed, radiation protectors are compounds that scavenge the free radicals and prevent the damaging effects of radiation on vital molecules (Hosseinimehr 2007; Brown et al. 1982; Jagetia 2007). Our research group showed that after injection of Bi_2S_3@BSA-FA-CUR nanoparticles into mice, tumors were completely eliminated in three weeks (Nosrati et al. 2019b). Moreover, in some studies, BSA was used for surface modification of radiosensitizers to increase stability and prolong blood circulation time (Abhari et al. 2020).

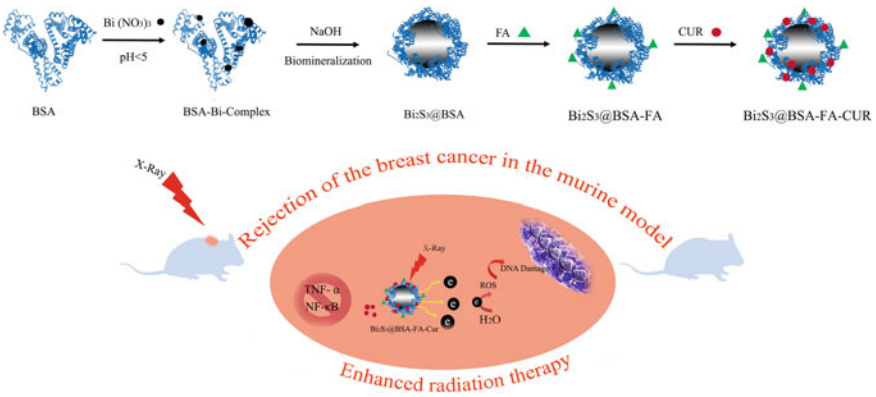

Fig. 9.3 Schematic illustration of the synthesis of Bi_2S_3 @BSA-FA-CUR and tumor ablation in the murine model. Reproduced from (Nosrati et al. 2019b) with permission from American Chemical Society

In a study conducted by Song et al. in 2016, HSA conjugated with folate was used to carry curcumin (HSA–CUR), and the therapeutic effects of the nanodrug were investigated in vivo and in vitro (Song et al. 2016). Zhang et al. designed and developed a new method for orally delivering curcumin in order to increase the mucoadhesion of curcumin in the gastrointestinal tract. They modified BSA nanoparticles with N-acetyl cysteine for curcumin delivery (CUR–NBSA). It was found that the synthesized nanoparticles increased mucoadhesion (Zhang et al. 2019). In another study Gong et al. loaded curcumin into albumin nanoparticles (HSA–CUR) and examined their accumulation at tumor sites and the antitumor effects (Gong et al. 2015).

9.3.3 Albumin as Carrier of Doxorubicin

Doxorubicin (also known as Adriamycin) is one of the chemotherapeutic drugs used to treat many cancers (Fig. 9.4). It is a member of the anthracyclines antibiotic family. Doxorubicin is highly lipophilic and has a long half-life (Arcamone 2012; Frederick et al. 1990; Thorn et al. 2011). The mechanism of action of this drug on cancer cells involves intercalation into DNA and inhibition of topoisomerase 2, which eventually disrupts DNA repairment and stops DNA replication. Another mechanism is the production of free radicals that damage the genome and eventually lead to cell death (Tacar et al. 2013; Pommier et al. 2010; Taymaz-Nikerel et al. 2018). However, one of the major limitations of this drug is the cardiotoxicity (Open 2018).

In the treatments of liver cancers, the image-guided intra-arterial treatment plays a crucial role. Poor results obtained from clinical experiments suggested the need of novel multipurpose drug delivery platforms for 1—increasing the drug concentration and 2—minimizing side effects (Lee et al. 2019). Perhaps one of the factors accounting for the poor results is the limited access of the drug to the desired site (Liang et al. 2013; Gaba et al. 2016; Sheth et al. 2013). Side effects are another challenge in clinics. Even if drugs are injected via the inferior alveolar (IA) method, the blood concentrations of the drugs are still high. Thus, it is very important to reduce

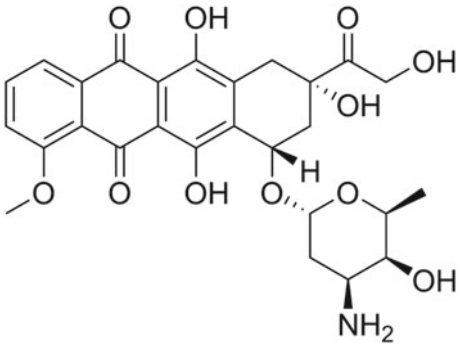

Fig. 9.4 Chemical structure of doxorubicin

the concentrations of drugs in the circulation via specific targeting delivery in order to minimize side effects. Real-time tracking of the drug delivery is one of the needs in the clinic that must be accomplished. Hence, development of specific targeting drug vehicles and multimodal imaging technologies to track the vehicles in vivo is very important. Recently, nanoparticles have been studied to address the mentioned challenges in order to improve the performance of drugs (Lee et al. 2019). In recent years, researchers have used ultrasound to create transient pores in cell membranes in order to increase drug penetration (**sonoporation**) (Moghimi et al. 2001; Vancraeynest et al. 2005). In sonoporation, cavitation microbubbles were employed to form transient pores in plasma membranes and subsequently facilitate specific drug delivery (Meng et al. 2019; Wang et al. 2018). In a study conducted in 2019, Lee et al. generated an HSA–doxorubicin nanodrug coupled with microbubbles (ADMB) to improve the therapeutic power by sonoporatio under ultrasound. Albumin–doxorubicin particles were synthesized by the desolvation method and attached to the surface of microbubbles. The size of the ADMB complex was at 2.33 ± 1.34 μm. The drug-loading efficiency was 82.7%. The echogenicity and cavitation of ADMB complex were created with the aid of ultrasound at a frequency of 2–9 mHz. This study aimed to investigate the anticancer effects of the ADMB complex with ultrasound microbubble activation in an animal model. Rabbit bearing *VX2* tumor was used as a model. It was observed that the ADMB complex improved the treatment efficacy under ultrasound as compared to free doxorubicin. With IA injection of the ADMB complex into the animal model with microbubble activation under ultrasound, both drug delivery and treatment efficacy can be monitored simultaneously. IA injection of the ADMB complex resulted in a five-fold inhibition of tumor growth. Overall, this study showed that tumor growth inhibition could be effectively increased by applying ultrasound with ADMB complex (Lee et al. 2019).

One of the main difficulties in chemotherapy is the low susceptibility of cancer cells to anticancer drugs after prolonged use, which can be caused by several factors, such as low drug penetration through plasma membranes, lysosomal escape, or multiple drug resistance (MDR) (Motevalli et al. 2019). One strategy to solve this problem is to simultaneously deliver multiple drugs to cancer cells with nanoparticles. In this regard, Motevalli et al. used albumin nanoparticles encapsulated with curcumin and doxorubicin to treat MCF-7 breast cancer cells and evaluated the therapeutic ability. Cells were treated with albumin–curcumin, albumin–doxorubicin and albumin–curcumin–doxorubicin. They found a higher intracellular accumulation of doxorubicin and a lower intracellular accumulation of curcumin in the simultaneous drug co-administration. It was shown that this result was due to the accumulation of curcumin in lysosomes upon fast release of it from the albumin–curcumin complex (Motevalli et al. 2019).

It was reported that transporter-mediated drug efflux like ATP-binding cassette (ABC) transporters plays a major role in drug resistance. To overcome this resistance, Onafuye et al. (2019) studied the effects of HSA–doxorubicin nanoparticles on UKF-NB-3 neuroblastoma cells (UKF-NB-3rVCR1 and UKF-NB-3rDOX20) and the expression of ABCB1. It was found that HSA–doxorubicin nanoparticles showed greater anticancer effects on UKF-NB-3rVCR1 and UKF-NB-3rVCR1 as

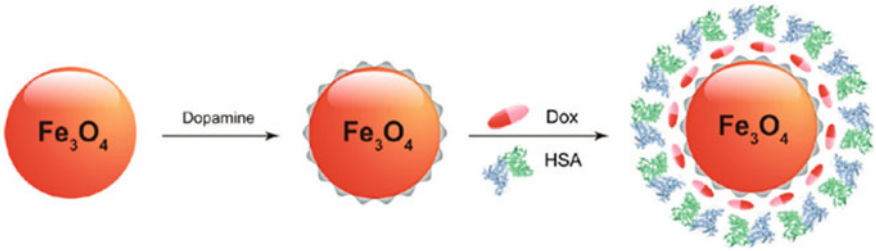

Fig. 9.5 Schematic illustration of the encapsulation of Fe_3O_4 nanoparticles and DOX into HSA. Reproduced from (Quan et al. 2011) with permission from American Chemical Society

compared to free doxorubicin, while inhibition of ABCB1 expression by zosuquidar had a similar effect. This indicated that albumin–doxorubicin may have inhibited the efflux effects. This study demonstrated that the use of HSA nanoparticles as a drug carrier can be an alternative method to overcome the resistance induced by the transporter (Onafuye et al. 2019). In a study in 2019, the anticancer effects of HSA–doxorubicin were also investigated (Kimura et al. 2019). Hao et al. bonded folic acid modified dextran into BSA and further linked the complex with superparamagnetic nanoparticles (Fe_3O_4–BSA–DEX–FA), and examined its theranostic effects against tumor (Hao et al. 2014). (Fig. 9.5). The anticancer effects of doxorubicin-albumin magnetic nanoparticles were also investigated on prostate and lung cancer cells by Zeybek et al. (Zeybek 2012). Recently, theranostic application of nanoparticles has been developed as a novel approach in medical nanotechnology. In a study conducted by Quan et al., iron oxide–dopamine nanoparticles coated with human albumin were used to deliver doxorubicin and to investigate the transanesthetic application. In this study, a complex of iron oxide nanoparticles, dopamine, and doxorubicin were encapsulated in HSA (Quan et al. 2011).

9.3.4 Albumin as Carrier of Paclitaxel

Paclitaxel (Fig. 9.6) is one of the drugs used in chemotherapy. It is an herbal alkaloid extracted from Pacific Yew tree bark. It is used to treat lung, ovarian, breast, and other cancers. This drug targets the tubulin in the cytoskeleton. Mitotic spindle assembly, chromosome segregation, and cell division are disrupted by paclitaxel. Paclitaxel is a microtubule inhibitor that controls the formation of microtubules and stabilizes them. This stabilization inhibits the identification of microtubule network which is critical for interphase and mitosis. Unlike other cytoskeleton-related drugs such as colchicine which inhibits microtubule assembly, paclitaxel stabilizes microtubule and prevents its disintegration resulting in an inaccessibility of chromosomes to the metaphase spindle (Kampan et al. 2015; Weaver 2014; Horwitz 1994; Xiao et al. 2006; Nosrati et al. 2019c). Albumin has become an efficient carrier for delivering hydrophobic drugs to cancer tissues because of its unique properties. There

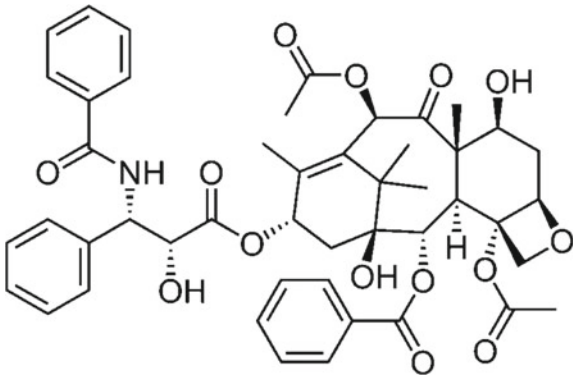

Fig. 9.6 Chemical structure of paclitaxel

are several specific and nonspecific sites on the surface of albumin molecules for hydrophobic molecules to attach to, which allows the binding of several drugs, such as paclitaxel (Kratz 2008; Purcell et al. 2000; Paál et al. 2001). Interestingly, albumin is absorbed by proliferating cancer cells and used as an energy supply for tumor growth after decomposition. It also allows the development of drug delivery systems for delivering anticancer drugs to cancer tissues due to their accumulation in cancer tissues (Stehle et al. 1997; Commisso et al. 2013; Kremer et al. 2002). Despite the potential anticancer effects of paclitaxel, its use in the clinic has been limited due to its high hydrophobicity and difficult preparation. Albumin–drug (nab) strategy is an efficient system for delivering hydrophobic drugs to target regions. Albumin–Paclitaxel (Abraxane) was the first approved nanodrug developed by Abraxis BioScience company. Albumin binds non-covalently to paclitaxel via more than six specific and nonspecific sites with different levels of affinity (Paál et al. 2001).

Recently, many studies have investigated the anticancer effects of paclitaxel loaded nanoparticles. For example, Yang et al. prepared paclitaxel loaded albumin nanoparticles and investigated the therapeutic effects in vivo. In this study, two prodrugs [one was sensitive to oxidative/reductive (Ox/Re) and the other was not] with a maleimide group that binds to albumin were designed and synthesized. Result show that the Ox/Re sensitive prodrug have remarkable anticancer effects in animal models (Yang et al. 2018). Zhang et al. (2011) investigated the antitumor effects of BSA loaded with paclitaxel and sorafenib (Zhang et al. 2011). In another study, gold nanorods (AuNRs) and paclitaxel were encapsulated in HSA, and the complex was examined for photothermal and chemotherapy against 4T1 breast cancer cells (Fig. 9.7) (Peralta et al. 2015). Ge et al. prepared nanoparticles of crushed human albumin encapsulating paclitaxel and investigated the anticancer effects and toxicity against cancer cells (Ge et al. 2018).

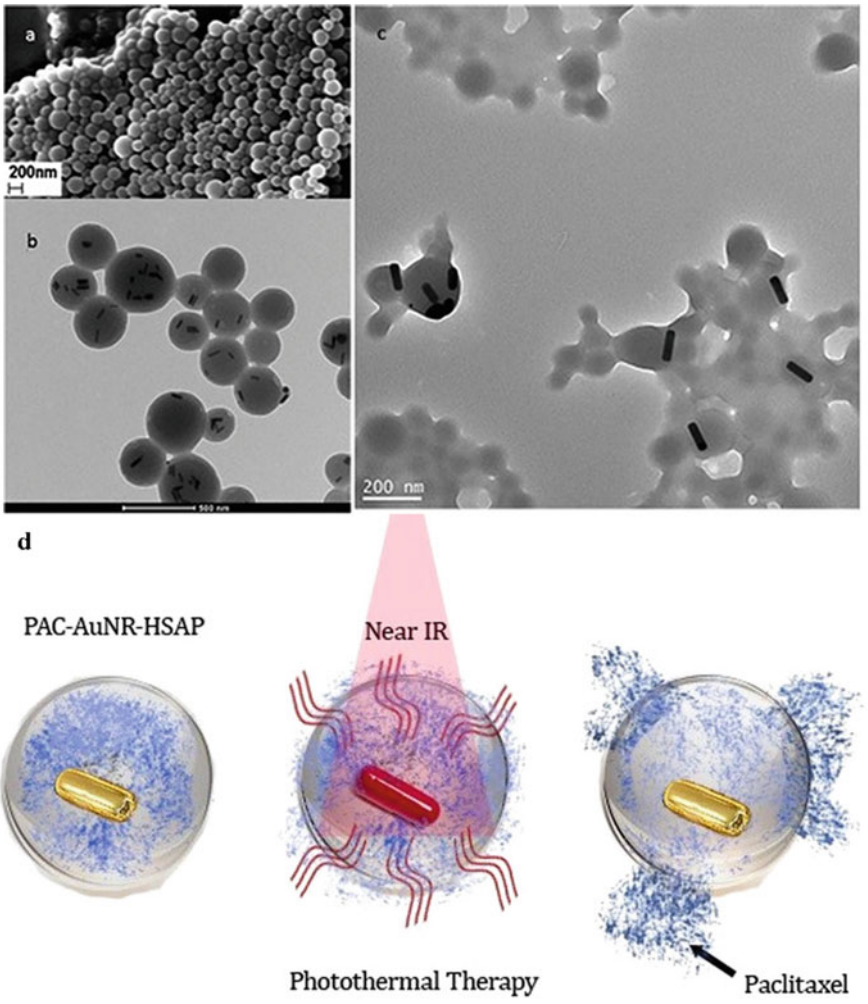

Fig. 9.7 (a) FESEM image of human serum albumin nanoparticles coated gold nanorods (AuNR–HSAPs). (b) TEM image of AuNR–HSAPs. (c) TEM image of AuNR–HSAPs used in in vitro cell studies. Reproduced from (Peralta et al. 2015) with permission from American Chemical Society

9.3.5 Albumin as Carrier of Methotrexate

Methotrexate (also known amethopterin; Fig. 9.8), a chemotherapy agent for a variety of cancers and an immunosuppressant, was used in chemotherapy for childhood acute leukemia in 1948 (Farber et al. 1948). It interferes with the growth of different cells in the body, including fast-growing cells such as bone marrow and cancer cells. This chemotherapy drug is used to treat many cancers such as rheumatoid arthritis, severe psoriasis, breast cancer, etc. (Weinblatt 2013; Hagner and Joerger 2010). It is also an

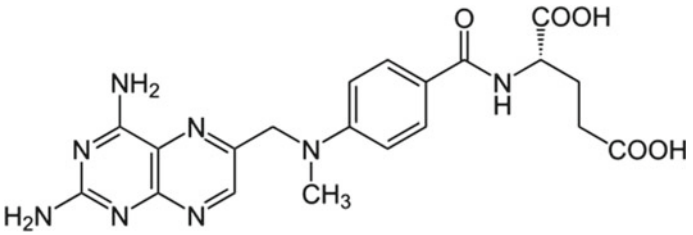

Fig. 9.8 Chemical structure of methotrexate

antifolate that inhibits the production of folic acid through a competitive inhibition mechanism. The mechanism of action of this drug is to prevent the conversion of dihydrofolate to tetrahydrofolate by binding to dihydrofolate reductase (DHFR). Folic acid is necessary for the synthesis of purine and pyrimidine bases and thus deficiency of folic acid leads to the disruption of the production of nucleic acids and eventually cell destruction (Chan and Cronstein 2013; Inoue and Yuasa 2013).

In a study conducted by Fiehn et al., the therapeutic effects of methotrexate–albumin prodrug were investigated against arthritis model mice. The synthesized prodrug complex (AWO54) specifically bound to cysteine-34 albumin to delivering methotrexate to target tissues. It was found that protein-degrading enzymes (cathepsin B and plasmin) which are highly expressed in rheumatoid arthritis broke down the prodrug complex and released methotrexate. In this study, the therapeutic effects of free methotrexate and the methotrexate–albumin prodrug on collagen-induced arthritis in mice were investigated. It was observed that the albumin–methotrexate prodrug had significantly greater therapeutic and inhibitory effects as compared to free methotrexate (Fiehn et al. 2008). In another study, methotrexate–HSA nanoparticles were prepared and investigated for their therapeutic effects on cancer cells. Methotrexate was conjugated to HSA by EDC to form nanoparticles with a size of 90–150 nm. The therapeutic effects of these nanoparticles (HSA–MTX) and free methotrexate on T47D breast cancer cell lines were investigated. It was observed that HSA–MTX nanoparticles showed a greater toxicity against the cells with a reduced IC50 as compared to free methotrexate. Nosrati et al. conjugated methotrexate to albumin (BSA–MTX), loaded curcumin into the complex (MTX–BSA–CUR) and investigated its anticancer effects in vitro (Nosrati et al. 2019d). In another study, HSA–MTX was investigated for anticancer effects on cancer cells (Wosikowski et al. 2003).

9.3.6 Albumin as Carrier of 5-Fluorouracil

5-Fluorouracil (Fig. 9.9) is also a chemotherapy drug commonly used in cancer treatment. It is an antimetabolite and an analog of pyrimidine bases that inhibits thymidine synthesis and disrupts genome replication. One of the limitations of this drug is

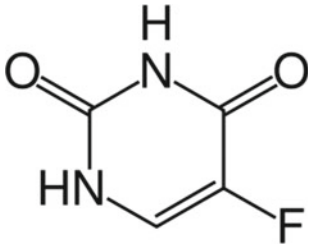

Fig. 9.9 Chemical structure of 5-fluorouracil

the short circulation time which results in reduced therapeutic effects. Increasing the drug dose can enhance therapeutic effects but may also result in enhanced side effects (Zhao et al. 2017; Carrillo et al. 2015; Miwa et al. 1998). In a study, an albumin-5-fluorouracil prodrug was synthesized and examined for its anticancer effects and circulation time. To prepare the albumin-5FU prodrug, 1-alkylcarbonyloxymethyl 5-FU was linked to a maleimide group and combined with BSA (BSA–EMC–5-FU). It was shown that BSA–EMC–5-FU complex was stable in acidic and neutral conditions but unstable in alkaline conditions. After intravenous injection of EMC–5–FU into SD rats, t1/2 and AUC values were significantly increased as compared to the rats injected with free 5-FU. It was observed that EMC–Cy5 specifically accumulated in the tumor site. It was also found that the inhibitory and anticancer effects of EMC–5-FU were much greater than those of 5-FU in H22 tumor model mice. Overall, this study addressed the challenges of short circulation time and nonspecific delivery, so provided a prospective drug delivery system for clinical applications (Zhao et al. 2017). Santhi et al. developed 5-fluorouracil–BSA nanoparticles by the pH-coacervation method and investigated their antitumor effects on mice bearing DLA tumor. They observed a high inhibitory effect on tumor growth (Santhi et al. 2002). To create an anti-hepatocarcinoma system for targeting the liver, Cai et al. conjugated 5-FU to galactosylated HSA (GHSA–5-FU) (Cai et al. 2006). Misak et al. synthesized albumin–5-Fu magnetic **nanocomposites** and investigated their antitumor activities (Misak et al. 2013).

9.4 Albumin as Coating Agent

Albumin has also been used as a coating material in various studies. For example, albumin-coated gold nanoparticles (BSA–Au) were prepared and investigated for their anticancer effects on different cancer cells using photothermal methods (AL-Jawad et al. 2018). Nosrati et al. used BSA as a coating material of bismuth sulfide and gold (Bi_2S_3–Au) heterodimers (Abhari et al. 2020). Also, Nosrati et al. used albumin to coat bismuth sulfide nanoparticles to prepare a radiosensitizer (Nosrati et al. 2019b). Najafi et al. used albumin to coat iron oxide nanoparticles. It was found that the non-biocompatibility as one of the major challenges in using iron oxide nanoparticles can be overcome by BSA coatings. The compatibility and non-toxicity

of BSA–iron oxide nanoparticles were confirmed in vitro (Najafi and Kouchakzadeh 2019). Hajshafii et al. coated Fe_3O_4 nanoparticles with BSA and dimercaptosuccinic acid (DMSA). Albumin-coated nanoparticles were found to be less toxic as compared to DMSA-coated ones (Hajshafii et al. 2014). In a study, BSA and chitosan (CS) were used to coat silver nanoparticles, and the antibacterial effects of the system were studied (Espinosa-Cristóbal et al. 2015).

In order to specifically target cancer cells, Azizi et al. designed and synthesized a new nanodrug with silver nanoparticles in 2017. After coating silver nanoparticle with albumin, the therapeutic effects were evaluated against MDA-MB-231 breast cancer cell lines. It was found that these nanoparticles (negatively charged with a size of 90 nm) were specifically absorbed by the cancer cells and caused cell death by inducing apoptosis. Azizi et al. observed that the LD_{50} of the synthesized nanoparticles against cancer cells was approximately 30-fold higher than that of the healthy white blood cells. These nanoparticles killed cancer cells via apoptosis and reduced tumor size, and therefore are advantageous in cancer treatments (Azizi et al. 2017a). In 2017, Azizi et al. coated copper nanoparticles with albumin and studied the anticancer effects on MDA-MB-231 breast cancer cells. Albumin-coated nanoparticles were found to decrease the viability of a significant portion of the cancer cells and exhibit a low toxicity against healthy cells (Azizi et al. 2017b). Recently, albumin was used as a coating agent for chalcopyrite nanoparticles used in chemodynamic therapy (Chen et al. 2019). Albumin has been used to coat nanoparticles in several studies of gene delivery. For example, Piao et al. used human albumin to coat lipid nanoparticles for the delivery of siRNA to breast cancer cells and in an animal model. They found that the delivery system successfully delivered siRNA to the target region and showed an excellent therapeutic performance (Piao et al. 2013).

9.5 Summary and Outlooks

In this chapter, we present the latest developments in albumin-based delivery systems for anticancer drugs and the associated challenges. The high biocompatibility, nontoxicity, low immunogenicity, and vast availability of albumin make it as a good drug carrier. In addition, albumin-based carriers have overcome some of the limitations of chemotherapeutic drugs. Albumin-based prodrugs can not only decrease side effects but also increase half-life of the loaded drugs. In recent years, a large number of formulations based on nanoparticles have been explored for clinical usage, but many of them failed to be approved. However, Abraxane, an antineoplastic medication, is an albumin-based nanodrug that has been approved by the FDA in 2005. Also, some of the albumin-based nanodrugs have been in clinical trials. Albumin-based formulations are prospective for clinical applications. Moreover, literature results will encourage future applications of albumin-based carriers.

Important Notes

- Albumin-based drug carriers can increase retention time of the drug in the bloodstream.
- Albumin-based nanoparticles have provided possibilities of clinical applications of similar drug delivery systems.
- Albumin can be used not only as a drug carrier but also as a stabilizer of many colloidal systems.
- In some cases, albumin is used in theragnostic systems.

Questions for Future Research

- **What are the potential side effects of albumin-based carriers?** Because of the tiny sizes and higher surface areas of nanoparticles, there is a fundamental need in determining their adverse effects on organisms. In addition, the long-term effects of albumin-based nanoparticles in the body should be evaluated.
- **How to extend the application potential of albumin-based systems in systemic delivery in terms of tackling cancer and treating other age-associated diseases?** Oral administration may be a future trend. Further investigation on the mechanisms of drug delivery mediated by albumin-based systems should be performed. Albumin-based systems also require an easy preparation approach to scale up the production.

Glossary

Albumin is the most abundant plasma protein that has many important roles, such as transporting fatty acids, thyroid hormones, bile acids, etc. Albumin nanoparticles have been used as drug carriers in the treatment of many diseases such as cancers.

Alkylating agent an anticancer compound that prevents the transcription of DNA molecules into RNAs and eventually the protein synthesis by linking the alkyl group to the guanine bases of DNA molecules.

Apoptosis is the programmed cell death that plays a critical role in the elimination of unnecessary cells such as damaged cells and virus-infected cells.

Curcumin is the main component of turmeric. Curcumin has many properties such as antioxidant, anticancer, anti-inflammation, etc.

Desolvation a method that can be used to prepare albumin nanoparticles by adding a solvent (such as ethanol or acetone) dropwise to an aqueous solution of albumin proteins under a magnetic stirrer in order to dehydrate the proteins.

Nanocomposite a compound consisting of two or more materials of which at least one is nanosized.

Radioprotector agents that protect healthy cells from radiation when tumor cells are irradiated.

Radiosensitizer agents that increase the sensitivity of tumor cells to radiotherapy.

Sonoporation a biophysical method for the formation of transient pores in plasma membranes which facilitates specific targeting drug delivery.

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Chapter 10
Exosomes as Vehicles for Systemic Drug Delivery

Magnolia Muk-Lan Lee, Wing-Yan Wong, Brandon Dow Chan, and William Chi-Shing Tai

Abstract Naturally occurring compounds (e.g., lipids and proteins) can serve as constituents for carrier production; biological entities per se may also function as ready-made carriers for systemic delivery. Examples of these entities include bacteria, viruses, erythrocyte ghosts, and exosomes. While many of these entities have been extensively reviewed in the literature on therapeutics delivery, comparatively little discussion has focussed on exosomes, a subset of extracellular vesicles released by all types of cells which are involved in local and systemic intercellular communication. In fact, exosomes carry a wide range of cargos (including nucleic acids, proteins, and lipids derived from their cell of origin) to recipient cells. This cargo-carrying systemic delivery system has already been exploited in the literature to transport therapeutics to target cells. In this chapter, we will provide an overview of the function, activity, and mechanisms of exosomes, methods for exosome isolation and cargo loading, and strategies for specific targeting of cells. The considerable potential for exosomes as a delivery method for therapeutics in disease will also be highlighted.

Keywords Exosome · Non-viral delivery · Exocytosis · Biogenesis · Extracellular vesicles

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10.1 Introduction

Drugs have long been used for maintenance of health, treatment of disease, and extension of lives. To date, a broad range of drugs have been identified to be effective toward different major diseases, including cancer, inflammatory and cardiovascular diseases, and diabetes. However, despite the huge number of efficient chemical entities, drawbacks including low solubility, instability, low absorption, and toxicity hinder development of these chemical entities into drugs for the treatment of disease. As drugs are mostly administered via enteral, parenteral, and topical application, development of novel drug delivery systems for sustainable, biocompatible, and targeted drug delivery could potentially address a host of difficulties currently encountered during drug development.

Extracellular vesicles (EVs) are secreted by a variety of cell types. This conserved secretory process was originally seen as a mechanism for removal of unwanted materials from cells; however, increasing evidence suggests cells utilize EVs for information exchange and communication, initiating a new direction for EV studies. In comparison with other drug delivery systems, EVs exhibit various advantageous properties, including small size, biocompatibility, and low toxicity. Considering requirements for the development of efficient and safe drug delivery systems, EVs can be viewed as an ideal delivery tool for further advancement.

In general, EVs can be categorized into three major subtypes depending on their size, biogenesis, secretory pathway, and function: (1) **exosomes** (30–150 nm), (2) microvesicles (100 nm–1 μm), and (3) apoptotic bodies (50 nm–5 μm) (Doyle and Wang 2019). Due to their size, exosomes are considered to be the most promising of the EVs for development as drug delivery tools.

10.2 Exosome Biogenesis

Exosomes are 30–150 nm intraluminal vesicles (ILVs) generated via the endosomal system. They can be excreted from various mammalian cells (including B cells, T cells, dendritic cells, platelets, and reticulocytes), and contain a wide diversity of proteins (e.g., integrins, tetraspanins, MHC molecules, RAB5 and 7, RAP1B and annexins, and **cytoskeleton**-associated molecules) (Fig. 10.1) (Darband et al. 2018). Biogenesis of exosomes begins with the maturation of early **endosomes** into late endosomes, followed by introversion of the endosomal membrane to produce ILVs in multivesicular bodies (MVBs) (Fig. 10.2) (Barile and Vassalli 2017). When transported to the near proximity of the plasma membrane, fusion of MVBs to the plasma membrane leads to the release of exosomes into the extracellular environment through exocytosis (Doyle and Wang 2019; Hessvik and Llorente 2018).

As exosomes belong to the class of ILVs, they utilize the same mechanisms and sorting machineries, one of which is the endosomal sorting complex required for transport (ESCRT). The ESCRT consists of four complexes (ESCRT-0, -I, -II,

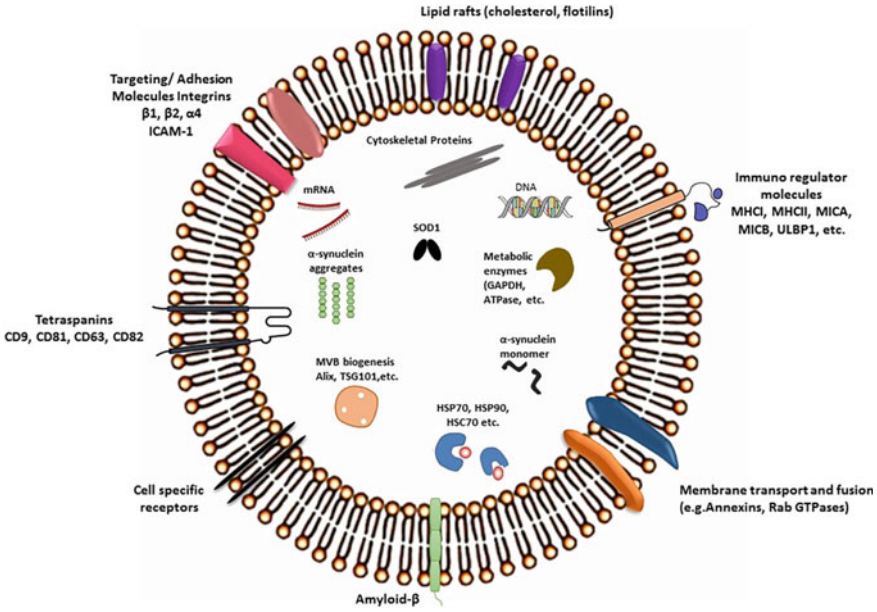

Fig. 10.1 Key components of a typical exosome. Reproduced from Darband et al. (2018) with permission from Elsevier B.V.

-III) associated with VPS4, VTA1, and ALIX, with different complexes involved in different stages of ILV biogenesis (Henne et al. 2011). During exosome biogenesis, ESCRT-0/I proteins (HRS, STAM1, and TSG101) determine the secretion, size, and contents of exosomes. Studies have shown that silencing of these genes not only resulted in reduction of exosome secretion, but also altered the size and contents of exosomes. **Small hairpin RNA** (shRNA) silencing of HRS slightly increased the proportion of smaller vesicles without altering CD63 and major histocompatibility complex class II (MHC II) contents in exosomes, whereas silencing of STAM1 increased the proportion of larger vesicles without altering CD63 and MHC II expression. Silencing of TSG101 neither altered the sizes of vesicles nor their expression of CD63 and MHC II. In contrast, ALIX depletion led to an increase in secretion of larger vesicles together with higher MHC II levels (Colombo et al. 2013).

Additionally, the composition of the EV membrane can also be altered depending on the membranes of their originating cells (Zaborowski et al. 2015). EV membranes consist of a lipid bilayer similar to the plasma membrane in cells, which when compared with the cellular plasma membrane are enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine (Fitzner et al. 2011), and differences in the contents of inner and outer leaflets have also been observed (Laulagnier et al. 2004; Llorente et al. 2013). Differences in the lipid content of the membrane lipid bilayer mostly affects the rigidity of exosomes, which may influence their resistance

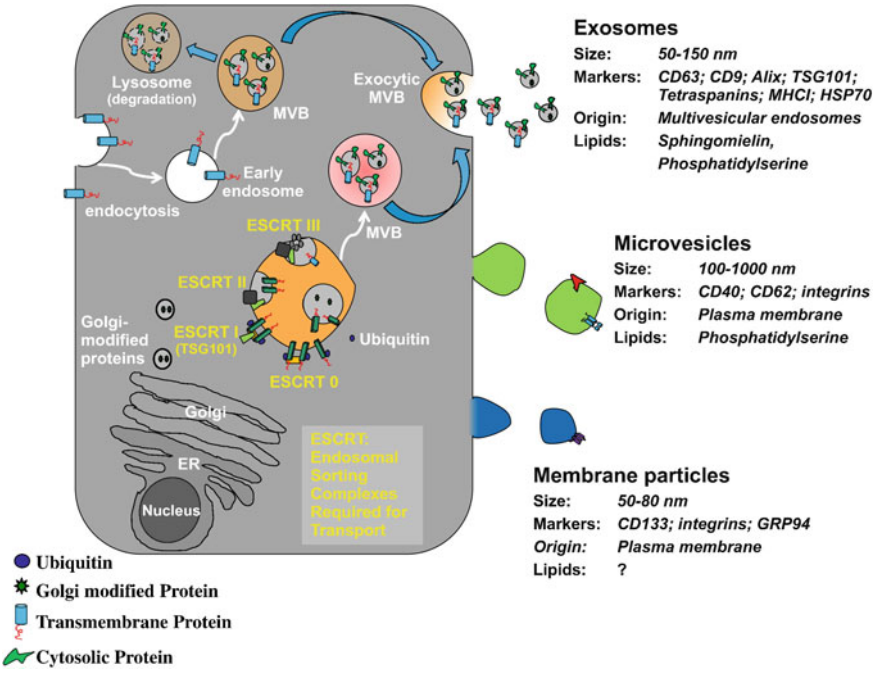

Fig. 10.2 Overview of exosome biogenesis. Reproduced from Barile and Vassalli (2017) with permission from Elsevier B.V.

to degradation and increase their stability during communication (Skotland et al. 2017).

10.3 Exosome Recognition

Once released into the extracellular space, exosomes can be recognized by other cells for internalization. In general, exosome cellular recognition can be achieved through (1) free floating, (2) adhesion, and (3) antigen recognition (McKelvey et al. 2015). Free floating refers to circulation of exosomes in body fluids via the circulatory system upon secretion, and recognition of exosomes by immune cells through this means may occur through opsonization. Opsonins, including lysophatidylcholine, phosphatidylserine, and lactoadherin present on the surface of exosomes may be recognized by phagocytic cells and facilitate **phagocytosis** (Blanc et al. 2007; Saunderson et al. 2014; Zhuang et al. 2011; Hanayama et al. 2002). In addition, expression of chemokines in exosomes also act as chemoattractants for the chemotaxis of immune cells, which in turn increase their migration to target sites (Chen et al. 2011).

Adhesion is the most commonly adopted method for exosome T cell recognition. The presence of ICAM-1, αvβ3/β5 integrins, and tetraspanins (e.g., CD4, CD63, CD81) in exosomes facilitate their adhesion with integrins present on the surface of their target cells, and the subsequent priming of T cells (Conde et al. 2005; Heijnen et al. 1999; Imai et al. 1995; Théry et al. 2001).

Another mechanism utilized by exosomes for recognition is antigen recognition, and two major molecules, MHC I and MHC II, have been shown to be involved in this mechanism (Blanchard et al. 2002). MHC I inhibits cytotoxic T cells and the NK cell response through binding with inhibitory receptors on target cells, whereas MHC II stimulates T helper cells upon binding. Therefore, depending on the functions of exosomes and their target cells, various molecules are present in exosomes to facilitate their recognition.

10.4 Exosome Uptake

After recognition, exosomes can be internalized into recipient cells through (1) phagocytosis, (2) **macropinocytosis**, (3) **clathrin**-mediated endocytosis, (4) caveolin-dependent endocytosis, and (5) membrane fusion. Phagocytosis is a receptor-mediated process, which as mentioned previously, involves the recognition of opsonins on exosomes and is mainly conducted by phagocytic cells. Additionally, the actin cytoskeleton and PI3K are important mediators of exosome internalization in this mode of uptake (Feng et al. 2010). Similar to phagocytosis, macropinocytosis also involves the formation of protruded ruffles through extension of the plasma membrane, but without direct contact of exosomes. Although actin and PI3K are also known to be involved in macropinocytosis, Na^+/H^+ exchange activity plays a major role in the regulation of macropinocytosis (Fitzner et al. 2011; Doherty and McMahon 2009; Kerr and Teasdale 2009).

Clathrin-mediated endocytosis occurs upon cell recognition of clathrin-coated vesicles, followed by fusion with endosomes into the cell after clathrin removal. In this mode of exosome uptake, clathrin plays an important role in uptake of clathrin-coated vesicles. It has been shown that treatment of cells by chlorpromazine, a cationic amphipathic drug, led to a dose-dependent suppression of exosome uptake (Kahlert et al. 2014). On the other hand, caveolin-mediated endocytosis, also known as clathrin-independent endocytosis, is a process similar to clathrin-mediated endocytosis, which involves internalization of **caveolae** into the cells. Caveolae are components of the plasma membrane rich in lipids, and depletion of cholesterol impairs caveolin-mediated endocytosis (Nabi and Le 2003). During exosome uptake, membrane fusion is required. Membrane fusion refers to direct fusion of the exosomal membrane with the plasma membrane of the recipient cell, leading to release of exosomal contents into the recipient cell. The process requires close

proximity of the exosome and the cell plasma membrane, leading to direct contact of both lipid bilayers, and fusion of the membranes (Jahn et al. 2003; Chernomordik and Kozlov 2008).

10.5 Exosome Composition and Functions

In general, exosomes contain proteins, lipids, DNA, mRNAs, and microRNAs, and their composition differs according to their cell of origin (Fernando et al. 2017). However, there are still proteins common to exosomes. As exosomes are generated through the ESCRT and will later potentially be taken up by their target cells, proteins associated with the ESCRT, including ALIX, TSG101, MHC II, CD63, and CD81 can be identified in most exosomes, and thus these proteins are regarded as "exosome markers" (Colombo et al. 2013; Chen et al. 2011; Conde et al. 2005; Heijnen et al. 1999; Imai et al. 1995; Blanchard et al. 2002).

Upon their release into the extracellular space, exosomes are exposed to different challenges, and degradation. However, in comparison to direct contact with the extracellular space, contents encapsulated in exosomes are more stable and possess higher water solubility, suggesting their potential function as carriers for information transduction (Mandal 2017). The stability of exosomes is influenced by the duration of storage, temperature, pH, and freeze–thaw cycles, where short storage durations, low temperature, physiological pH, and limited freeze–thaw cycles can positively affect the stability of exosomes (Lee 2016; Maroto et al. 2017; Cheng et al. 2019).

Exosomes were originally regarded as a mechanism for eliminating waste from cells; however, increasing evidence has suggested that exosomes participate in multiple biological processes. Depending on their cellular origin and their contents, exosomes are involved in cell-to-cell communications, modulation of the immune response, antigen presentation, cell death, cell proliferation, cell differentiation, and angiogenesis. In addition, due to their unique functions, exosomes have also been utilized in biomarker development and diagnosis (Zeringer et al. 2015; Li et al. 2017).

10.6 Exosome Isolation

Various methods are currently employed for the isolation of exosomes, and development of these methods has been based on the characteristics of exosomes. Currently, exosomes can be isolated by (1) differential ultracentrifugation, (2) size-exclusion chromatography (SEC), (3) immunoisolation, and (4) polymer precipitation.

Differential ultracentrifugation consists of a series of centrifugation steps comprising differential increases in speed and duration, where exosomes are isolated at high centrifugal forces of up to 1,000,000 × g. This method remains the most widely employed method and the gold standard for the isolation of exosomes, not only due to the limited requirements for technical expertise, but also due to its low

cost. In addition, depending on the degree of purity required, an extra centrifugation step with a 30% sucrose cushion can be included. The addition of this process can better eliminate contaminants when compared with using ultracentrifugation steps alone, yielding exosome preparations of extra high purity (Théry et al. 2006).

Another method for size-based exosome isolation is SEC, which separates exosomes by passing biological fluids along a porous stationary phase. The time for the particles to be eluted depends on their size; the smaller the particles, such as exosomes, the faster they will be eluted. Through collection of different fractions, exosomes can be isolated. Compared to ultracentrifugation, SEC is faster and has been proposed to better retain the size and vesicular characteristics of exosomes (Li et al. 2017; Gámez-Valero et al. 2016).

Exosome isolation can also be achieved by immunoisolation. This method provides an isolation method based on immunoaffinity interactions, which utilizes specific interactions between proteins on exosomes and antibodies on magnetic beads, and the subsequent capture of exosomes (Théry et al. 2006). Typical exosomal markers including CD9, CD63, and CD81 have been utilized for the isolation of exosomes, and through this method, specific subsets of exosomes can be isolated with high purity.

Polymer precipitation is an emerging technique for exosome isolation, especially for the isolation of exosomes from small volumes of biological fluid. This precipitation method relies on the application of water-excluding polymers to biological fluids, in order to force out the less soluble particles from the biological fluids. Afterwards, low speed centrifugation is applied for isolation of exosomes (Zeringer et al. 2015).

10.7 Exosome Sourcing

Immature dendritic cells (imDCs) have been suggested as a potential source for therapeutic exosomes. imDC-derived exosomes have been identified as a favorable for therapeutic use, as they do not possess surface markers such as MHC I/II, CD40, CD86, etc., which lowers the potential for immunological cross-reactivity. These exosomes can be isolated from $CD34^+$ cells from patient blood samples. Alternatively, mesenchymal stem cells (MSCs) can be obtained from the bone marrow, fat, and other tissues of patients. MSCs have been suggested as having great potential for mass production of exosomes, as they are prolific producers of exosomes which have also been shown to have immunosuppressive activity (Yeo et al. 2013).

Exosomes derived from cancer cells have been demonstrated to be involved in the progression of disease via transfer of their oncogenic contents. Cancer-derived exosomes have been shown to increase cell transformation, cell proliferation, angiogenesis, and metastasis (Othman et al. 2019). On the other hand, the tumor-targeting ability of cancer-derived exosomes can provide an advantage. In one study, breast cancer-derived exosomes loaded with doxorubicin were seen to be more effective in reducing tumor burdens in mouse models of breast and ovarian cancer than treatment

with free doxorubicin alone. Doxorubicin contained in the exosomes was found to be more stable and had greater accumulation in tumors (Hadla et al. 2016a). Additionally, cancer-derived exosomes have also been shown to be able to activate dendritic cells, and induce cytotoxic T lymphocyte-dependent anti-cancer immune responses (Wolfers et al. 2001).

Recently, plants have also been suggested as an alternative source for therapeutic exosomes. Studies have suggested that, due to their specific lipid and miRNA contents, plant-derived exosomes can exert important biological functions against inflammatory diseases and cancers (Rome 2019). Exosomes isolated from grapefruits were coated with targeting modifications and then loaded with doxorubicin and curcumin. Grapefruit exosomes carrying the therapeutic cargo had improved effects when compared to exosomes or chemotherapeutics alone (Wang et al. 2015). Plant-derived exosomes have also been demonstrated to be strong candidates for the delivery of therapeutics as they can cross mammalian barriers without inducing the host immune response or necrosis (Rome 2019). In addition, in comparison with cell-derived or synthetic exosomes, plant-derived exosomes could be more easily scaled up for mass production.

10.8 Drug Loading Methods

The loading of drugs into exosomes can be split into two major categories: pre-loading, and post-loading methods. In pre-loading methods, parental cells are treated with the drug to be incorporated, or the parental cells are modified to produce the drug. Thus, the exosomes produced by the parental cells already contain the drug of interest. In post-loading methods, drugs are loaded into exosomes after their isolation from cell culture media, or biological fluids such as plasma, serum, milk, etc.

10.8.1 Pre-loading Methods

Pre-loading methods are especially applicable when the drugs of interest are nucleic acid or protein based, and where donor cells can be programmed to produce the wanted molecules to be packaged and released in exosomes. Here, donor cells are transfected with miRNA/siRNA/plasmid DNA to overexpress a certain gene product (RNA or protein), which will be packaged by the cell into the luminal space or membrane of secreted exosomes (Antimisiaris et al. 2018). Studies have also utilized pre-loading methods to produce exosomes loaded with chemotherapeutics. In one study, mesenchymal stromal cells were treated with the anti-cancer chemotherapeutic paclitaxel. Exosomes were isolated from the conditioned cell culture medium, and using HPLC and ATR-FTIR spectroscopy methods, it was confirmed that the exosomes contained paclitaxel. The paclitaxel-loaded exosomes produced a strong

anti-proliferative effect on CFPAC-1 human pancreatic cells when compared with control exosomes from untreated cells (Pascucci et al. 2014).

10.8.2 Post-loading Methods

Several post-loading methods have been demonstrated, including simple incubation, electroporation, sonication, extrusion, and freeze–thaw cycling. In the simple incubation method, isolated exosomes are mixed together with drugs, and drugs diffuse along the concentration gradient into the exosomes. In one study, exosomes were incubated with curcumin in PBS at 22 °C for 5 min. In the purified exosomes, researchers found that the curcumin was incorporated into the lipid bilayer via hydroscopic interactions, which resulted in increased drug stability (Sun et al. 2010). However, the loading efficiency of the simple incubation method depends on the hydrophobic interaction between the lipid layer of the exosomes and the drug to be loaded, and a low loading capacity is considered to be a major shortcoming of this method.

Another post-loading method is electroporation. Temporary pores are created in the phospholipid bilayer membranes of exosomes through application of an electric field, and when mixed with drugs, the drugs can travel into the lumen of the exosomes. Electroporation has been well utilized for the loading of miRNAs and siRNAs into exosomes as these nucleic acids are relatively large and hydrophilic in nature, and thus do not diffuse into exosomes spontaneously. In previous studies, researchers have shown that electroporation produced better results in terms of loading of RNAs when compared to another major method of introduction of nucleic acids into cells, chemical transfection (Shtam et al. 2013; Wahlgren et al. 2012). However, electroporation has also been said to have low efficiency, potentially cause negative effects on the integrity and stability of exosomes, and lead to aggregation or instability of RNA cargo (Kooijmans et al. 2013). In response, other studies have shown that the use of optimized buffers and electroporation parameters could improve the integrity and stability of exosomes and the cargo within (Johnsen et al. 2016).

Another method for loading cargo into exosomes is sonication. In this method, ultrasonic waves cause deformations in the membrane of exosomes in suspension. When mixed together with the target drugs, drugs can take advantage of the disruption in the exosomal membranes and diffuse into the lumen of the exosomes. This process has been shown to have no significant negative effects on exosomal cargo or exosomes; the membrane integrity of exosomes was shown to be restored within an hour of the sonication process. Researchers also observed that sonication could lead to incorporation of cargo not only into the lumen of exosomes, but also into the phospholipid bilayer of the membrane. This can produce to a two-phase drug release, the first, a burst from the release of the luminal contents, and the second, a slow release from the drug incorporated into the membrane, raising interesting possibilities for the use of sonication in exosomal drug delivery (Meel et al. 2014).

In the mechanical extrusion method, a mixture of exosomes and drugs are loaded into a syringe-based lipid extruder and extruded through 100–400 nm pore membranes. Through this process, the membranes of the exosomes are disrupted and mixed with the drug, resulting in cargo loading of the exosomes. Few studies have been conducted on this method of cargo loading, and with mixed results. In one study, porphyrins were loaded into exosomes via 31 rounds of extrusion. Results showed that this method altered the zeta potential of the vesicles, and the extruded exosomes caused cytotoxicity in treated cells, whereas porphyrin containing exosomes loaded via alternate methods did not (Fuhrmann et al. 2015). In contrast, in another study, where 10 rounds of extrusion was used to load catalase into RAW264.7 cells, the extrusion-loaded exosomes were shown to have no cytotoxicity and performed better than freeze–thaw-loaded and simple incubation-loaded exosomes (Haney et al. 2015).

In the freeze–thaw cycling method of cargo loading, a mixture of drugs and exosomes are subjected to several cycles of freezing at −80 °C or in liquid nitrogen followed by thawing at room temperature. At least three freeze–thaw cycles are performed to ensure incorporation of the drug into the exosomes (Sato et al. 2016). However, the efficiency of drug loading using this method has been shown to be lower in general than that of sonication or extrusion.

10.9 Exosome Delivery

Exosomes express on their plasma membranes certain lipids, cell adhesion molecules, and ligands to target certain recipient cells. While studies have shown that the targeting ability of exosomes is based on their donor cell, there is also great potential in the engineering of ligands in or on exosomes for specific targeting. A commonly applied approach is to introduce a plasmid expressing genes encoding targeting proteins into the donor cells, and targeting proteins are consequently incorporated into the secreted exosomes. In one study, a fusion protein of iRGD (which specifically recognizes αv integrins on the surface of tumor cells) and Lamp2b (an exosomal membrane protein) was overexpressed in immature dendritic cells. Exosomes isolated from these dendritic cells were loaded with doxorubicin. Treatment with these engineered therapeutic exosomes showed highly efficient targeting and drug delivery to αv integrin-positive breast cells, and in vitro and in vivo efficacy (Tian et al. 2014). In another study, engineered exosomes were used to deliver miRNA cargo to cancer cells. Specific targeting was accomplished through engineering of the donor cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide, which binds specifically to EGFR. Exosomes isolated from these donor cells expressed the GE11 peptide on their surfaces and when loaded with let-7a miRNA could specifically target and inhibit EGFR-expressing breast cancer cells in a mouse xenograft model (Ohno et al. 2013).

Exosomes can also be modified post-isolation to achieve specific targeting. Copper-catalyzed azide alkyne cycloaddition “click” chemistry has been used to

conjugate ligands to the surfaces of exosomes. Click chemistry is ideal for the bioconjugation of small molecules or macromolecules to the surface of exosomes, as it is rapid, efficient, has high specificity, and is compatible with aqueous buffers (Smyth et al. 2014). In one study, click chemistry was used to add the cyclo(Arg-Gly-Asp-D-Tyr-Lys) [c(RGDyK)] peptide, which possesses high affinity to the integrin αvβ3 upregulated in ischemia, to the surface of mesenchymal stromal cell (MSC)-derived exosomes. cRGDyK-exosomes were shown to target the ischemic lesion regions of the brain and could enter microglia, neurons, and astrocytes. Treatment of mice with curcumin-loaded cRGDyK-exosomes could suppress inflammation and apoptosis in ischemic brain lesion regions more effectively than curcumin or exosome treatment alone (Tian et al. 2018). Click chemistry may affect exosome targeting through the bioconjugation of a range of targeting moieties with azide-terminated groups (Smyth et al. 2014; Wang et al. 2014). In addition to click chemistry, other methods have been employed which can improve delivery of exosomes and cargo. Researchers devised a method to improve the cellular uptake of exosomes and release of their internal cargo via the formation of an exosomal complex with cationic lipids and a pH-sensitive fusogenic peptide. The cationic lipids of these complexes conferred a positively charged surface potential to enhance binding and uptake into recipient cells, while the pH-sensitive peptide enhanced intracellular cargo release (Nakase and Futaki 2015). In another study, transferrin-conjugated superparamagnetic nanoparticles were bound to the surfaces of blood-derived exosomes, via targeting of native transferrin receptors present on the membrane of the exosomes (Qi et al. 2016). Despite the above successful examples, many obstacles still stand in the way of attaining exosome targeting via surface modification. For example, reactions must be conducted in such a way that does not disrupt the function of the therapeutic exosome, avoiding exosome disruption, aggregation, stability, etc. (Armstrong et al. 2017).

The major routes of exosome administration are via intravenous or intraperitoneal injection or via oral gavage, although intranasal administration has been employed for exosome delivery to the brain. Engineered exosomes were administered to the brain of transgenic reporter mice using the nasal route to test for intracellular protein delivery in vivo. This resulted in the transport of engineered exosomes predominantly to recipient neurons in a number of brain regions, including the olfactory bulb, cortex, striatum, hippocampus, and cerebellum. The ability to engineer exosomes to deliver biologically active proteins across the blood–brain barrier represents an important step for the development of therapeutics to treat brain diseases (Sterzenbach et al. 2017). In another study, exosomes delivered through intranasal administration could improve the motor symptoms of Parkinson's disease (PD) mice and reduce the upregulated tyrosine hydroxylase expression associated with PD in the model (Narbute et al. 2019).

10.10 Use of Exosome in Disease Treatment

Exosomes are naturally adapted for the transport and intracellular delivery of proteins, mRNAs, miRNAs, various non-coding RNAs, mitochondrial DNA, and genomic DNA. In recent decades, the focus has shifted from synthetic drug compounds to the delivery of biological drugs (e.g., proteins and nucleic acids); therefore, the use of exosome-based vehicles for delivery of various therapeutic cargoes has increasingly gained attention (Sun et al. 2010; Haney et al. 2015; Tian et al. 2014; Jang et al. 2013; Hadla et al. 2016b; Yang et al. 2015; Pascucci et al. 2014; Kim et al. 2016; Qu et al. 2018; Aqil et al. 2016; Munoz et al. 2013; Ohno et al. 2013; O'Brien et al. 2015; Lu et al. 2017; Rutter and Innes 2017; Pan et al. 2012; Didiot et al. 2016; Wahlgren et al. 2012; Banizs et al. 2014). A summary of selected studies conducted on the use of exosomes as drug delivery systems is detailed in Table 10.1. Several studies are focused on cancer, using exosomes or exosome-like vesicles to deliver anti-cancer agents such as doxorubicin (Tian et al. 2014; Jang et al. 2013; Tacar et al. 2013) and paclitaxel (Tian et al. 2014; Yang et al. 2015) to facilitate an inhibitory effect, reduce dose-related toxicity, or increase drug sensitivity. Results showed that the efficacy of doxorubicin loaded into exosomes was greatly enhanced when compared to doxorubicin delivered by other delivery systems, and significantly fewer adverse effects were observed on major organ systems, especially the heart, implying that delivery via exosomes might diminish major shortcomings of this chemotherapeutic drug. Other studies addressed the function of exosomes as carriers of anti-inflammatory agents (Sun et al. 2010), bringing compounds across the blood–brain barrier for the treatment of Parkinson's disease (Haney et al. 2015). Studies also evaluated the possibility of using exosomes for the therapeutic delivery of miRNA (Ohno et al. 2013, Munoz et al. 2013), siRNAs (Wahlgren et al. 2012; Banizs et al. 2014; Andaloussi et al. 2013), hsiRNAs (Didiot et al. 2016), shRNAs (Pan et al. 2012), and proteins (Haney et al. 2015; Lu et al. 2017).

Besides the examples listed in Table 10.1, there are several planned or ongoing therapeutic clinical studies related to exosome-based vesicles. In a phase-I trial, researchers isolated dendritic cells of patients with advanced melanoma and pulsed them with tumor antigen. They then purified exosomes-presenting tumor antigens and intradermally and subcutaneously injected these in patients. The exosome administration was tolerated for up to 21 months. Results showed that a mild inflammatory reaction at the site of injection was observed in some patients, and one of 15 patients exhibited a specific melanoma antigen T cell response and a reduction in tumor size (Escudier et al. 2005). Another phase-II trial evaluated IFN-γ-DC-derived exosomes loaded with MHC I/II restricted cancer antigens as a maintenance immunotherapy after induction chemotherapy in patients bearing inoperable non-small cell lung cancer. Though the primary endpoint of 50% non-progressors by four months post-chemotherapy was not met, these exosomes were found to be able to boost NK cell function in a fraction of treated patients, highlighting a potential avenue for further exploration (Besse et al. 2016). The notion of using exosomes as a

Table 10.1 Summary of studies conducted using exosomes as drug delivery system

Type of cargo	Cargo	Source of exosomes	Loading method	Route of administration	Aim/outcome	References
Small molecule	Curcumin	EL-4 (mouse lymphoma cell line)	Incubation	Intraperitoneal	Exosomes deliver curcumin, an anti-inflammatory agent, to activated myeloid cells in vivo	Sun et al. (2010)
Small molecule	Doxorubicin	Mouse immature dendritic cells (imDCs)	Electroporation	Intravenous	Exosomes modified by targeting ligands can be used for the delivery of doxorubicin to tumors	Tian et al. (2014)
Small molecule	Doxorubicin	Monocyte or macrophage	Incubation	Intravenous	Use bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors	Jang et al. (2013)
Small molecule	Doxorubicin	Breast and ovarian tumor	Electroporation	Intraperitoneal	Exosomes increase the therapeutic index of doxorubicin in breast and ovarian cancer mouse models	Hadla et al. (2016b)

(continued)

Table 10.1 (continued)

Type of cargo	Cargo	Source of exosomes	Loading method	Route of administration	Aim/outcome	References
Small molecule	Paclitaxel, Doxorubicin	Brain endothelial bEND.3 cells	Incubation	Microinjection using a Nanoject IITM Auto-Nanoliter Injector	Exosomes deliver anti-cancer drugs which crossed the blood–brain barrier and entered into the brain	Yang et al. (2015)
Small molecule	Paclitaxel	Pancreatic adenocarcinoma	Incubation	N/A	Exosomes can deliver Paclitaxel to inhibit in vitro tumor growth	Pascucci et al. (2014)
Small molecule	Paclitaxel	Macrophage	Incubation, sonication, electroporation	Intranasal	Overcome drug resistance; inhibition of tumor growth	Kim et al. (2016)
Small molecule	Dopamine	Blood	Incubation	Intravenous	To deliver dopamine to the brain for better treatment of Parkinson's disease	Qu et al. (2018)
Small molecule	Celastrol	Bovine milk	Incubation	Oral	Exosomal formulation enhances therapeutic response of celastrol against lung cancer and reduces dose-related toxicity	Aqil et al. (2016)

(continued)

Table 10.1 (continued)

Type of cargo	Cargo	Source of exosomes	Loading method	Route of administration	Aim/outcome	References
miRNA	Cy5-tagged anti-miR-9	Mesenchymal stem cell-derived exosomes	–	–	Delivery of functional anti-miR-9 to glioblastoma multiforme cells enhanced chemosensitivity	Munoz et al. (2013)
miRNA	Let-7a	HEK293 cells	Incubation	Intravenous	Exosomes can deliver microRNA (miRNA) to epidermal growth factor receptor (EGFR)-expressing breast cancer cells	Ohno et al. (2013)
miRNA	miR-134	Breast cancer cell (Hs578T cell lines, and its isogenic subclone Hs578Ts(i)8 cells)	Pre-transfection	NA	Reduced migration and invasion; enhanced chemosensitivity	O'Brien et al. (2015)
Protein	Catalase	Monocytes and macrophage	Incubation, permeabilization with saponin, freeze–thaw cycles, sonication, or extrusion	Intranasal or intravenous	Deliver catalase across blood–brain barrier to treat Parkinson's disease	Haney et al. (2015)

(continued)

Table 10.1 (continued)

Type of cargo	Cargo	Source of exosomes	Loading method	Route of administration	Aim/outcome	References
Protein	α-fetoprotein	Dendritic cells	Pre-overexpression	Intravenous	Inhibited tumor regression in autochthonous hepatocellular carcinoma mouse models	Lu et al. (2017)
Protein	Stress response protein	Aplastic fluids of Arabidopsis leaves	NA	NA	Intracellular communication	Rutter and Innes (2017)
shRNA	shRNAs	Huh7 cells	Incubation	Intravenous	Both human and mouse hepatic cells exchange small silencing RNAs, partially mediated by shuttling of exosomes	Pan et al. (2012)

(continued)

Table 10.1 (continued)

Type of cargo	Cargo	Source of exosomes	Loading method	Route of administration	Aim/outcome	References
hsiRNA	Hydrophobically modified siRNAs (hsiRNAs)	U87 glioblastoma cells	Incubation	Microinjections	Exosome can mediate the delivery of hydrophobically modified siRNA for huntingtin mRNA silencing	Didiot et al. (2016)
siRNA	siRNA	Plasma exosomes	Electroporation	NA	Exosome vesicles derived from humans can deliver short interfering RNA (siRNA) to human mononuclear blood cells	Wahlgren et al. (2012)
siRNA	Nucleic acids	Primary endothelial cells isolated from the aorta of C57BL/6 $ApoE^{-/-}$ mice	Electroporation	Culture	Exosomes can act as carriers for small interfering ribonucleic acid delivery	Banizs et al. (2014)

vehicle is promising and inspiring. However, there are issues that must be considered before taking advantage of exosome-based vehicles, which are discussed below.

10.11 Summary and Outlook

Exosomes are a new frontier in drug delivery and promise great clinical value as they offer distinct advantages as delivery vectors due to their strong biocompatibility, small size, and the flexibility to cross major biological barriers such as the blood–brain barrier. Great progress has been made on the application of exosomes in disease therapy; however, a number of challenges still remain, as highlighted in the *Questions for Future Research* box, and below. Before the full potential of using therapeutic cargo and functional exosomes in drug delivery is realized, a clear understanding of the therapeutic efficacy, long-term safety, and technical issues of isolation, stability, drug loading, and assembly must be reached (Kooijmans et al. 2012). In addition, large-scale production and quality control of exosomes carrying therapeutic cargo remains an obstacle.

First and foremost, as exosomes can be secreted from many types of cells and thus carry a diverse range of cargo derived from their parental cell (Kahlert et al. 2014; Valadi et al. 2007; Thakur et al. 2014), it remains to be studied which cells are most suitable for the isolation of exosomes. Choosing the correct cell line for therapeutic exosome production is of great importance. Exosomes comprise heterogeneous components and may show immunogenic (immunostimulatory or immunosuppressive) effects based on the nature of their parental donor cells. Tumor exosomes exhibit potential problems, including induction of apoptosis in activated cytotoxic T cells, impairment of monocyte differentiation, induction of myeloid-suppressive cells and T regulatory cells, suppression of lymphoid activation signaling molecules, and induction of a pro-inflammatory microenvironment (Taylor and Gercel-Taylor 2011). There is also the concern that exosomes may be involved in disease progression. A study showed that exosomes that carry caspase-3 may inhibit cell death by apoptosis or enhance tumor cell survival by preventing chemotherapeutics drug accumulation (Turturici et al. 2014). Another study demonstrated that serum exosomes isolated from dextran sulfate sodium induced acute colitis mice could activate the MAPK signaling pathway and trigger a pro-inflammatory response in naïve macrophages in vitro (Wong et al. 2016).

In addition, the precise components of natural exosomes that are critical for therapeutic delivery are yet to be pinpointed. There remains a large knowledge gap on the key components of exosomes, which likely differ among exosomes with varying functions and target cells, raising safety concerns for the application of exosomes. Extensive proteomic studies have identified more than 4000 proteins and more than 1500 miRNAs in exosomes from various sources, but integrative studies are required to elucidate the biological functions of these components (Antimisiaris et al. 2018). To overcome the problem, one potential approach is to design artificial exosomes or exosome mimetics that can overcome unwanted immune reactions (Aryani and

Denecke 2016). Avoiding the selection of tumors as donor cells for the source of exosomes has also been suggested (Liu and Su 2019). An attempt to classify vesicles derived from different sources (different species, types of cells, biological fluids, etc.), in terms of their organotropism, immunogenicity, and biodistribution, following administration by enteric and parenteral routes should be made. Such classifications will be particularly beneficial for the optimal selection of vesicle sources for each specific drug targeting application (Antimisiaris et al. 2018). Proper cell choice can also dictate the native population of exosomal surface proteins that might ensure a desirable ligand–receptor interaction with proposed target cells. Studies focusing on investigating optimal producer–target cell combinations should be conducted and are vital to producing exosomes for therapeutic application.

Beyond the uncertainties regarding the safety of exosomes in drug delivery, there are also technical issues to be concerned with. Currently, there is no distinct optimal technique for the isolation of exosomes with high purity (Petersen et al. 2014). An additional challenge governing the assembly of functional exosomes is the loading of therapeutic cargo, where it is important to take into account the structural and biological functions of the substance to be incorporated, such as lipids which come in various sizes and shapes and can influence the behavior of the exosome in biological environments (e.g., fusion and stability). Another challenge to be addressed is the controversy on whether exosome uptake is cell type-specific (Feng et al. 2010) and whether it involves membrane fusion or endocytosis (Conde et al. 2005; Parolini et al. 2009). In addition, while it is crucial to verify the preservation and stability of exosomal cargo, we must also confirm that the contents of the exosomes are removed without significantly altering the native structure and composition and biological function of exosome in the offloading process, especially in long-distance communication. Furthermore, during the processing and modification of engineered exosomes, changes to donor cells and exosomes may affect exosome content or protein composition. This may affect the bioactivity and efficacy of engineered exosomes. Therefore, it is necessary to select a transformation method with little influence on the morphology and composition of exosomes. Standardization and quality control for either derived exosomes or modified exosomes for drug delivery systems could be employed to address the aforementioned problem. Studies have recommended the addition of an extra step to the standard procedure where the vesicles are stripped of their initial contents prior to the loading step. The effects of isolation and modification of the parental cells on the quality and function of the released exosomes should also be elucidated. Detailed characterization should be conducted on the different types of extracellular vesicles released and their role in transferring signals that may propagate or limit disease (Akuma et al. 2019). Another study has suggested using clinical-grade, purified synthetic lipids and recombinant proteins in the development of exosome mimetics, manufactured and assembled using controllable procedures (Barile and Vassalli 2017). Needless to say, a better understanding of exosome biology together with standardized methods for exosome quantification, isolation and storage, molecular characterization, and potency assays will greatly enhance the future promise for exosome-based therapeutic applications.

Lastly, achievement of large-scale production of exosomes for clinical use is no doubt one of the most important challenges (Meel et al. 2014). There have been reports of rapid purification of therapeutic exosomes on a large scale, including via differential ultracentrifugation and sucrose density gradient ultracentrifugation, size-based separation (ultrafiltration), and exosome precipitation (Théry et al. 2006; Heinemann and Vykoukal 2017; Niu et al. 2017). However, these techniques still require expansion to different cell types. In addition, there are high financial costs associated with the large-scale production of exosomes required for clinical studies and post-drug approval (Taylor and Shah 2015).

To conclude, exosomes are poised to play a vital role in the treatment of various diseases, especially cancer and inflammatory disease, in the future. To bridge the gap between bench work and clinical trials, through further studies, we must develop a better understanding of the biogenesis and biochemical characteristics of exosomes, functions of exosomes in disease, as well as elucidate the specificity, efficacy, and safety of exosomes. Importantly, clinical trials are on the horizon for the use of exosomes as a drug carrier for different diseases. Last but not least, continued research to develop standardized identification and evaluation of exosomes and feasible approaches for large-scale production is essential to increase opportunities for the clinical translation of exosomes.

Important Notes

- Exosomes are nanosized particles released by a variety of cells. Because of their small size, biocompatibility, stability, specificity, and low toxicity, they can be seen as an excellent method for drug delivery.
- Exosomes have been shown to be involved in cell-to-cell signaling and transportation through transmission of their luminal contents from their cells of origin to target cells. We can co-opt this activity to develop therapeutic exosomes that deliver specific drugs to target cells in disease.
- Several aspects, including exosome origin, isolation methods, cargo loading methods, and targeting are important factors to evaluate when considering the therapeutic use of exosomes as a delivery method.
- Recent cell line and animal research has shown the potential efficacy of exosomal delivery of drugs in several diseases, mainly the delivery of chemotherapeutics and miRNA/siRNA/shRNAs in cancer.
- Several clinical studies have also commenced to investigate the safety and efficacy of exosomal drug delivery in disease.

Questions for Future Research

- **How can we ensure a consistent biological source for proper exosome isolation and a reproducible method of loading drug molecules effectively into exosomes?** Isolation methods for exosome isolation should be further developed and improved to increase yields, purity, and lower costs. Similarly, existing methods for cargo loading into exosomes should be closely examined and compared to identify optimal methods. At the same time, these methods must be continually improved or novel alternate methods of cargo loading devised.
- **How can we manufacture exosome-based vehicles/therapeutic exosomes on a large scale, while keeping in mind issues of efficacy, storage, and stability?** As exosomes can be isolated from many different cell types, the possibility of mass production techniques attracts interest. However, whether those techniques can be applied across the board is an issue that should be addressed.
- **Most of the reported exosome-based therapy studies focus on carrying chemotherapeutic drugs to treat cancer, can exosomes-based vehicles be used for a greater range of disease?** In fact, exosomes have already been demonstrated to be able to deliver miRNA, siRNA, and recombinant proteins, as well as synthetic small molecule drugs. What is next is to ensure the stability and integrity of exosomes after isolation and cargo loading, and to test the efficiency of different exosomes in different disease models.

Glossary

Apoptotic bodies Small cytoplasmic fragments which sometimes may also contain nuclear fragments.

Caveolae Flask-shaped invaginations of the plasma membrane.

Clathrin A cytoplasmic protein that plays an important role in endocytosis and intracellular trafficking.

Cytoskeleton A highly dynamic supramolecular network which consists of protein components (e.g., intermediate filaments, actin filaments, and microtubules). It involves in regulating diverse cell processes, ranging from cell motility to phagocytosis.

Endosomes Vesicular structures in which internalized materials intended for degradation are accumulated and concentrated.

Exosomes Small 30–150 nm vesicles released upon fusion of multivesicular bodies with the plasma membrane, involved in local and systemic communication.

Extracellular vesicles Membrane-bound vesicles secreted from a variety of cells, in organisms ranging from bacteria to humans.

Macropinocytosis An actin-dependent endocytic process by which cellular internalization of a substantial volume of fluid occurs.

Phagocytosis A receptor-mediated engulfment of particles with a diameter greater than 0.5 μm into plasma membrane-derived vacuoles known as phagosomes.

Small hairpin RNA A ribonucleotide sequence which forms a hairpin turn by itself. It can be used as a tool for gene silencing.

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Part IV
Systemic Delivery Techniques Based on Physical Means

Chapter 11
Use of Physical Approaches for Systemic Drug Delivery

Rajesh Vadlapatla, Zhijun Wang, Priyank Kumar, and Nina Pavuluri

Abstract Physical methods of drug delivery involve application of physical forces to deliver drugs using different approaches. These approaches combine forces (such as magnetic, thermal, ballistic, and electrical forces) with novel formulation techniques, for example, those in targeted drug delivery. The important approaches include iontophoresis, low-frequency ultrasound, electrophoresis, magnetic drug delivery, and the use of microneedles. Physical methods have been used for the treatment of various tumors, targeted delivery of drugs and genes, magnetic resonance imaging, cell purification, and hyperthermia. In this chapter, we will provide an overview of the strategies employed in physical methods of drug delivery. The working principles underlying ultrasound-, photo-, magnetic-, and electrical-based delivery methods, as well as the possible use of microneedles to control drug release, will be presented. Clinical applications relevant to these methods will also be discussed.

Keywords Physical methods · Magnetic · Ultrasound · Microneedles · Electrophoresis

11.1 Introduction

Conventional drug delivery systems are easier to manufacture and convenient to administer to patients. However, nonspecific and uncontrollable drug release from these systems leads to undesired toxicity and side effects in non-targeted cells and tissues. Since the introduction of the concept of the "magic bullet" as targeted drug delivery in the 1960s by Paul Ehrlich, targeted drug delivery (TDD) has rapidly evolved. TDD is an efficient method of selectively delivering drugs to disease areas such as tumors and inflammatory areas in the body. This selective accumulation

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of drugs in the disease areas for a prolonged period with high controllability often results in less systemic side effects compared to conventional drug delivery systems. Moreover, advantages such as lower drug dose, greater control over toxicity and bioavailability of the dose, extended duration of release, and the possibility of having different drug release kinetics make TDD a desirable delivery system.

Breakthroughs in biomedical engineering have provided materials such as nanoparticles, liposomes, micelles, and microspheres for efficient drug delivery. These materials are employed to deliver therapeutic agents to specific targeted sites in a controlled manner. The small size of these materials allows them to enter the host cell nuclei making them ideal for gene delivery. Barriers such as skin, blood–brain barrier, different physiological conditions, mucosal cells, and the nuclear membrane play an integral role as defense mechanisms to protect our body from different assaults (Mitragotri 2013). However, these barriers also act as a major obstacle against drug delivery. Physical forces including ultrasound, magnetic, light, thermal, ballistic, and electrical are used to overcome these barriers for the delivery of drugs and other agents. Delivery systems and devices based on physical methods are noninvasive, versatile, and adaptable (Fig. 11.1) (Rodriguez-Devora et al. 2012). Approaches such as **iontophoresis**, sonophoresis, low-frequency ultrasound, magnetic drug delivery, and **microneedles** have been extensively studied. Iontophoresis involves application of low-intensity electric current on biological membrane such as skin to deliver positively or negatively charged drug. Sonophoresis is a drug delivery method where ultrasound is used to increase the absorption of drugs and other agents into the epidermis, dermis, and skin appendages. During low-frequency ultrasound, transient cavitation occurs due to rectified diffusion (Schoellhammer and Traverso 2016). These unstable bubbles implode resulting in the formation of aqueous channels leading to an increased drug delivery. In magnetic drug delivery system, magnetic stimuli provide temporally and spatially controlled release of the magnetic carriers to the targets and release drugs under the influence of an external magnetic field. Microneedles are minimally invasive micron sized (less than 1 nm) hybrid devices arranged on a small patch. They allow the delivery of large hydrophilic molecules of high molecular weight across the stratum corneum. This chapter aims to introduce and describe

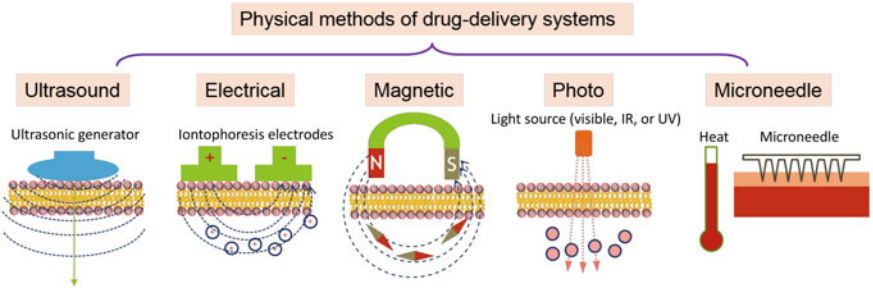

Fig. 11.1 Physical methods of drug delivery systems, which mainly include ultrasonic, electrical, magnetic, photo, and other (e.g., thermal and microneedle) techniques

the concepts involved in different types of physical methods and their influence on targeted drug delivery.

11.2 Methods and Applications

11.2.1 Ultrasound-Based Method

Ultrasound is defined as sound waves with higher frequencies than the audible limit of human hearing (above 20,000 Hz). It can transmit energy of pressure through a medium (air, liquid, or solid) and physically move the molecules in the medium. Ultrasound has been widely utilized to improve drug delivery of chemical drugs and biologicals into targeted tissues since the 1950s.

11.2.1.1 Mechanism of the Application in Drug Delivery

Two mechanisms have been reported, including direct oscillatory motion and cavitation, which are able to enhance drug transport by perturbation of drug carriers or by increasing cell permeability (Pitt et al. 2004).

Oscillatory Motion

The oscillating fluid driven by ultrasound increases the molecular diffusion and therefore leads to higher penetration of drug molecules. Although the direct oscillatory motion may increase the membrane permeability and/or perturb drug carriers, the acoustic streaming produced by ultrasound usually is too weak to be effective in enhancing drug transport in vivo. The mechanism of oscillatory motion was only applicable for preparation of microsphere, microdroplet, and nanoparticle formulations in pharmaceutical drug development (Patil et al. 2019; Shpak et al. 2013).

Ultrasound-Induced Cavitation

Cavitation is the formation of gas-filled cavities, also known as cavitating gas bubbles. It can be produced in a liquid medium exposed to ultrasound, which generates the pressure passing through the medium. Ultrasound-induced cavitation is much more effective in drug delivery compared to oscillatory motion. The drug transport can be enhanced by several orders of magnitude. Two mechanisms, microstreaming and sound pressure, were considered to contribute to the gas bubbles involved transport. Microstreaming is the creation of circulating eddies around the oscillating bubble,

which can transport drugs at a high rate by creating high shear rates close to the surface of the bubble. Shear rate is the rate of change of velocity at which one layer of fluid passes over the adjacent layer. The extremely high shear rate near the surface of the bubbles can stress the cells and drug vesicles, resulting in higher permeability and drug release. Sound pressure or acoustic pressure generated by ultrasound is the pressure to the surface of the oscillating bubble that is perpendicular to the sound direction. If the drug carriers are denser than the fluid, they can be pushed toward the bubble. On the contrary, if they are less dense, the drug carriers can be repelled away. Because most of the drug carriers such as liposomes, nanoparticles, and micelles are denser than the body fluid, they can be pushed toward the bubbles in the ultrasonic field. This leads to an increase in the dispersive transport of the drug carrier, especially when the shear rate is high.

Microbubbles are cavitating gas bubbles with smaller sizes between 0.5 and 10 μm. Microbubbles can pass the energy of pressure from ultrasonic waves and produce forces to permeabilize cell membranes or disrupt the drug carriers (Zhang et al. 2019a). Therefore, ultrasound-induced microbubbles are used widely for drug delivery including chemical drugs, proteins, and genetic materials such as DNA, microRNA, and siRNA.

11.2.1.2 Application in Drug Delivery

Perturbation of the Drug Carrier

Ultrasound waves can perturb the drug carriers and control the release of the encapsulated drugs. In the ultrasound field, the drug carrier can be pushed into the oscillating bubble. If the force exceeds the strength of the vesicle, the structure of the drug carriers can be ruptured and the drug molecules inside will be released. In addition, the collapse of the cavitation produces a shock wave that can drive the water wave. As this spike of dense fluid passes over a vesicle, shear stress is produced at the vesicle surface and ruptures the vesicle. For liposomes, the vesicle may be reformed into smaller sizes without changing the total surface area. Thus, partial interior solution will be forced to release. High-intensity focused ultrasound can generate direct heat (hyperthermia) changes in the tissue and cause thermal perturbations to the drug carriers or surrounding tissues. This can be used for the control of drug release or produce treatment effects directly (Ahmed et al. 2015; Tucci et al. 2019).

Micelles, nanoparticles, and liposomes are extensively applied to formulate chemical chemotherapeutic drugs. Their efficacy can also be improved by applying ultrasound. A type of multiporous lipid–PLGA hybrid microbubble was developed with doxorubicin as the model drug. It was found that ultrasound could trigger the destruction of the microbubbles and provide on-command controlled drug release, both in vitro and in vivo (Chen et al. 2019). Ultrasound-induced cavitation of microbubbles enhanced the uptake of doxorubicin in MDA-MB-468 cells and increased the extravasation of the liposomes. In vivo, ultrasound significantly increased the tumor uptake of doxorubicin by 66% (Thomas et al. 2019). High-intensity focused ultrasound could

enhance the delivery of intraperitoneal liposomal doxorubicin (Rezaeian et al. 2019). Although, high energy of ultrasound may cause damage to surrounding tissues, it could be ameliorated by using ultrasound absorption agents such as gold nanoparticles (Sadeghi-Goughari et al. 2019). Ultrasound-controllable and implantable release systems that utilized waterborne polyurethane and chitosan composite membrane as a drug carrier with a wide flexible loading capacity for doxorubicin were used.

Doxorubicin can be released efficiently from the drug-loading matrix and taken up by tumor cells (Feng et al. 2019). An ultrasound responsible drug-coated microcapsule was used to improve drug release of paclitaxel. It was found that ultrasound could increase the release rate of paclitaxel in the coronary artery and improve the drug concentration in the target vessel by using a porcine model (Song et al. 2019). Hematoporphyrin monomethyl ether is a lipophilic sonosensitizer, which was used for the ultrasound-triggered release of vincristine. Ultrasound showed improved targeting efficiency of the formulation (Lin et al. 2019).

Such technology can also be applied to biologicals, such as protein and DNA. An acoustically responsive scaffold system consisting of a bFGF-loaded monodispersed double emulsion embedded within a fibrin matrix was developed. Ultrasound was found able to spatiotemporally control release of basic fibroblast growth factor from a composite hydrogel (Dong et al. 2019). The delivery of DNA was greatly enhanced in various tissues including cardiac, vascular, skeletal muscle, tumor, and even fetal tissue by the way of microbubbles. In a study, DNA was directly attached to the microbubbles (Tran et al. 2019; Yin et al. 2014). The anticancer microRNA was packaged into nanoparticles using FDA-approved pegylated poly lactic-co-glycolic acid. The study showed ultrasound could deliver the microRNA to deep tissues safely and noninvasively (Rubinsky et al. 2007).

Increase in Membrane Permeability

Ultrasound may increase cell permeability by direct effect or cavitation. This is known as sonoporation. Cavitation makes cell membranes and capillaries more permeable to drugs. In the absence of cavitation, ultrasound has almost no effect on the structure of tissue cells, but if the cells are adjacent to a small cavitating bubble, the asymmetric bubble collapse can produce a jet of liquid with high speed which may rupture the cell membrane and increase the cell permeability (Fig. 11.2).

The utilization of physical force such as sonophoresis has been widely used to improve the permeability of the drug absorption (Park et al. 2019). It has been reported that sonophoresis increased the absorption of niacinamide and retinol by 402% and 292%, respectively, especially when combined with iontophoresis, where an increased absorption of glutamic acid by 240% was seen with a fabricated device. In MiaPaCa-2 cell treatment with nanobubbles, ultrasound enhanced cellular permeability, resulting in 2.5-fold higher uptake of liposome-encapsulated paclitaxel in comparison to only liposome treatment (Prabhakar and Banerjee 2019). Ultrasound enhanced the permeability of gold nanoparticles conjugated with an infrared marker (4-aminothiophenol) to fibroblast (NIH3T3 cells) (Domenici et al. 2019). Multidrug

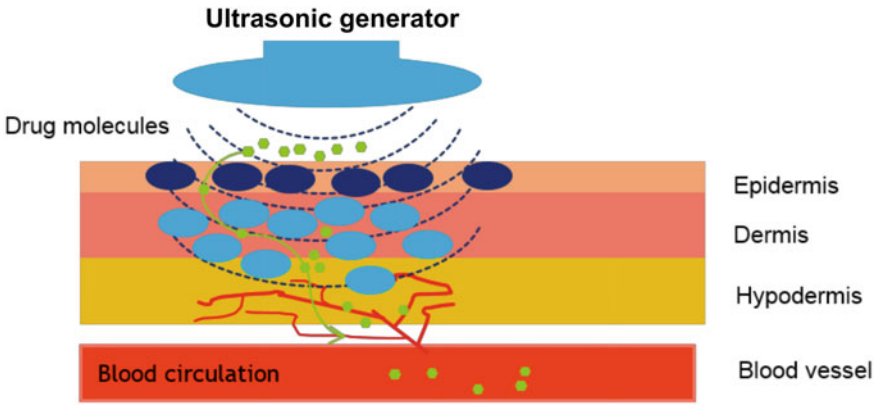

Fig. 11.2 Ultrasound enhanced drug delivery of transdermal formulation

resistance often leads to the failure of chemotherapy against cancer. The overexpression of **P-glycoprotein** (P-gp) is considered to be the main mechanism. Ultrasound increased the uptake and cytotoxicity of dual taxane and P-glycoprotein inhibitor loaded nanoparticles in drug resistant cells (Jackson et al. 2019). Yang et al. found a synergistic fungicidal effect of low-frequency and low-intensity ultrasound with amphotericin B-loaded nanoparticles on *C. albicans* in vitro (Yang et al. 2018). In this study, amphotericin B nanoparticles were prepared by a poly(lactic-co-glycolic acid) (PLGA) method. The results demonstrate that the application of ultrasound enhanced the antibacterial effectiveness of AmB-NPs ($P < 0.01$), and the antifungal efficiency increased significantly with increasing AmB concentration of drug-loaded nanoparticles under ultrasonic irradiation. Karki et al. used the sonoporation to deliver small interfering RNA (siRNA) into primary T cells. The sonoporation resulted in high efficiency transfection of siRNA into the mouse and human T cells.

Transdermal drug delivery offers many advantages as a topical drug administration route over other routes of drug administration. However, skin has low permeability and most of the drug molecules are not able to penetrate and diffuse through the skin. The low permeability of the skin is a significant barrier to successful drug delivery. Many technologies have been utilized to increase the drug absorption, such as nanoparticles, application of absorption enhancers (surfactant, PVP, DMSO, oleic acid), and physical enhancement (tape stripping or skin abrasion). However, these methods tend to cause local irritation. Certain nanoparticles such as gold nanoparticles with a size less than 40 nm in diameter have shown good penetration through the epidermis. The combination strategy of transdermal physical enhancement methods is advantageous in terms of a decline in energy density, thereby reducing skin irritation. Zimon used ultrasound to enhance skin permeability with quaternized starch (Q-starch) to deliver miR-197, a miRNA to treat psoriasis, a common auto-inflammatory skin disease (Lifshiz Zimon et al. 2018). This resulted in decreased expression of the miR-197 target proteins and a significant reduction in psoriatic activity markers. Ultrasound-enhanced delivery of proteins has also been applied in transdermal insulin

administration. Ultrasound-induced cavitation can reversibly disrupt the structure of the stratus corneum to allow transport of macromolecules.

11.2.2 Electrical-Based Method

Reverse electroporation, a technique of reversible permeabilization of cell membrane, has been long studied in the field of cellular fusion, genetic, and drug delivery (Weaver and Chizmadzhev 2005; Chang et al. 2012). A brief, high voltage, electric impulse is utilized to disrupt the homeostasis of cells which in turn forms nanopores in cell membrane (Rubinsky et al. 2007; Edd et al. 2006). Not only for the drug delivery but also for the delivery of other therapeutic agents such as proteins, tracers, dyes, RNA, DNA, antibodies, etc., electroporation has been beneficial (Golzio et al. 2004). Electroporation (EP) is gaining decent popularity in the field of molecular genetics (Rodriguez-Devora et al. 2012).

A report by Marshall and coworkers (2010) has shown the application of reverse EP mediated therapy in the treatment of coronary artery disease. In this report, EP has been utilized as a tool to deliver naked DNA expressing VEGF protein to the porcine heart (Marshall et al. 2010). Hollow nanoneedles also called as nanostraws are emerging as a powerful tool to manipulate and monitor the regular intracellular behavior (VanDersarl et al. 2012). Nanostraws are designed to have an inner fluidic channel extending through the substrate, allowing manipulations of intracellular environment (Wen et al. 2019). Integration of low voltage EP with nanostraws has made it possible to achieve effective cell perforation with marginal cell damage (Xie et al. 2013). Nanostraw-EP integrated platforms offer a strong potential to deliver various biomolecules into a varied range of cells. Cao and colleagues have reported the significance of nanostraw-EP platform for efficient intracellular delivery into cells that are hard to transfect, for example, neurons, cardiomyocytes, and stem cells along with the insertion of cas-9 ribonucleoprotein expression system for genome editing (Cao et al. 2017). Not only delivery but also this platform has a potential to, repeatedly and non-destructively, extract cellular content (Xie et al. 2013).

Immune cells should be genetically modified prior to adoptive immunotherapy. For example, in CAR-T therapy, patient's immune cells are transgenically tailored *ex-vivo*, to express tumor-related antigen receptors, to specifically target the tumor cells. The caveat to this technology is limited yield due to non-viral plasmid transfection methods such as bulk EP or nanocarriers (Shi et al. 2018). A recent report demonstrated that **dielectrophoresis**-mediated 3D nano-EP platform assisted transfection of CAR-T cells in NK-92 cells resulted in significantly improved efficiency (Roth et al. 2018).

SiRNA- or MiRNA-associated knockdown of **proto-oncogenes** has proved to be an effective therapeutic tool. Tabernero et al. in 2013 has vetted the effectiveness of RNAi therapeutics in treating liver cancer patients overexpressing VEGF and KSP (Tabernero et al. 2013). To deliver the oligonucleotide at the target site, previous research has utilized lipid nanoparticle-based delivery system. However, due to its

stochastic nature, the lipid nanoparticle-based delivery of oligonucleotide results in non-uniform delivery. Hence, to address the issue of non-uniform delivery of oligonucleotide, nanochannel-EP platform may be used for RNAi-based therapy to ensure precision control (Gao et al. 2014).

Gene editing is emerging as a research hotspot, driven by the discovery of novel editing tools such as engineered nucleases. Cas9, CRISPR-associated protein, which is being widely used as a gene-editing tool, has an immense potential to advance the progress of molecular therapeutics (Sander and Joung 2014). The gene-editing cargo of CRISPR/Cas9 is delivered by utilizing EP (Shi et al. 2018).

Both preclinical and clinical studies have proven the efficiency of EP successfully recorded (clinicaltrials.gov). Cancer therapeutics has embraced the potential of EP demonstrating relevant applications, for example, study of melanoma treatment where safe, effective, reproducible, and titratable administration of IL-2 and IL-12 by electroporation has demonstrated its anticancer potential (Daud et al. 2008). Additionally, developments in the field of prostate cancer have also demonstrated the potential of EP (Roos et al. 2006, 2008; U.S. 2014). These studies show that EP has a therapeutic advantage over several other methods of drug delivery. Besides all the advantages of EP, there remains a few limitations of this technique. For example, miniaturized EP is still not able to transfect large enough cell population analogous to the conventional delivery methods.

Another method of noninvasive drug delivery is iontophoresis. Charged drug is delivered through the biological membranes under the influence of very mild electric current. The iontophoretic transport depends on electromigration and electroosmosis (Roustit et al. 2014). Iontophoresis has been successfully used to treat skin tumors, head and neck cancers, alopecia, local anesthesia, and atopic dermatitis (Taveira et al. 2009; Gelfuso et al. 2011; Santana et al. 2017; Gratieri and Kalia 2014; Río-Sancho et al. 2017; Kigasawa et al. 2010). In 2006, a fentanyl-based patient controlled transdermal iontophoretic system was approved for the management of acute moderate to severe postoperative pain. However, due to technical considerations and lack of positive clinical trials data, marketing authorizations of this product were suspended by the European medicine agency (Viscusi et al. 2004).

Lidosite® from Vyteris Inc., Fair Lawn, NJ, USA, a combination of Lidocaine and epinephrine, was approved by FDA in 2004. A randomized, open label, cross over study reported that the nociception achieved by 10 min Iontocaine® (Abbott Laboratories, Abbott Park, IL, USA) in children undergoing repeated procedures requiring peripheral iv access is similar to the nociception achieved after 60 min of local application of lidocaine/prilocaine cream (Galinkin et al. 2002). However, a randomized double-blind study reported mild **erythema** at iontophoresis site in 50% of adults as well as 60% of children (Zempsky et al. 2004). Despite minor adverse effects, iontophoresis is successfully used in the clinics to induce local anesthesia.

11.2.3 Magnetic Based Delivery Method

The magnetic based drug delivery (MBDD) method was first introduced in the 1950s as a targeted drug delivery system (Liu et al. 2019). Since then the interest in MBBD has been on the rise due to the availability of stronger magnets, superior magnetic probes, introduction of rare-earth magnets, and intelligent nanomagnetic material. Different magnetic drug carriers such as nanoparticles, microspheres, and liposomes have been developed over the years for site-specific targeting of drugs, antibodies, and radionuclides (Gul et al. 2019; Wu and Huang 2017; Koppisetti and Sahiti 2011). The availability of the intelligent drug carriers coupled with the availability of stronger and superior magnets led to a renewed interest in the field of MBBD.

The MBDD method involves incorporation of drugs into the magnetic material followed by injection and guidance of these particles to the target site under the influence of a localized magnetic field (Fig. 11.3) (Mody et al. 2014). These materials are expected to stay at the target site until the completion of therapy and are then subsequently removed from the body (Gul et al. 2019). During magnetic drug targeting, the magnetic material carries the drug, increases its dissolution rate, selectively delivers the drug to its target site, and achieves high concentration of the drug in the areas around the target site through controlled drug release. Typical magnetic drug delivery includes loading the drug in biocompatible carriers such as nanoparticles, emulsions, liposomes, and micelles and transferring to bodies for treating various diseases.

Magnetic based drug delivery can be either passive or **active targeting**. In the passive targeting, the drug delivery occurs by passive accumulation through enhanced permeability and retention effect via leaky vasculature of endothelium at the target site (i.e., tumor) leading to an increased localization of drug-loaded particles. In active drug targeting, the magnetic carrier particles interact with targeted cells that express specific epitopes or receptors. During active drug targeting, a higher concentration of drug within the target site is achieved using magnetic particles.

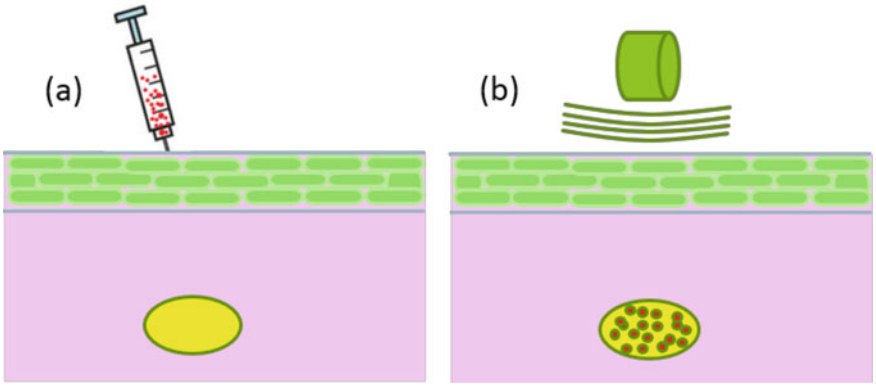

Fig. 11.3 Steps involved in drug delivery mediated by using the magnetic based method. **a** Injection of drug-loaded magnetic carriers. **b** Drug delivers into the target

11.2.3.1 Types of Magnets

Based on magnetic behavior, the magnetic materials are classified into six major types, i.e., diamagnetic, paramagnetic, ferromagnetic, antiferromagnetic, superparamagnetic, and superferromagnetic (Njagi et al. 2011). Paramagnetic materials lose magnetic momentum when the external field is removed. Magnetic nanoparticles (size less than 10 nm) behave like a superparamagnetic material only under the influence of external magnetic field and revert back to their nonmagnetic behavior by removing the external field (Xiong et al. 2018). Supermagnetism is a type of magnetism which appears in small ferromagnetic or ferrimagnetic nanoparticles where magnetization can randomly flip direction under the influence of temperature.

Proper selection of a magnet plays a crucial role in drug targeting as the magnetic field determines the direction of movement of the drug in the body. The magnetic systems used for magnetic based delivery are divided into static field magnet systems and varying field magnet systems. In the static field magnet system, the magnetic field remains constant over time, whereas in the varying field magnet system the magnetic field changes with time.

11.2.3.2 Magnetic Carriers

Magnetic carriers can deliver high concentrations of potent drugs near the target sites without affecting the normal surrounding tissues. They are differentiated into magnetic nanoparticles, **magnetic microspheres**, **magnetic liposomes**, magnetic emulsions, and magnetically modulated systems and devices.

Magnetic Nanoparticles

Magnetic nanoparticles (MNPs) are of submicron range (less than 100 nm) containing organic or polymer coating; drug and ferromagnetic particles such as iron, nickel, cobalt and their oxides such as magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), cobalt ferrite (CoO . Fe_2O_3) and chromium di-oxide (CrO_2) (Wu and Huang 2017). Their small size, large surface area, biocompatibility, and ability to target specific sites as well as their imaging and therapeutic properties have made them widely used carriers for drug delivery (Gul et al. 2019). The loading capacity, biocompatibility, and improving molecular imaging quality of the nanoparticles can be altered by surface functionalization or surface modification method (Thiruppathi et al. 2016; Deng et al. 2018). A variety of approaches including noncovalent binding, covalent conjugation, and surface coating is employed for altering functionalization of nanoparticles (Deng et al. 2018). Drug loading and targeted release of nanoparticles are achieved by employing pH or temperature-sensitive materials. Nanoparticles coated with dextran and poly(ethylene glycol) (PEG) show improved solubility, biocompatibility, longer circulation time, and enhanced uptake. Furthermore, functionalization exhibits excellent near-infrared optical properties for targeted imaging

of tumors through photoacoustic imaging and near-infrared fluorescence imaging (Deng et al. 2018).

MNPs are prepared by biomineralization, physical methods, and chemical methods. In biomineralization method, living organisms such as bacteria prepare magnetic particles to use for navigational purpose (Biehl et al. 2018). The physical methods of MNP preparation involve top-down method using particle reduction by milling or bottom-up method using laser evaporation technique (Biehl et al. 2018). The chemical methods include co-precipitation, microemulsion, thermal decomposition, and solvothermal methods.

Magnetic Microspheres

These supramolecular hollow spherical particles in the micrometer size range are comprised of different materials such as proteins or synthetic polymers. They can successfully deliver a variety of substances such as cells, medications, antibodies, nucleic acids, and enzymes. Their small size (less than 4 microm) allows them to easily pass through capillaries without producing embolic occlusion (Farah 2016). They can be dragged into the adjacent tissues by placing a suitable magnet with a magnetic field of 0.5–0.8 T. Magnetic microspheres are prepared by either the phase separation emulsion polymerization method or by the solvent evaporation method.

Magnetic Liposomes

Liposomes are vesicular, colloidal particles with a lipid bilayer structure. The magnetic liposomes are prepared by entrapment of ferrofluid within the core of liposomes. They can also be prepared by covalent attachment of ligands to the surface of the vehicles or by incorporation of target lipids in the matrix of structured phospholipids. These amphiphilic magnetic liposomes can incorporate both hydrophilic and lipophilic drugs. The hydrophilic drugs are incorporated into the aqueous layer, and the lipophilic drugs are incorporated into the lipid bilayer. Application of magnetic liposomes includes site-specific targeting, cell sorting, and as magnetic resonance contrast exchanging agents. Pradhan et al. studied the effects of folate-targeted doxorubicin temperature-sensitive magnetite-sensitive liposomes (Pradhan 2010). The doxorubicin release from these thermosensitive liposomes can be controlled by selective heating by the electromagnetic fields.

Magnetic Emulsions

These are magnetically responsive colloidal particulate systems in which the particle carrying the drug are in the submicron size range (i.e., 10–100 nm). The main components of these nanoemulsions are oil, emulsifying agents, and water. The ferrofluids containing the magnetic particles constitute the internal phase. With the application

of an external magnetic field, magnetic emulsions can also be selectively localized to a specific target site, much like other magnetically modified systems such as microspheres and nanoparticles. These magnetic nanoemulsions can be prepared either by high-energy emulsification or by low-energy emulsification methods.

Magnetically Modulated Delivery Systems and Devices

These delivery systems and devices are gaining popularity as they can deliver drugs at higher rate on demand. One example is the magnetic polymer matrix system in which the ethylene vinyl acetate copolymer is combined with magnetic beads. These magnetic beads are made with iron (79%), chromium (17%), carbon (1%), manganese (1%), silicon (1%), molybdenum (0.75%), and phosphorous (0.04%) or a small amount of samarium cobalt magnets. Drug release from the magnetic polymer matrix can be controlled by an oscillating external magnetic field. Another example is polymer–magnetic composite fibers. This biocompatible controlled-release system is a recent development that can magnetically actuate and release the drug at a specific target location. Perera and others studied the drug release of a magnetically actuated acetaminophen delivery system based on polyvinyl alcohol (PVA) − MNP fibers, generated via infusion gyration (Perera et al. 2018). They demonstrated the ability of PVA–MNP fibers to be actuated via an external magnetic field, and characterized their physicochemical properties.

Apart from the examples mentioned above, magnetic modulated implantable drug delivery devices have been extensively studied for magnetic based delivery. This type of system consists of a small implantable device composed of a silicone-based sponge impregnated with magnetic carbonyl iron particles. Drugs are injected into the polymer-coated sponge layer. Since this device can be actuated when an external magnet is applied, no battery is required. In a recent study, Lee et al. implanted a small volume device in living animals that is enabled with on-demand, pulsatile drug release when an external magnetic field is applied (Lee 2018). They demonstrated that this device delivered a varied amount of drug by using different outlet ports, and the drug is only released when there is an external magnet.

11.2.3.3 Applications of Magnetic Targeted Drug Delivery

Rapid progress in magnetic drug delivery has led to the development of a wide range of applications in biomedical and biophysical fields such as targeted delivery of drugs and genes, MRI, cell purification, and hyperthermia (treating tumors with heat). The clinical trials on magnetic drug delivery were first conducted in 1996 using a single permanent magnet placed on the surface of the skin near the tumor site (Lübbe et al. 1996). In addition, theranostics therapy such as combination of magnetic hyperthermia and radiation therapy are currently in clinical trials for the treatment of brain and prostate cancer (Rahbar et al. 2018).

Magnetic nanoparticles have displayed great potential in targeted drug delivery due to their magnetic core intrinsic capabilities. They deliver drugs under the influence of magnetic fields and are manipulated by different material such as iron, nickel, cobalt, and their oxides. The application of MNPs includes treatment of tumors, magnetic resonance image (MRI), vascular contracting agents, diagnostic agents, targeting of genes, tissue engineering, bioseparation, cell tracking, and theranostic agents.

When MNPs are exposed to an external high-frequency alternative magnetic field, they transform electromagnetic energy to heat due to magnetic hysteresis of the material. This released heat reduces the viability of cancer cells by killing or making cancer cells susceptible to the effects of radiation and/or antineoplastic drugs (El-Boubbou 2018). The clinical trials are ongoing for such treatment in Europe, completed enrollment and treatment for Stage 1, and preparing for next stage single-arm study for the ablation of intermediate risk prostrate cancer with nanotherm therapy (Magforce 2019).

In photodynamic therapy (PDT), light-sensitive drugs (photosensitizers) are injected systemically. Approximately 24–72 h after the injection, these drugs only stay in tumor cells but not on normal cells (National Cancer Institute 2011). These drugs are photo exited in the tumor tissue using light of appropriate wavelength and power producing reactive oxygen species (Hou et al. 2016). These reactive oxygen species cause cell damage in the tumor. Photofrin® (porfimer sodium, Axcan Scandipharm Inc. USA) for injection is a photosensitizing agent used in PDT to treat tumors and of high-grade dysplasia (HGD) in Barrett's esophagus (BE) (Axcan Scandipharm Inc 2006).

Photothermal therapy (PTT) uses an optical absorbing agent (photosensitizer) to absorb energy and convert it into heat upon stimulation by an electromagnetic radiation such as radiofrequency, microwaves, near-infrared irradiation, or visible light. In vivo, the released heat may cause hyperthermia, which leads to tumor cell death (Eskiizmir et al. 2018).

11.2.4 Photo-Based Method

Photo-based drug delivery is a technology that uses ultraviolet (UV), visible, or near-infrared light (light in the near-infrared region with the wavelength from 750 to 2500 nm, NIR) for drug delivery. Visible light has seen application in medical use since ancient times. Until recent centuries, photodynamic therapy has been developed by using photosensitizers or photosensitizing medicinal treatment. Photosensitizers are molecules that can produce chemical changes in other molecules when exposed to light and can be used to enhance drug transport. Recently, photo-responsive nanomaterials for targeted and controlled-release drug delivery systems have attracted more research interest due to their ability to precisely and accurately control the drug release in specific cells or tissues. The main mechanisms involved include

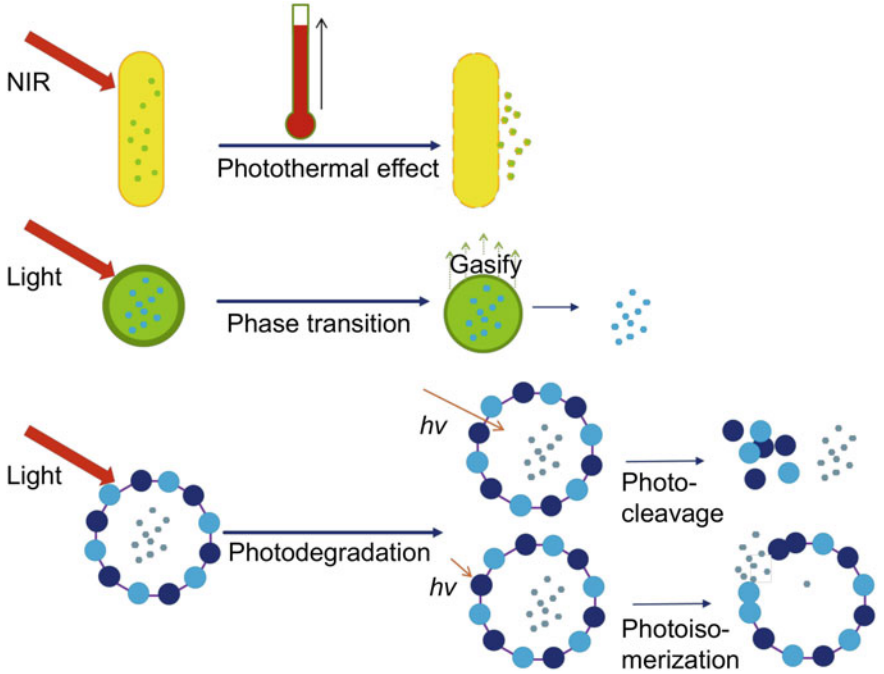

Fig. 11.4 Mechanisms of photo-based drug delivery system: photothermal effect, phase transition, and photodegradation

photothermal effect, phase transition, and photodegradation (photolysis, photoisomerization, photo-cross-linking/un-cross-linking, and photoreduction) (Fig. 11.4). Because of the poor tissue penetration of visible light and high phototoxicity of UV light, nanomaterials have been developed which are more responsive to low-energy photon irradiation, particularly for NIR light (Zhao et al. 2019).

11.2.4.1 Photothermal Effect

Photothermal effect is the production of heat by light irradiation, including visible light and light in NIR light. Noble metals such as gold and silver are resistant to corrosion and oxidation. Gold-based nanoparticles exhibit a strong photothermal effect in the presence of light in NIR, due to its unique property of light interaction with light. The heat can then trigger the polymer to collapse and release the drugs (Rodriguez-Devora et al. 2012; Deng et al. 2018).

Gold nanorods are rod-shaped nanoparticles with gold inside, which generates plasmonic heat induced by NIR light. Therefore, they have been frequently used in photo-based drug delivery systems (Pérez-Juste et al. 2005). Gold nanorods were incorporated into mesenchymal stem cells which can be used as natural drug carriers.

Vincristine was encapsulated into the gold nanorods and delivered to the cancer cells via mesenchymal stem cell carriers. The drug was released upon NIR laser irradiation. This gold nanorod-based formulation represents a promising therapy for the improved treatment of cancer (Muslimov et al. 2019). A thin layer of hyaluronic acid was immobilized to the gold nanorod and functionalized with a macrocycle molecule together with a drug that interacts with this molecule. This formulation was able to conjugate retinoic acid and release the drug within a few minutes after exposure to a NIR laser (Francisco et al. 2019). A hybrid nanocarrier based on human serum albumin/chitosan was used to encapsulate free docetaxel and doxorubicin-modified gold nanorods simultaneously. The results showed that the release of doxorubicin can be promoted by NIR light irradiation. In addition, a significant synergistic effect of docetaxel and doxorubicin was observed under NIR light exposure (Villar-Alvarez et al. 2019).

Due to the photothermal property of Fe/FeO, Fe/FeO-based polymer is another type of photothermal material. PLGA polymer matrix coated with Fe/FeO core–shell nanocrystals were also used for light-responsive formulation (Wang et al. 2019a). Indocyanine green, a cyanine dye used for medical diagnostics, also shows a photothermal effect. Cisplatin was prepared into nanoparticles using human serum albumin, called human serum albumin-indocyanine green-cisplatin nanoparticles. Cisplatin release can be triggered by using NIR light (Wang et al. 2019b).

Certain synthesized polymers have photothermal properties. The folic acid-polyethylene glycol (FA-PEG) modified polydopamine nanoparticles (FAPPs) were synthesized which are type of photothermal polymer. Nitric oxide (NO) loaded nanoparticles were developed, which release NO gas under NIR light irradiation. NO could reverse MDR by inhibiting P-gp activity, and the amount of NO could be regulated by adjusting the action time. The inhibition of P-gp leads to a significant chemosensitizing effect on doxorubicin (Wei et al. 2019).

11.2.4.2 Phase Transition

Phase transition is the transformation from one phase of matter to another in a thermodynamic system, such as transitions between solid, liquid, and gaseous states by absorption or emission of heat. The phase transition can also be triggered by light irradiation.

Perfluorocarbons (such as perfluoropentane (C_5F_{12}) and perfluorohexane) are chemically inert and non-toxic substances. When induced by light or acoustic energy, perfluorocarbons can undergo phase transit from liquid to gas. This property could be applied for controlled release by ultrasound or laser irradiation. The doxorubicin-encapsulated perfluorocarbon nanodroplets were developed which could release the drug in an on-demand manner for suppression of angiogenesis (Yuan et al. 2019). Perfluorocarbons can also be used for penetration of the blood–brain barrier. It was used to deliver extravasation of dye into brain tissue, demonstrating the potential to be a noninvasive, cost-effective, and efficient image-guided delivery (Hallam et al. 2018).

In another study, gold nanorod-coated doxorubicin-encapsulated perfluorocarbon nanodroplets were developed as light-activated on-demand drug delivery carriers. The gold nanorods on the perfluorocarbon nanodroplets could resonate with a laser wavelength and generate plasmonic heat on the gold nanorods leading to the vaporization of the nanodroplets to gas bubbles (phase transition) and the release of the encapsulated drug from the nanodroplet core. This study proved that the light-activated nanodroplets could be used for on-demand targeted therapy (Yuan et al. 2019).

11.2.4.3 Photodegradation

Photodegradation is the alteration in the chemical structure of drug carriers by light including photoisomerization and photocleavage (photolysis, oxidation, and reduction).

Photoisomerization

Azobenzene is a chemical compound composed of two phenyl rings which exhibits reversible trans-to-cis isomerization by light exposure. The interaction between azobenzene and β-cyclodextrin can be utilized to prepare polymers as light-responsive drug carriers. Camptothecin was incorporated into nanoparticles with azobenzene and β-cyclodextrin. This formulation showed improved cytotoxicity under the stimuli of light (Pang et al. 2019). Another unimicelle system was constructed based on a robust host–guest recognition between the β-cyclodextrin-grafted hyperbranched conjugated polymer and azobenzene-functionalized poly(ethylene glycol). The photoisomerization could be triggered by NIR light leading to the disassembly of micelles and the release of doxorubicin, which showed improved cytotoxicity in Hela cells (Huang et al. 2019).

Spiropyran is a chemical compound with photochromic properties. It has two heterocyclic groups and can be converted to its isomer (merocyanine) under UV light. A nanogel of poly(acrylic acid-co-spiropyran methacrylate) cross-linked by N,Nbis(acryloyl) cystamine was proven to be light responsive. By exposure to UV light in an acidic solution, spiropyran (hydrophobic) converts to its hydrophilic form (merocyanine) and the nanogel is swelled up. It was used to encapsulate doxorubicin and showed different behaviors based on presence or absence of UV light (Chen et al. 2017).

Photocleavage

Certain polymers are light degradable with photocleavable species conjugated to the polymer side chains as protecting groups. Upon light irradiation, photocleavage of the light-responsive units leads to the deprotection of these functional groups,

which can initiate a consecutive degradation process of polymer main chains and thus release the payload of polymer nanoparticles.

PLGA (poly(lactic-co-glycolic acid)) is a copolymer approved by the Food and Drug Administration (FDA) as an excipient for formulation development, which has good biodegradability and biocompatibility. A modified PLGA polymer was prepared through the protection of hydroxyl, thiol, and amine side groups with oNB groups. Under the UV irradiation, oNB groups can undergo cleavage resulting in rapid degradation of PLGA (Olejniczak et al. 2015).

Using the similar strategy, a new light-responsive aliphatic polycarbonate was developed to deliver a photosensitizer 5,10,15,20- tetrakis (m-hydroxyphenyl) chlorin. Multiple photocleavable oNB groups were conjugated to the side chains of this polymer, which can be cleaved under UV exposure. This polymer underwent rapid degradation via intramolecular cyclization. The light-induced reaction then led to the burst release of the photosensitizer (Sun et al. 2019a, b). Photosensitizers can enhance the transfection of DNA into living cells. The oxidation-responsive supramolecular polycationic assemblies were used to deliver p53. The reactive oxygen species from light can accelerate the disassembly of polymer and DNA complexes, facilitating the release of DNA from the complexes and improving the subsequent transfection (Zhang et al. 2019b).

Polyurethanes based on serinol with o-nitrobenzyl pendant groups are light-responsive polymers. Upon irradiation with UV light, the polymers undergo rapid degradation (Sun et al. 2019a).

Coumarin is an aromatic lactone compound found in natural plants that shows strong light absorption property. The carbonyl group of coumarin derivatives can be hydrolyzed by UV light. Coumarin has been used to prepare coumarin-containing photo-responsive carriers for drug delivery. Coumarin was conjugated to octadecyltrimethoxysilane (C18)-modified hollow mesoporous silica nanoparticles to obtain NIR light-responsive polymers. This system was used to incorporate doxorubicin. Under excitation by NIR light at 800 nm, the drug molecules were released due to the degradation of the polymer. This drug release can be effectively controlled by exposure to light (Ji et al. 2013). Puromycin caged with the photo-responsive 7-diethylaminocoumarinyl protecting group carbamate was developed. This system represented an efficient photo-activatable antibiotic for in-cell applications (Herzig et al. 2017).

The disulfide group is a very popular functional group in the biological system which can be reduced by light irradiation leading to photolysis. Disulfide-nanogated multifunctionalized mesoporous silica nanoparticles were used to load doxorubicin. The uptake in MCF-7 cells was visualized via two-photon fluorescence imaging and showed the improved cytotoxicity of doxorubicin. The results indicated that it can be used as a potential medical nanocarrier for targeted delivery (Croissant et al. 2015).

11.2.5 Microneedles

Microneedles have been studied by various researchers as novel physical method of systemic drug delivery. Microneedles or microneedle arrays are micron sized hybrid devices of hypodermic needles and transdermal patches. A microneedle array is composed of an array of micron size needles that are arranged on a small patch. Microneedles allow the delivery of large hydrophilic molecules of high molecular weight across the stratum corneum, as they place the drug molecules directly into the epidermis or upper dermis, from where the drug molecules enter the systemic circulation. This painless mechanism of drug delivery (as MNs are not long enough to reach skin nociceptors) with its potential for self-administration allows for better patient compliance and is known to result in faster onset of action (Xie et al. 2017). Microneedles are available in different sizes and are formulated using different materials such as silicon, metal, ceramic, silica glass, carbohydrates (maltose), and a variety of dissolving and biodegradable polymers (Larrañeta et al. 2016). The needles are typically up to 1500 μm long to facilitate the placement of the drug in the epidermis, up to 250 μm wide and have tip thickness up to 25 μm. Longer microneedles are also available to facilitate the release of the drug in the dermis region of the skin. There are different types of microneedles with their unique mechanism of drug delivery. Solid microneedles which use poke with patch approach are used for pre-treatment of the skin, whereas coated microneedles use coat and poke approach; a coating of drug solution is applied on the needle surface. Dissolving microneedles are made of biodegradable polymers, while hollow microneedles are filled with the drug solution and deposit the drug in the dermis (Ita 2015). Hollow microneedles have the primary advantage of being able to deliver large doses directly into the dermis layer.

Microneedles have been studied for application and efficacy in oligonucleotide delivery and compared with iontophoresis approach of drug delivery by Bora et al. and found to be effective (Bora et al. 2008). They have also been studied extensively in vaccines, peptides, and hormone delivery (Li et al. 2017). Administering lidocaine through microneedle was shown to cause less pain as compared to hypodermic injection and thus showed better patient compliance (Li et al. 2017). Meloxicam-loaded polymeric microneedles were prepared using polydimethylsiloxane molds. The in vitro permeation studies showed approximately 100% drug release in 60 min. The drug deposition was found to be 63.37% and improved transdermal flux of 1.60 μg/cm2/hr was observed. The permeation increased 2.58 times compared to free drug solution (Amodwala et al. 2017). Dissolvable microneedles were explored for treating neuropathic pain. These delivered selective calcitonin gene-related peptide (CGRP) antagonist showed high specificity against the receptors. The analgesic microneedle patch showed no skin irritation and side effects. About 75% microneedle dissolved within 20 min on the application (Xie et al. 2017). Self-degradable microneedles were investigated for melanoma treatment by delivering anti-PD-1 (aPD1) in a sustained manner. Anti-PD-1 and glucose oxidase loaded pH-sensitive dextran nanoparticles were delivered through microneedle (Naguib et al. 2014). Bhatnagar and coworkers investigated the delivery of chemotherapeutic agents, tamoxifen

and gemcitabine, through microneedles for the treatment of breast cancer. Localized delivery of these drugs would help to reduce the side effects (Bhatnagar et al. 2018). Macroflux® is a novel transdermal microneedle system that is developed by Alza Corporation. Therapeutic peptides, proteins, and vaccines such as desmopressin, human growth hormone (HGH), TH 9507 (a human growth hormone releasing factor analog), and ovalbumin (45,000 Da protein) are in the developmental stage for transdermal delivery by Macroflux® (Morgan et al. 1998).

Till now, many microneedle products have been launched into the market in recent years. Soluvia® is a hollow microneedle product that is attached to a syringe that is developed and marketed by Sanofi Pasteur Europe to deliver influenza vaccination. Valeritas developed h-patch® to deliver insulin. 3 M has several microneedle transdermal patches that are used to deliver biologics and other small molecules. 3 M's hollow microneedles can be used to administer some hundreds of milligrams of proteins, which go directly into the systemic circulation (Burton et al. 2011).

11.2.6 Other Permeation Enhancer Based Systems

Temporary barrier improvement with permeation enhancer based systems including physical permeation enhancing techniques like ablative fractional resurfacing using lasers has recently been explored to enhance systemic drug delivery (Lee et al. 2001; Hædersdal et al. 2010).

11.2.6.1 Ablative Fractional Resurfacing or Laser-Assisted Drug Delivery

Lasers are used for the treatment of dermatological conditions such as acne, where the laser radiation destroys the target cells over a short frame of time (~300 ns), and for drugs targeted for systemic absorption which require deeper channels to access the dermal vascular plexuses. Such direct and controlled exposure of the skin to laser radiation results in ablation of the stratum corneum without significant damage to the underlying epidermis. Removal of the stratum corneum by this method has been shown to enhance the delivery of lipophilic and hydrophilic drugs (Lee et al. 2001).

11.2.6.2 Suction Ablation

Suction ablation technique involves the removal of epidermis while leaving the basal membrane intact. Vacuum or negative pressure is applied to form a suction blister. This mechanism of drug delivery is also referred to as skin erosion by avoiding dermal invasivity, thereby avoiding pain and bleeding. Cellpatch® manufactured by Epiport Pain Relief in Sweden is a commercially available suction blister device, which delivers morphine.

11.3 Summary and Outlooks

Physical methods of drug delivery possess a great promise in treatment of cancer, inflammation, skin disorders, and other diseases. The development of this type of drug delivery system has accelerated greatly in recent years due to advances in nanotechnology, material science, and molecular cell biology. Although physical methods of drug delivery have shown great promise both in diagnosis and therapy, there are challenges to be overcome before these methods can be used practically in intervention execution. For instance, while microinjection enables delivery of therapeutics in vitro, it can hardly be applied to all cells in a living body. This also applies to particle bombardment, which can be applied for gene delivery in vitro but can hardly be translated in vivo. Other challenges include the toxicity associated with unremoved magnetic materials in the body, as well as the poor efficiency and lack of specificity in vivo, also affect the wide applications of physical methods in execution of anti-aging interventions. Nevertheless, with rapid advances in technologies, it is expected that some of these challenges would be overcome someday in the future. In addition, a synergistic combination of multiple techniques may enhance the delivery efficiency mediated by physical means. This is also a direction that is worth exploring for future research on physical methods of drug delivery.

Important Notes

- Theranostics therapy such as combination of magnetic hyperthermia and radiation therapy is currently in clinical trials for the treatment of cancer.
- Ultrasound-induced cavitation can reversibly disrupt the structure of the stratus corneum to allow transport of macromolecules.
- Integration of low voltage EP with nanostraws has made it possible to achieve effective cell perforation with marginal cell damage.
- Ultrasound increases membrane and cell permeability by direct effect or cavitation. Perturbation of drug carriers such as nanoparticles, liposomes, and micelles by ultrasound leads to controlled release of the encapsulated drugs.
- Reactive oxygen species from light can accelerate the disassembly of polymer and DNA complexes, facilitating the release of DNA from the complexes and improving the subsequent transfection.

Questions for Future Research

- **How to deliver sufficient energy to the target sites without safety concerns when ultrasound based methods are adopted?** When ultrasound is applied, the intensity of ultrasound can be significantly attenuated by bone. The modern technique is, therefore, needed to deliver ultrasound through the bone tissues. In addition, the safety of ultrasound still needs to be further evaluated, although ultrasound is generally considered to be safe.
- **How to optimize and control the penetrating ability of light in the body when a photo-based method is adopted?** NIR can deliver more photonic energy than visible light because of the lesser light attenuation in tissues. However, tissue heating is a risk of higher-energetic infrared light sources. UV light contains higher energy than visible light and NIR, but it is not as permeable as NIR. The safety of UV light is another concern. The development of new photosensitizers or nanomaterials which are sensitive to light is a potential area to be further explored, which can improve the utility of photo-based drug delivery system.
- **How to enhance the clinical translation of research on magnetic based drug delivery methods?** Right now, although several therapeutic nanoparticle platforms such as liposomes and albumin nanoparticles have been approved for cancer and other disease treatments, the decomposition and long-term toxicity of such nanoparticle platforms in magnetic based drug delivery systems in clinical setting has not been proven. Another concern with magnetic based drug delivery system is the interaction between the magnetic carrier material and ferromagnetic substances such as pacemaker present in the human tissue. Such an interaction may lead to serious health consequences. The application of magnetic targeted drug delivery to disease locations near the body surface is well documented. However, such applications in clinical use beneath the tissues and anatomical barriers is not yet well understood. Furthermore, none of the magnetic based drug delivery systems have been currently approved by the US FDA. Rigorous safety and toxicological tests are needed before they are tested in large number of patients.

Glossary

Active targeting Selective transport of a carrier to specific cells/tissues by modifying the carrier with a chemical group that can be recognized specifically by those target cells/tissues.

Dielectrophoresis Motion of a neutral particle caused by polarization effects in a non-uniform electric field.

Erythema Abnormal redness of the skin or the mucous membrane caused by capillary congestion.

Iontophoresis A method in which the charged drug is delivered through the biological membranes under the influence of very mild electric current.

Magnetic liposomes Vesicular, colloidal particles prepared by entrapment of ferro fluid within the core of liposomes.

Magnetic microspheres Supramolecular magnetic hollow spherical particles in the micrometer size range. They are comprised of different materials such as proteins or synthetic polymers.

Microneedles Miniature needles fabricated from lithographic techniques for transdermal drug delivery.

P-glycoprotein A 170-kDa plasma membrane protein which is thought to be responsible for the occurence of multidrug resistance in mammalian cells.

Proto-oncogenes Genes that show the potential to be changed into active oncogenes.

Reverse electroporation A technique that involves temporary permeabilization of the cell membrane under the influence of a short electric impulse. This technique can be utilized to deliver a wide range of therapeutic agents including, dyes, tracers, antibodies, and oligoneucleotides.

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Chapter 12
Inhalation as a Means of Systemic Drug Delivery

Tomasz R. Sosnowski

Abstract In the preceding chapter, Vadlapatla and co-workers have presented an overview of different types of physical methods for systemic therapeutics delivery. In this chapter, we will focus specifically on how the use of physical devices (inhalers) and drug formulations can facilitate systemic delivery via the oral/nasal route using aerosols. In fact, systemic drug delivery via inhalation is challenging because the drug has to be aerosolized to extra-fine particles or droplets during a short period of patient inspiration. The drug carriers converted to aerosol cloud must contain medicines in the form that allows them to be easily absorbed from lung surface to the circulation, with the simultaneously reduced pulmonary clearance due to the specific interactions on the lung surface. This chapter will present the above aspects and will show that the required action of inhaled medicines may be obtained by adjusting the properties of drug particles forming powders or liquid formulations (suspensions) that undergo aerosolization. The possible use of electronic inhalers or add-on control systems to achieve an improved aerosol dosing to perform the inhalation maneuver correctly will also be discussed.

Keywords Inhalation · Aerosols · Lung deposition · Smart inhalers

12.1 Introduction

Inhalation is a convenient way of drug delivery to the respiratory system, and it is frequently used as first-choice method in the treatment of pulmonary diseases (asthma, COPD, bronchiolitis, pneumonia, etc.) (Newman 2009; Pirożyński and Sosnowski 2016). The method seems to be natural and simple to use (although it is not always true—see e.g., Fink and Rubin 2005), painless, and almost free of side effects. Indeed, due to low dose of active pharmaceutical ingredient, API, delivered in such a treatment, the systemic side effects are absent even for drugs which

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induce them after oral application (e.g., steroids, anticholinergics). Although inhalation is much safer than other methods of drug delivery, minor local symptoms are sometimes encountered after steroid inhalation, such as throat irritation, candidosis, or dysphonia (Kobayashi et al. 2006). Some drugs used in treatment of bronchial diseases (e.g., short- and long-acting beta$_2$-agonists: SABA and LABA) may also cause minimal systemic effects such as tremor or tachycardia (Sears 2002).

Inhalation therapy (or: **aerosol** therapy) is the rational strategy in the treatment of respiratory diseases since it is capable of drug delivery directly to the sites on the lung surface that require curing and quickly respond to the therapeutics. It was found that only several micrograms of API can result in the local dose in the lungs comparable to almost one hundred milligrams applied in form of a tablet or injection (Alangari 2014). This allows to effectively fight airway inflammation, fluidize the bronchial mucus, or trigger/calm the receptors of the epithelial cells. To achieve these goals, drugs must be carried to the lung surface as aerosol with defined properties. The most important features include the suitable particle size distribution and the correct inhalation maneuver for maximization of intrapulmonary penetration and **lung deposition** of drug particles. If drug or dosing system (i.e., formulation and inhaler) are not optimally designed, then inhaled aerosol will deposit mainly in the throat and central airways or penetrate too deep to the gas-exchange regions (alveoli). With incorrect inhalation technique, significant amounts of inhaled aerosol can be also exhaled. Obviously, in such cases drugs are not delivered to the regions of the respiratory tract that need pharmacotherapy. On the other hand, looking closer at the situation of suboptimal drug delivery in the treatment of respiratory diseases, one may find it beneficial in the view of systemic drug delivery, i.e., for addressing the inhaled drugs to the alveolar region where the ultrathin air/blood barrier (<0.1 μm) and very large surface area of the mass exchange (~90 m^2) create good conditions for fast diffusion and absorption of the drug from the airspace to the bloodstream. In this work, we try to discuss the essential technical requirements of the effective systemic delivery of drugs via this route.

12.2 Rationale for Delivery of Systemic Drugs via the Respiratory System

Table 12.1 summarizes pros and cons of inhalation as a method of drug delivery. Delivery of systemic drugs through the lungs may be attractive for several reasons. Primarily, it allows to avoid the first-pass effects related to the liver metabolism of drugs delivered via GI tract. Following the inhalation, drugs are absorbed directly to the circulation via the air/blood barrier in a non-invasive and painless way.

The pharmacokinetic profile for inhaled drugs may be similar (but usually not identical) as in the case of subcutaneous injection, however, the drug bioavailability may be lower (Gonda 2006). The inhalation method is well suited for drugs that are not easily absorbed from the GI tract and for fast-acting small molecules (e.g.,

Table 12.1 Benefits and drawbacks of inhalation drug delivery in general

Pros	Cons
1. The method of drug delivery is non-invasive, pain-free, and easy-to-use	1. Dose delivered to the target area is only a fraction of the metered dose
2. Enzymatic drug deactivation in the lungs is low, so the delivered dose can be minimized	2. The efficiency of local drug delivery depends on many technical factors and can be patient-dependent
3. Low side effects due to minimal mass of drugs delivered outside the target	3. Difficulty in definition and manufacturing the required drug formulation and aerosolizing device (inhaler)

painkillers). However, the major efforts have been spend to adapt inhalation as a method of delivery of proteins and peptides, such as insulin or hormones. Some concepts of vaccine delivery via inhalation have been also considered (LiCalsi et al. 1999; Griffin 2014).

It may be noted that aerosolized drugs may be also delivered to the nasal cavity and absorbed from there, however, in this situation the properties of aerosol and delivering devices should be very different than those used in pulmonary inhalations. Therefore, administration of systemic drugs via the nose will be not discussed here. The reader may find information on the nasal drug delivery elsewhere (Türker et al. 2004; Ghadiri et al. 2019).

12.3 Inhalation Aerosol as a Drug Carrier

In contrast to other methods of drugs administration, inhalation never delivers to the target areas the full dose of drug loaded into the delivery device (an inhaler), since.

(i) some amounts of the drug are always retained in the device,
(ii) part of released aerosol is deposited in the mouth-and-throat region, and
(iii) part of inhaled aerosol is removed from the lungs during exhalation.

Generally, it is expected that only particles with aerodynamic diameter d_a smaller than 5 μm can penetrate to the lower respiratory tract (bronchial tree), however, even smaller particles are required to maximize the dose deposited in the pulmonary alveoli (Boer et al. 2015). Recently, Sosnowski (Sosnowski 2016) has proposed the numerical parameter which takes into account both the amount of inhaled drug expected to deposit in the alveolar region (the target) and the amount of "wasted" drug due to the deposition in other parts of the respiratory system which do not contribute to the systemic delivery (upper airways and bronchi). This parameter may be called Pulmonary Targeting Index, PTI, and expressed as

$$\mathrm{PTI} = \frac{D_\mathrm{P}}{D_\mathrm{ET} + D_\mathrm{TB}} \tag{12.1}$$

where D_P denotes pulmonary (alveolar) deposition fraction, D_{ET}—extra-thoracic deposition fraction, D_{TB}—tracheobronchial deposition fraction. Since the total deposition fraction D_{TOT} (i.e., aerosol deposited minus aerosol exhaled) is a sum of D_{ET}, D_{TB}, and D_P, we can write

$$\mathrm{PTI} = \frac{D_P}{D_{TOT} - D_P} \tag{12.2}$$

Taking into account the deposition efficiency curves in different parts of the respiratory system (calculated using, e.g., ICRP or NCRP deposition models (International Council for Radiological Protection 1995; Council and for Radiological Protection 1997)), one can find the dependence of PTI on particle diameter. Such relationship is depicted in Fig. 12.1 in the normalized form of PTI_{norm} (where $\mathrm{PTI}_{norm} = \mathrm{PTI}/\mathrm{PTI}_{max}$, therefore it takes values only between 0 and 1). The graph shows, that the optimum size of particles for systemic drug targeting during inhalation is between ~0.8 and 2.5 μm ($\mathrm{TI}_{norm} > 0.75$). It is also clear that particles larger than ~3 μm are poorly targeted to the alveoli, so they cannot be considered as effective carriers of such drugs. The rapid drop in *PTI* is also observed for nanoparticles.

In the same work (Sosnowski 2016), two strategies of increasing the delivery of systemic drugs via inhalation were indicated. The first one has been already highlighted above and it is related directly to the maximization of alveolar deposition of inhaled aerosol particles. It can be achieved by some technical means, when:

(i) particle properties, such as size, shape, and structure, are properly designed,
(ii) the inhalation airflow required for deep penetration of inhaled aerosol can be achieved using a given delivery device.

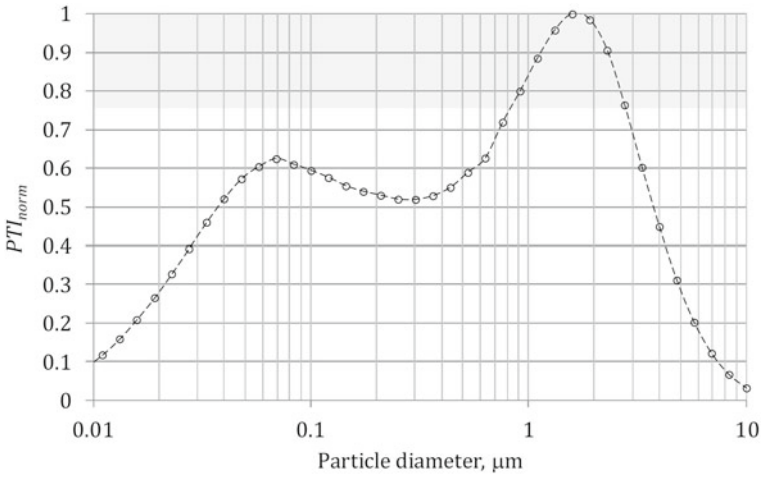

Fig. 12.1 Calculated normalized Pulmonary Targeting Index, PTI_{norm} as a function of particle size

Both issues are within the domain of aerosol technology and inhaler design, and they will be discussed in more details in Sects. 12.4 and 12.5. The second strategy addresses the increased bioavailability and drug release rate after deposition of the particles that carry the drug. The required properties can be achieved by careful design of particle solubility, surface characteristics, and internal structure. These "particle engineering" (or: "powder engineering") problems should consider also the possibility of reduction of the clearance of aerosol deposits from the alveolar region which should extend the residence time of drug on the pulmonary surface. We will focus on that in the following section.

12.4 The Significance of Drug Formulation

Typically, particles used in inhalation therapy are compact in shape (spheres, cube-like particles, etc.) and may be either dense or porous. Until now a little attention has been paid to the elongated fiber-like particles which are known to better penetrate to the deep lungs even if such particles are longer than the size commonly considered effective for lung delivery (Lippmann 1990; Ikegami et al. 2002). Elongated particles may avoid clearance by phagocytosis because they are often too long for the effective uptake by alveolar macrophages. Due to a higher surface area compared to compact particles with the same mass, elongated particles also can release drug faster, according to the known mass transfer law:

$$N = kA\Delta c \tag{12.3}$$

where N—mass transfer rate (kg/s), k—mass transfer coefficient (m/s), A—surface area (m^2), Δc—difference in the solute concentration (kg/m^3), being the driving force for the mass transfer process between two adjacent regions. The increase in the surface area of the mass exchange can be also obtained using nanometer-size particles, however, their dispersion in inhalers and further delivery to the respiratory system is often not effective (Fig. 12.1). A promising solution may be proposed by drug carriers composed as a few micrometer-size particles with the defined submicro or nanostructure. After good penetration and deposition in the lungs, such particles can decompose to the primary "building blocks" (i.e., nanoparticles) due to the interactions with the pulmonary fluids. Structured particles can be obtained by a variety of methods of powder engineering, including controlled spray drying from nanocolloidal precursors, spray-freeze drying, or particle precipitation from supercritical fluids (Abdelwahed et al. 2006; Okamoto and Danjo 2008; Lee et al. 2009; Gradoń and Sosnowski 2014; Jabłczyńska et al. 2018). This concept of systemic drugs nanocarriers was proven by Jabłczyńska et al. (2015) who demonstrated the possibility of nanoparticle release by taking up water by micrometer-sized particles of inhalable powders. The nanostructured powders were produced

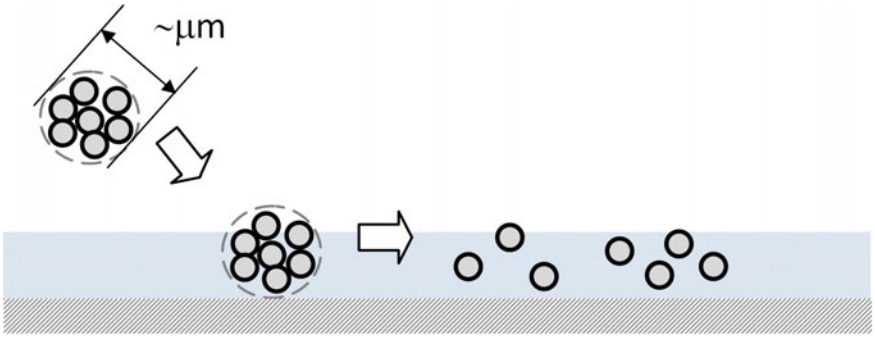

Fig. 12.2 Formation of drug nanoparticles by disintegration of microsized nanostructures due to their hydration on the pulmonary surface

from polysacharide (polyaldehyde dextran, PAD, and dialdehyde carbomethylcellulose, DACMC) nanosuspensions via the controlled spray drying process. Inhalable nanostructures formed from biocompatible polysaccharides are promising candidates for drug carriers in systemic diseases and anti-cancer therapies (Wasiak et al. 2016). The reconstitution of nanoparticles is schematically illustrated in Fig. 12.2, although other scenarios of the behavior of nanostructured microcomposites after their contact with the pulmonary liquid were also proposed (Sosnowski 2015). As already mentioned, the advantages of submicron drug particles are their increased solubility and dissolution rate as compared to larger structures (Muller et al. 2001).

Particle contact with the lung liquids is an important step in the drug particle-lung interaction. In the gel-like layer of bronchial mucus (which is, however, of less importance in the systemic drug delivery), the diffusion of drug molecules to the cells may be enhanced by reducing mucus viscosity (Odziomek et al. 2012). In the alveolar region, drug interactions with the **lung surfactant** may determine the particle retention time which, in turn, will define drug bioavailability.

The lung surfactant is essential for the regulation of lung mechanics, but also important for the rate of alveolar clearance (Notter 2000; Gradoń and Podgórski 1989; Sosnowski et al. 2000). The mass transfer processes in the lungs and macrophage activity are enhanced by **Marangoni effects** that are created during periodical compression and expansion of pulmonary liquid interface during breathing. The Marangoni convection is driven by the variations in the surface tension caused by the surfactant (Sosnowski 2018). Accordingly, if these effects are disturbed, particles deposited on the alveolar surface will stay longer in the deposition area. In the case of systemic drugs, this should create better conditions for drug absorption to the circulation. Sosnowski et al. (2003) suggested that inhalable drug carriers with increased surface area available for adsorption of lung surfactant molecules may reduce the surface tension gradients in alveoli and increase the residence time of particle in the alveolar zone. This provides better conditions for drug dissolution and permeation to the blood.

12.5 The Significance of an Inhalation System

As discussed earlier, the effective drug delivery by inhalation requires the simultaneous fulfillment of several conditions:

(i) aerosol particles must be within a certain size range to avoid inertial or gravitational separation in the upper airways and large bronchi,
(ii) patients must inhale in a certain way to push aerosol particles to the required depth of the respiratory system,
(iii) physicochemical properties of inhaled particles must be specially adjusted to assure their effective uptake by the organism after deposition on the lung surface.

The efficient inhalation device (inhaler) is needed to meet two last requirements from the above list. The inhaler should be easy in use and allow to obtain a cloud of aerosolized particles with the required quality (Fig. 12.3). Individual patient's characteristics are also important and the knowledge of it allows to define the requirements of personalized inhalation therapy (Kadota et al. 2020). Drug loaded into inhaler can be a powder, as discussed in the previous section, or liquid: either drug dissolved in water or aqueous suspension of micronized drug particles, if the drug is insoluble. Liquids must be atomized to produce fine mists of droplets that are required for the efficient pulmonary deposition.

Atomization of liquid medicines is done with nebulizers, which are operated applying several principles:

(i) in jet nebulizers, droplets are formed due to interactions of liquid with the stream of air delivered from the compressor,
(ii) in mesh nebulizers, droplets are formed by ultrasonic vibrations ($f < 200$ kHz) of the microperforated plate made of metal or polymer,
(iii) in classic ultrasonic nebulizers, droplets are ejected from the surface jet produced by ultrasonic energy ($f > 2$ MHz).

Each class of nebulizers has some drawbacks, for instance, problems with the high residual volume (in jet and ultrasonic nebulizers), membrane clogging (mesh nebulizers), or drug heating/chemical decomposition (ultrasonic nebulizers). However,

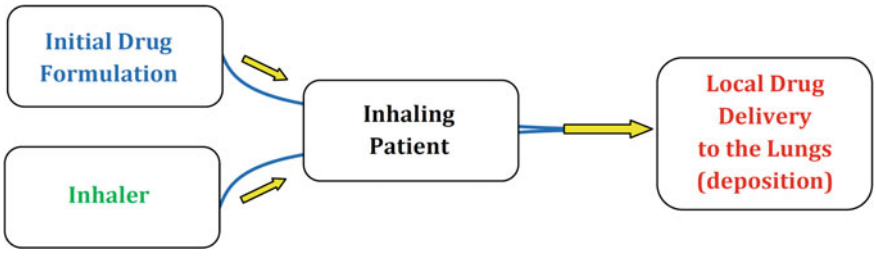

Fig. 12.3 Interplay between drug formulation, inhaling device, and the user (patient) in providing conditions of effective delivery of aerosolized drug to the lungs

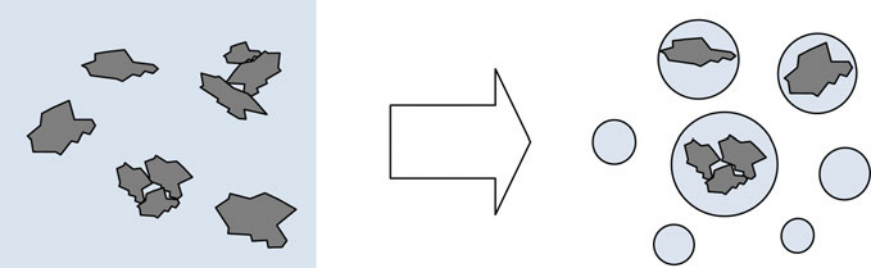

Fig. 12.4 Schematic explanation of formation of drug-free small droplets in the case of large insoluble drug particles (or aggregates) present in the atomized liquid suspension

the main advantage of all nebulizers is that they do not require special inhalation maneuvers since drugs are inhaled by patients during tidal breathing. It makes the inhalation treatment easy, however, not necessarily effective. Nebulizers deliver the prescribed dose during a few-minute period of inhalation (i.e., not during a single breath). All classic nebulizers operate in the continuous mode so in the devices without uni-directional valves, aerosolized drug released during exhalation is wasted. Recent studies questioned the suitability of nebulizers (of any kind) for the effective delivery of drugs atomized from suspensions, e.g., steroids (Sosnowski 2019). It has been argued that if the insoluble drug particles are big (~2–3 μm) or they form agglomerates of such sizes, they probably are not present in the small droplets released from nebulizers. Accordingly, small aerosol droplets may contain only the solvent (drug-free droplets shown in Fig. 12.4), and this obviously reduces the mass of the API delivered to the lung surface. Moreover, in mesh nebulizers with micrometer-sized orifices in the vibrating membrane, these apertures may be blocked by solid particles of a steroid. This discussion shows that not all drug formulations are equally good for use in the **nebulization**.

Other types of classic inhalers [viz., the **pressured Metered Dose Inhaler** (pMDI) and the **Dry Powder Inhaler** (DPI)] can deliver the whole drug dose in single inhalation which is an advantage but requires a special inhalation maneuver. Therefore, a problem of patient "adherence" to the inhalation therapy arises (Makela et al. 2013). The efficient use of pMDIs is mainly related to the lack of coordination that is required to fire the spray in the right moment of inspiration. Errors in the coordination lead to a poor drug delivery due to the significant aerosol losses in the mouth and throat (Giraud and Roche 2002). Powder aerosolization in DPIs relies on the aerodynamic energy of air drawn through the device by inhaling patient (such DPIs are known as passive inhalers). During inhalation a powder dose is dislodged, fluidized, and de-aggregated into fine aerosol particles that are carried to the lungs by the same breath. Different mechanisms of aerosol formation from powders are used. The very common one is based on dispersion of powder from a perforated capsule which rotates or vibrates due to the airflow inside the inhaler (Moskal and Sosnowski 2012). The difficulty and uncertainty in the delivery of a given dose from passive DPIs are related to the requirements of an energetic inspiration followed by a breath

hold. Errors in doing this maneuvers may restrict the effective use of DPIs by, e.g., children, disabled, or older patients. A variability in the energy of inspiration results in a low reproducibility of the fraction of **fine particles** released from DPIs. Moreover, many DPIs are flow-dependent and they also differ in the internal aerodynamic resistance (low-, medium-, and high-resistant devices), which additionally increases inter-subject variability of drug dosing (Hejduk et al. 2018).

The problem of the inhalation dynamics in the relation to the moment of aerosol release from the inhaler is also essential. Inhalation produces a variable airflow of a quasi-sinusoidal profile, Fig. 12.5. The aerosol cloud must be produced and be ready for inhalation during the phase of increasing flow (e.g., point A in Fig. 12.5) since the particles must be transported with the airflow down to the lung periphery. When the aerosol enters the mouth too late (point B in Fig. 12.5), it cannot be not delivered to the deepest parts of the respiratory system because of the inspiratory phase of breathing terminates very soon. Therefore, the effective DPIs must be capable of releasing aerosol with the required particle size distribution already in the early stage of inhalation. It is not always obtainable since the **powder fluidization** and entrainment may start already at low airflow rates, i.e., when the aerodynamic energy is insufficient to effectively break-up powder clusters and to release fine particles. It is also possible that when the airflow is approaching the maximum value (PIFR—peak inspiratory flow rate), so it is capable of inducing good aerosolization of the powder, there may be already no drug in the inhaler. To avoid this situation some

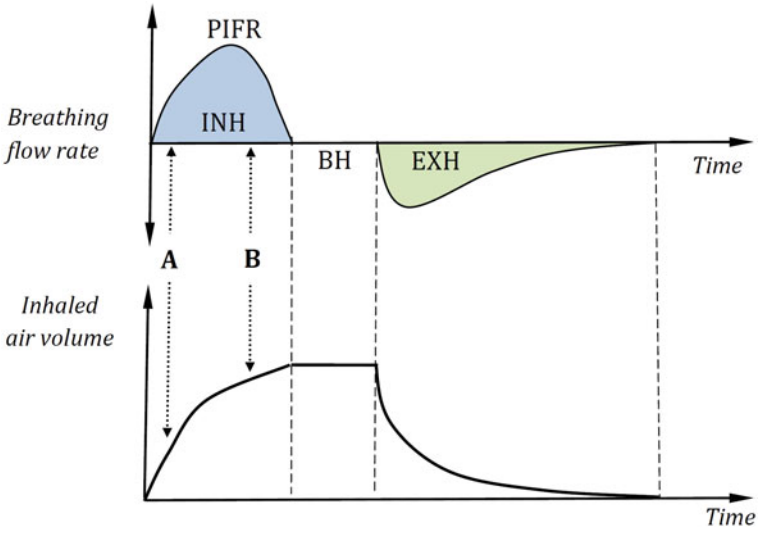

Fig. 12.5 The inhalation curves: airflow rate and lung volume vs. time. For deep lung deposition aerosol with the required particle size distribution should be available in moment A. If the aerosol is released inhaled in moment B it cannot be carried to the lung periphery, because the inhalation ends very soon. Abbreviations: INH, inhalation; BH, breath hold; EXH, exhalation; PIFR, peak inspiratory flow rate

novel concepts of powder excitation have been tested, and, so-called, active DPIs have been proposed (LiCalsi et al. 1999; Crowder 2004; Sosnowski et al. 2014; Boer et al. 2017).

12.6 Current and Future Concepts of Systemic Drug Delivery via Inhalation

Adapting lungs as a portal for systemic drug delivery has been considered already for a more than a decade (Agu et al. 2001; Patton et al. 2004), and the most attractive application has been related to treating diabetes (Mastrandrea and Quattrin 2006) although other concepts have been also proposed (Gonda 2006). The problem of methods to enhance drug absorption from the lung surface has been recently addressed [e.g., (Ghadiri et al. 2019; Islam and Ferro 2016)] and it will be not discussed in this work since it is focused mostly on technical aspects of inhalation delivery of systemic drugs. Historically, the absorption of nicotine or other neuroactive compounds via the respiratory system was realized by inhalation of smoke from burning leaves of plants. Modern electronic nicotine delivery systems, ENDS, i.e., e-cigarettes, continue this concept in a (probably) less harmful way (Sosnowski et al. 2018). As shown in Sect. 12.3 by using PTI parameter (Eq. 12.1), the efficient systemic drug delivery requires that the formulation and the inhaling device allow to obtain the cloud of extra-fine particles (1–3 μm) with the reduced amount of submicron particles that are poorly deposited and mostly exhaled (Fig. 12.1). Sosnowski and Kramek-Romanowska (Sosnowski and Kramek-Romanowska 2016) investigated ECs as a potential platform for drug delivery by inhalation. They showed that aerodynamic resistance of ECs is much higher than of any common passive DPI, and that ECs produce aerosol (mist, typically called "a vapor") composed of submicron droplets with the mass median aerodynamic diameter, MMAD, close to 400 nm. Such droplets are also visible in the exhaled vapor what confirms that inhaled aerosol is not considerably captured inside the respiratory tract. Numerical simulations presented by these authors confirmed that the maximum deposition efficiency of EC droplets is only ~35% for a very deep and slow inhalation of the vapor followed by a few-second breath hold.

The idea of aerosol generation by the principle analogous to ECs has been already developed for systemic drug delivery. The device known as Staccato® inhaler (Dinh et al. 2011) has been designed to produce aerosol particles with a neurological drug (Loxapine®) by utilizing the principle of thermally driven **sublimation** of the solid drug and the consecutive re-sublimation, i.e., vapor cooling and condensation to fine solid particles. In contrast to ECs, the particles have the MMAD of 2–3 μm (Berkenfeld et al. 2015) which assures their effective inhalation and deposition in distal lung regions. It must be noted though, that this kind of drug delivery platform is useful only for APIs which do not lose their therapeutic activity when heated up to more than 300 °C.

Systemic drugs can be delivered from passive DPIs. Another neurological drug has been granted FDA US marketing approval in the early 2019. Levodopa for Parkinson's disease is available here in form of DPI product Inbrija® (Acorda Therapeutics, Ardsley, NY). It delivers 42 mg of the drug in a single inhalation with the passive capsule-type DPI, with the operating principle similar to Turbospin/Podhaler. The drug-containing powder has been engineered as porous, low-density particles (Patel and Jimenez-Shahed 2018).

Insulin is the systemic drug most often considered in systemic delivery via the lungs. After unsuccessful earlier concepts of the Exubera® DPI [see, e.g., (Santos and Edelman 2014)], a new delivery system known as Afrezza® has been proposed more recently. Afrezza® (MannKind Corp., USA) consists of a passive DPI (Dreamboat® inhaler) and specially designed powder formulation with rapid-acting insulin. The powder is loaded into disposable cartridges which contain 4, 8, or 12 inhalation doses. The basic innovation of this product is a specially designed powder carrier based on fumaryl diketopiperazine, FDKP [Technosphere® particles (Rosenstock et al. 2015)]. The carrier FDKP particles are prepared by controlled crystallization at acidic pH that leads to the formation of aggregated nanocrystals. These aggregates form microparticles with MMAD of ~2.5 μm, and insulin is non-covalently incorporated within them (Pfützner et al. 2002; Pfützner and Forst 2005). The Dreamboat® inhaler is a compact passive DPI, i.e., it still requires a rather forceful inspiratory flow to fluidize and de-aggregate the powder into fine aerosol particles.

As mentioned earlier, the known problem in the effective use of any inhaler is patient "adherence" or "compliance", i.e., the capability of proper operation of the given device. According to the literature (Lavorini et al. 2008), more than 90% of patients use their inhalers incorrectly. This results in the poor control and increased costs of inhalation therapies (Makela et al. 2013). Therefore, the idea of using inhalers for delivery of systemic drugs must take into account this issue as well. Many novel inhalation systems which have been proposed or are still under development, seek an improvement in patients adherence to the inhalation therapy. Recent years have brought new ideas of **smart inhalers**, which have become an important contemporary trend in the inhalation therapy in general (George and Bender 2019; Kikidis et al. 2016). The most common developments in this field rely on pressure measurement in the inhaler mouthpiece or monitoring the sounds of air flowing through the inhaling device. These data allow to optimize the inhaler use by a better control over the dynamics of inspiration phase of the breathing cycle (Taylor et al. 2018). In this work, we mention only selected systems that have been proposed to improve the reproducibility of locally delivered dose of systemic drugs. They allow both to maximize the target dose and to reduce the mass of wasted drug, which is extremely important in the case of the potent or expensive molecules delivered by inhalation.

(i) **Dance 501®** (Aerami Therapeutics—earlier: Dance Biopharm) is the name for inhalation system composed of a hand-held mesh nebulizer electronically synchronized with breath and liquid formulation of rapid-acting recombinant human insulin (Fink et al. 2017; Zijlstra et al. 2019). Aerosol emission is done only during a short period during inhalation phase of breathing, where the flow

rate is in the range of 7–14 L/min. This assures a better aerosol penetration and optimizes the delivery of the dose to the target areas in the lungs.

(ii) **I-neb® AAD®** (Adaptive Aerosol Delivery—Philips Respironics) is a battery-operated vibrating mesh inhalation system. It can be used to deliver hormones, e.g., prostacyclin/iloprost (Ventavis®—Actelion Pharmaceuticals US, Inc.) for the treatment of pulmonary arteliar hypertension, scleroderma and Raynaud syndrome. The device has electronic interface that measures pressure changes in the airflow and then sets the duration of aerosol pulse to deliver the drug only in the first phase of inspiration. The system also continually adapts aerosol delivery to changes in breathing pattern, which allows to maximize the delivered dose and minimize the amount of wasted drug (Denyer et al. 2004; Denyer and Dyche 2010).

(iii) **Akita® Jet** (Vectura) is the electronic system used to optimize drug delivery from the jet nebulizer. The electronics guides the patient to inhale with a pre-set inspiratory flow rate, and at volume and time that helps to achieve the precise and targeted drug delivery to the lungs (Fischer et al. 2009).

(iv) **AERx®** (Aradigm) is an innovative drug delivery platform generating fine mist aerosol and providing the breath control technology. It is a hand-held device which allows to deliver drug dose typically in one or two breaths. The drug is packed in the single-use AERx Strip which contains the mesh in the form of the array of laser-machined nozzles. The AERx device initiates the release of the aerosol early during the person's breath, and then controls the inhalation rate to ± 30 LPM, which helps to target the aerosol to deep lung regions. The aerosolization process is gentle, so it does not degrade sensitive molecules (e.g., proteins). AERx was successfully tested in intrapulmonary hormone delivery (Davison et al. 2005). It is also claimed that the system may offer the platform for personalized aerosol therapy (Cipolla et al. 2010).

Besides these complete inhalation platforms/systems, other interesting electronic add-on devices have been proposed to better target the inhaled drugs:

(v) **Bluhale®** add-on electronic accessory has been proposed to improve the compliance of using Dreamboat® DPI in Afrezza®—the inhalable insulin product described earlier. Bluhale® is a small electro-acoustic device that measures the amount of insulin taken via the sound generated during use. This indicates the pressure of inhalation, flashing a red light if the Afrezza® is inhaled improperly and a green light if the inhalation is done correctly.

(vi) **Digihaler®** (available with AirDuo® and ProAir® drugs—Teva Pharmaceuticals) contains a built-in electronic module that records and stores information about using the DPI (Airmax® inhaler). Digihaler® may be used with a mobile application where it transmits the information.

(vii) **FindAir®** (FindAir, Poland) is an electronic module attached to the top of pMDI. It contains sensors transmitting the information regarding the inhaler use to the mobile phone app. This allows to better control the use of the inhaler by a patient and to correlate the symptoms and therapeutic effectiveness of the drug with the frequency of drug administration from the pMDI.

Several other technical solutions of smart inhaler technology may be found in the sources on the Internet, e.g., Hailie® sensor (Adherium, NZ), Propeller sensor (Propeller Health, Madison, WI), 3 M™ Intelligent Control Inhaler (3 M Corp.), myAirCoach system (The myAirCoach Consortium). This list certainly will be extended in the near future.

12.7 Summary and Outlooks

Drugs targeting during inhalation is challenging since it requires the availability of aerosol with a defined particle size distribution that is formed at a certain time-window of the patient's inspiration. It is of special importance in systemic drug delivery via the respiratory system when the deposition of drug particles in the alveoli should be maximized with the simultaneous reduction of drug delivery to the upper airways and fast-cleared ciliated bronchi.

The best strategies to deliver inhaled systemic drugs effectively is to apply them as extra-fine aerosol particles (between ~1 and ~3 μm) precisely dosed at the initial phase of the inhalation which is done in a controlled way. Primary drug particles of this size may be obtained using "particle engineering" approach, and they may be formed, for instance, as aggregated nanostructures which disintegrate on the lung surface. This will increase the mass exchange rate in the lungs, hence allow a faster drug absorption from the lung surface to the circulation. Particle properties may be also adjusted in a way which extends their residence time in the alveolar region by promoting the specific interactions with the lung surfactant. The required timing of aerosol supply can be enhanced by electronic systems of flow monitoring during inspiration which should help the patient to do the inhalation correctly. Such systems become available nowadays and may be adapted also for inhalable systemic drugs. All these reveal that understanding of various technical requirements of aerosol delivery to the pulmonary region is indispensable for the efficient delivery of inhaled drugs to the blood.

Important Notes

- Systemic drug delivery requires aerosol targeting with simultaneous reduction of drug deposition in other locations. Mathematical modeling and calculation of Pulmonary Targeting Index (PTI) for given inhalation dynamics helps to determine such conditions.

- Adequate drug quality is obtained by simultaneous adjustment of the initial drug formulation (physicochemical properties), inhaling device (aerosolization principle), and patient adherence to the proposed method of drug delivery. Each factor needs a careful elaboration by technical (engineering) approach.
- Novel inhalation systems allow to obtain a better patient compliance in drug delivery by inhalation. Many of them have been initially developed for dosing of pulmonary medicines, but they may be easily adapted to systemic drugs delivered via inhalation.

Questions for Future Research

- **How to enhance the efficiency of systemic inhalation delivery?** Systemic inhalation delivery requires aerosol targeting with simultaneous reduction of drug deposition in other locations. Better understanding of drug targeting in the respiratory tract and particle interaction with the lung surface to obtain high efficacy of systemic drugs is still required in order to further enhance the efficiency of systemic delivery via inhalation methods.
- **How to facilitate the translation of inhalation technologies into clinical practice?** Many different inhalation systems have been developed for dosing of pulmonary medicines, but the use of those systems sometimes may not be easy to patients. More sophisticated dosing devices are needed to be developed in future research for improving patient compliance.
- **How research on systemic inhalation delivery can be applied to the execution of biogerontological interventions in practice?** Over the years, systemic inhalation delivery of various medicines are being developed (e.g., hormones, neuro-active drugs, etc.). Unfortunately, till now use of related technologies in anti-aging medicine is limited. Scientifically based concepts of effective inhalation systems dedicated to the cases of coexisting respiratory tract diseases (e.g., COPD) and systemic disorders are needed for future research. This issue is of high importance for older patients, particularly because aging is a systemic phenomenon that may lead to multiple disorders concomitantly.

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Glossary

Aerosol Two-phase system composed of fine particles or liquid droplets suspended in the air.

Dry powder inhaler A device which converts the powder into inhalable aerosol. Usually driven by patients inspiratory flow (passive DPI), but can be assisted by external power (active DPI).

Fine particles Inhalable aerosol particles with the aerodynamic size (diameter) below 5 μm. Extra-fine particles are smaller than approximately 3 μm, and they are considered most useful in the systemic delivery of inhaled drugs.

Lung deposition The process of drug particle settling on the surface of the respiratory system. It occurs due to several mechanisms which depend mainly on particle size and density (particle impaction, gravitational sedimentation, diffusion).

Lung surfactant A natural surface-active compound present in the alveolar region of the respiratory system. The surfactant forms a thin layer on the top of pulmonary liquid and acts as a barrier for inhaled particles that are deposited in this region.

Marangoni effects Liquid flows driven by surface tension gradients on the air/liquid interface. In the lungs they may occur on the alveolar surface due to the local variations of the lung surfactant concentration.

Nebulization An atomization process of liquid medication by converting into inhalable aerosol via various methods (jet, ultrasonic or vibrating mesh nebulizers).

Powder fluidization The process of powder lifting by air. It is often associated with deagglomeration of powder grains which helps to obtain inhalable aerosol.

Pressurized metered dose inhaler A device where drug is atomized due to decompression of a drug/carrier mixture released from a pressurized can via a dosimetric valve.

Smart inhalers Inhaling devices with built-in (or attached) electronic accessories for the measurement or control of airflow parameters and other conditions of inhaler use. They often also offer a visual or sound feedback to the user, and may be connected to mobile phone applications or computer programs.

Sublimation A process of material transformation from solid state directly to gas state (vapor). Re-sublimation is the reverse process.

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Part V
Modification of Carrier Properties for Systemic Delivery

Chapter 13
Use of Electrospinning to Enhance the Versatility of Drug Delivery

Marko N. Živanović

Abstract In Sections II, III, and IV, authors have introduced various chemical, biological, and physical strategies for systemic delivery, and have also discussed principles and parameters to be considered when drug carriers are designed. Although these strategies may facilitate the efficiency of systemic delivery per se, practical issues (e.g., repeated dosing) involved in clinical practice may not be fully addressed. To reduce the number of dosing, one commonly used strategy is to incorporate a carrier into a polymer matrix that enables sustained release of the carrier. By implantation of the matrix into a body site, sustained release of the drug carrier (or drugs per se) can reduce the number of dosing required. Electrospinning is one of the techniques widely applied to engineer such matrix. This chapter will give an overview of the possibilities to couple experiments with simulations in electrospinning, and examine, both from the experimental and numerical side, the ways to apply this process to drug delivery and to optimize the process in creation of microfibers, which can subsequently be used for applications such as sustained drug release and tissue engineering in anti-aging medicine. Because a number of electrospinning techniques have already been recognized as an effective method of drug application and delivery, the use of biodegradable polymers in the creation of nanofibers prepared for drug release enables application within the organism with precisely estimated action dynamics.

Keywords Drug delivery · Electrospinning · Finite element method · Neural networks

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13.1 Introduction

Electrospinning is technique that is used to produce micro and nanofibers using the process of polymer elongation based on electrostatic field. Because of a better ratio surface/volume, nanofibers have better mechanical properties (high porosity and permeability, ability to retain electrostatic charges…) than microfibers (Stepanyan et al. 2016). This is a multi-phase and multi-physics process which involves couple of phenomena–mass and heat transfer and diffusion, evaporation, **electrohydrodynamics**, etc. Main parameters that govern the whole electrospinning process are usually divided into three groups: solution parameters (solvent system, polymer concentration, conductivity, etc.), process parameters (applied voltage, flow rate, collector design, distance between needle tip and grounded collector), and the ambient parameters (relative humidity and temperature). All these parameters control the continuity, uniformity, and fibers diameter. Their application could be found in the fields of biotechnology and biomedical, environmental engineering, defense, and others that explore the advantageous mechanical characteristics of nanofibers Karra (2007). Some studies have been performed to use nanofibers for cancer diagnosis (Ke et al. 2015). Due to their physicochemical properties, electrospun nanofibers have also been explored as a tool for the architecture control of cardiovascular **tissue engineering** (Oh and Lee 2013). In addition, nanofiber meshes can act as protective clothing from biological and chemical agents due to their excellent potential for filtration (Gorji et al. 2017). One example of successful application of electrospinning is the creation of **scaffolds** in tissue engineering, particularly for the development of cardiovascular scaffolds. Currently, there are numerous research attempts in improving the surgical methods for coronary artery bypass grafts, and the tissue engineering route provides highly beneficial alternatives for the existing surgical methods. It is essential that the produced scaffold meets desired characteristics. As polymer-based fibers are the product of electrospinning, the main aim in this process is to optimize the fiber formation for the best possible scaffold development. Another possibility of using electrospinning is to create scaffolds in order to control drug release (Ye et al. 2019).

A typical electrospinning setup only requires a spinneret (syringe pump, syringe, and a flat tip needle), a high-voltage power supply, and a collector plate, which is usually a conductor. Fibers are formed from a polymer and solvent solution, and the properties of these materials reflect the fiber production. The solution is passed through a positive needle electrode toward a negative collector electrode, with a large electric field between the two. The negative electrode can either be a flat collector for initial fiber testing, as seen in Fig. 13.1a, or it can be a rotating collector, collecting the fibers in a cylindrical scaffold shape, as seen in Fig. 13.1b.

Many studies have been conducted to fully understand the electrospinning process, but they are mostly experimental and only a few mathematical models have been created to describe the mechanism of the process. Trial and error approach that is common in experiments used to determine parameters can be time-consuming and lead to wasted chemicals. As a result, numerical simulation of electrospinning

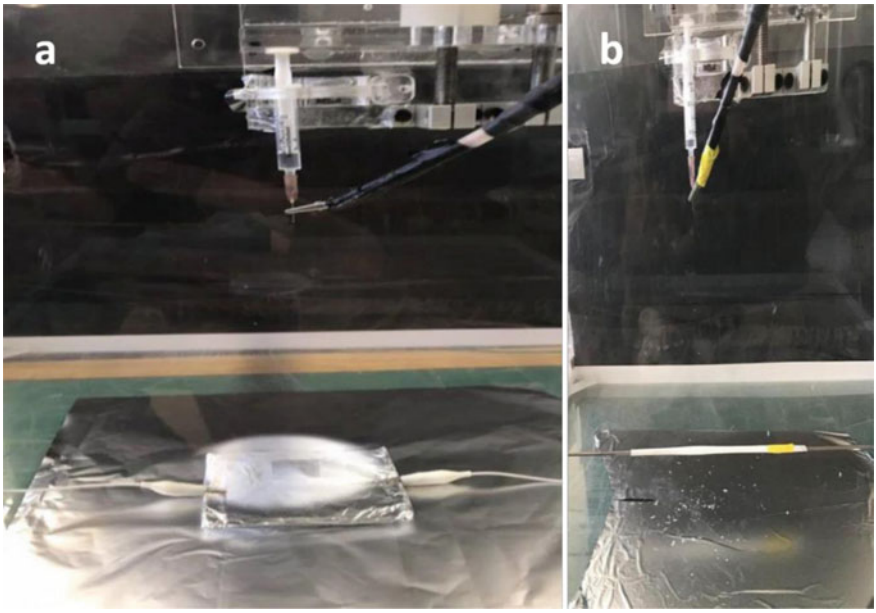

Fig. 13.1 Electrospinning setup with flat **a** and rotating **b** negative electrode

process, including prediction of the shape of the jet traveling from the needle to the collector will be useful in terms of optimization of the electrospinning process. This would reduce the trial and error approach, have beneficial effect on decreasing the amount of waste management, amount of chemicals, equipment maintenance costs, etc.

Chemical modification of the used polymer is required for the creation of electrospun nano and microfibers that are capable of carrying drug or another useful biomaterial. This modification can be performed by electrospinning a polymer already mixed with the drug or by applying the drug to an already formed fiber. The basic requirement of these procedures is (bio)compatibility of the drug and the polymer, as well as the drug and the solvents used. Today the possibilities of drug application using electrospun fibers are being explored using anti-cancer drugs, enzymes, saccharides, cytokines and growth factors, antibiotics, and more. For example, **polycaprolactone** (PCL) can be used quite successfully for controlled ampicillin releasee Sultanova et al. (2016) combination of PCL and **polyethylene glycol** (PEG) for incorporation and release of salicylic acid Yu et al. (2014), zein protein fibers for the release of hydrophobic ketoprofen drug (Yu et al. 2013), and many others. Another of many options is the application of drug-carrying nanoparticles on fibers. One of the simplest examples is the application of silver nanoparticles incorporated in chitosan polymer for wound treatment, i.e., for their rapid healing (Lee et al. 2014). The created fibers exhibited significant antibacterial activity against *P. aeruginosa* and methicillin-resistant *S. aureus* compared with unmodified fibers. Radmansouri

et al. showed loading of electrospun chitosan/cobalt ferrite/titanium oxide fibers carrying doxorubicin for hyperthermic tumor cell treatment and controlled drug release (Radmansouri et al. 2018).

13.2 Methodology for Coupling Between Experiments and Numerical Modeling

To the precise experimental measures, researchers are trying to create general simulation models of the electrospinning process. With these models, additional results can be obtained without the need to repeat experiments that are costly in time and materials consumption. However, electrohydrodynamic of the complete electrospinning process that includes both the steady jet and instability region is extremely complex. As a result, various mathematical models have been proposed till today to simulate the process of electrospinning, but none of them is steadfast to the theory (Rafiei et al. 2012). Taylor established the "leaky dielectrical model" that suits only few liquids with finite conductivity. Furthermore, Roozemond used and combined the "leaky dielectric model" and the "slender-body approximation" for modeling electrospinning viscoelastic jets (Kornev 2011). Some continuum simulations have examined only the steady jet region of the electrospinning with adequate equations for that regime–conservation of mass, the conservation of charge, the equation of motion, and the electric field balance (Gadkari 2017; Feng 2003). Another paper by Šušteršič et al. simulated electrospinning process and for that used FEM and FVM approaches with the commercial ANSYS and in-house **PAK software**. They showed that used computational approaches could be used to implicitly determine the homogeneity of the obtained electrospun fibers based on jet shape (Šušteršič et al. 2018).

Mathematical modeling of experimental setup regarding the electrospinning process usually can be divided into two main approaches:

1. **Finite element method** that solves the modified Navier–Stokes equations under the influence of electric field and the interface between the two fluids is determined by using the volume of fluids (VOF) method.
2. **Discrete models** such as Reneker, which assumes the jet as a system of beads connected by viscoelastic elements. Each bead is submitted to four forces: the Coulombs Force, the Viscoelastic Force, Electric Force Surface, and Tension Force.

In this chapter, we will focus on the second approach of creating discrete model of Reneker, applying Newton's second law and writing a series of differential equations to define motion of each bead, projected on x, y and z axis. Some models in literature were based on equations with mistakes or used coefficients with random values without explaining their meaning. There is no consistent explanation in definitions of equations, and most complete papers lacked details, especially on how to model the transition between the straight and the whipping phase of the jet. This lead to the main motivation to create a detailed and consistent simulation of the polymer jet

between the needle and the collector. We also compare it to the current state of art and discuss the influence of several parameters on the electrospun fibers.

Using the aforementioned Renerker's model, the jet is considered as a slender body, not affected by radial effects, and it is modeled as a system of beads connected by viscoelastic elements, with the same mass and the same charge and connected by viscoelastic elements (Fig. 13.2). The background electric field is assumed to be axial and uniform, all the properties are constant, the effects of gravity and air drag are negligible (Vught 2010). It must be emphasized that Reneker did not take under

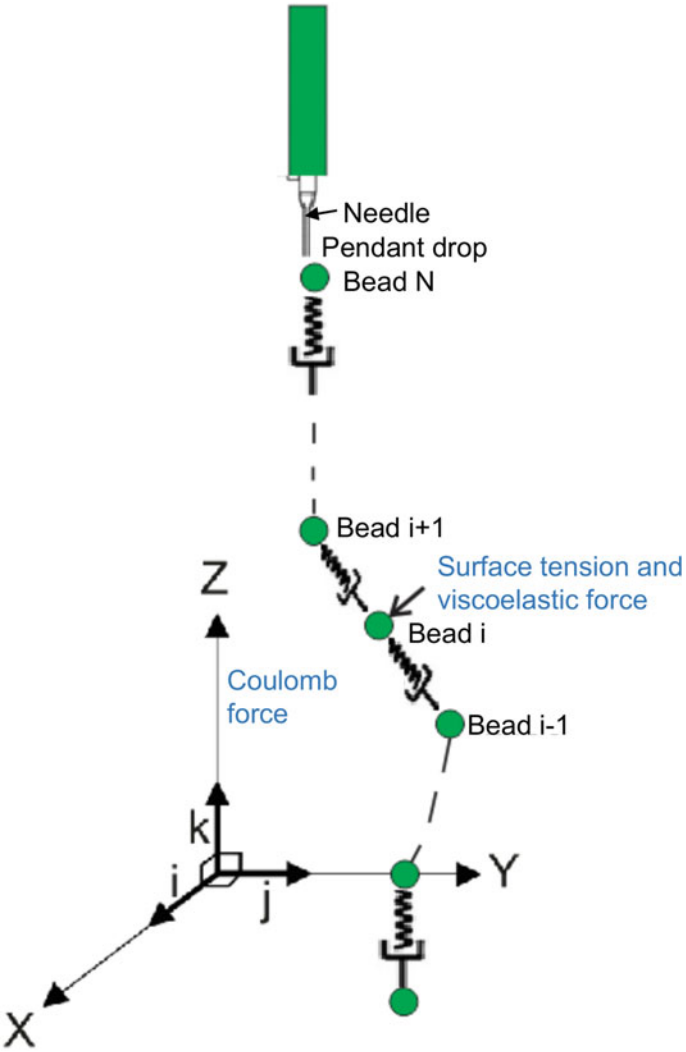

Fig. 13.2 Schematic representation of the jet during electrospinning

consideration the evaporation and solidification of the polymer and did not simulate the jet initiation zone and (Karra 2007). Other scientists have used a discrete model; they did not neglect the gravity nor the air drag (Lauricella et al. 2016). A detailed explanation of the model could be found in (Ferouka et al. 2018). In order to describe the movement of the jet, the trajectory of each bead is examined. The position of bead i is given by $r_i = \mathbf{i}x_i + \mathbf{j}y_i + \mathbf{k}z_i$ where i, j, and k are the unit vectors of the cartesian coordinate system's axes, x, y, and z. Each bead is submitted to four forces (Karra 2007; Feng 2003; Vught 2010). The first one is the Coulomb force, which is exerted by all the other charged beads and depending on their electrical charge and the distance that separates them from bead i.

$$F_{ci} = \sum_{\substack{j=1 \\ j \neq i}}^{N} \frac{e^2(r_i - r_j)}{l_{ij}^3} \tag{13.1}$$

where $l_{ij} = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2 + (z_i - z_j)^2}$ is the distance between the bead i and the bead j. The second force is the electric force. It is derived from the potential difference between the pendant drop and the collector and only applied in the z-direction.

$$F_{Ei} = \frac{-eV_0}{h}\boldsymbol{k} \tag{13.2}$$

The third one is the viscoelastic force, which is due to the stress σ. This stress acts on an element between two beads. The viscoelastic force resulting on this bead equals to the stress multiplied by the cross area. The force acting on bead i results from the sum of the force generated by bead $i + 1$, that is defined to be positive and bead $i - 1$, acting in opposite direction, thus negative.

$$\mathrm{F}_{VEi} = \pi a_{i,i+1}^2 \sigma_{i,i+1} \frac{r_{i+1} - r_i}{l_{i,i+1}} - \pi a_{i-1,i}^2 \sigma_{i-1,i} \frac{r_i - r_{i-1}}{l_{i-1,i}} \tag{13.3}$$

where σ is the stress pulling the bead I to bead $i + 1$ or $i - 1$. This stress is characterized by Maxwell's model of a spring-damper system:

$$\frac{\mathrm{d}\sigma}{\mathrm{d}t} = G\frac{\mathrm{d}l}{l * \mathrm{d}t} - \frac{G}{\mu}\sigma \tag{13.4}$$

In addition, $a_{i,i+1}$ is cross-sectional radius of the filament formed by the bead i and $i + 1$. Here it is worth noting that to calculate a_{ij}, we use the equality settled by the conservation of the volume $\pi a_0^2 L = \pi a^2 l$. The last force is the surface tension force, which is given as

$$F_{\mathrm{sf}i} = -\frac{\alpha\pi a_{av}^2}{\sqrt{x_i^2 + y_i^2}} K_i(|x_i|\mathrm{sign}(x_i)i + |y_i|\mathrm{sign}(y_i)j) \tag{13.5}$$

where α represents the surface tension coefficient (N m^{-1}) of the polymer; $K_i = \frac{1}{R}$ is the jet curvature calculated using the coordinates of beads $(i-1)$, i, and $(i+1)$, and the definition of the curvature for three points (Vught 2010):

$$\begin{aligned} R =& (((x_i - ((y_{i-1} - y_i) * (x_{i+1}^2 - x_i^2 + y_{i+1}^2 - y_i^2) \\ &+ (y_i - y_{i+1}) * (x_{i-1}^2 - x_i^2 + y_{i-1}^2 - y_i^2))/(2 * ((x_{i+1} - x_i) * (y_{i-1} - y_i) \\ &- (x_{i-1} - x_i) * (y_{i+1} - y_i))))^2 \\ &+ ((y_i - ((x_i - x_{i-1}) * (x_{i+1}^2 - x_i^2 + y_{i+1}^2 - y_i^2) \\ &+ (x_{i+1} - x_i) * (x_{i-1}^2 - x_i^2 + y_{i-1}^2 - y_i^2))/2 * ((x_{i+1} - x_i) * (y_{i-1} - y_i) \\ &- (x_{i-1} - x_i) * (y_{i+1} - y_i))))^2)^{1/2} \end{aligned} \tag{13.6}$$

and the meaning of "sign" is as follows:

- $\mathrm{Sign}(x) = 1$, if $x > 0$
- $\mathrm{Sign}(x) = -1$, if $x < 0$
- $\mathrm{Sign}(x) = 0$, if $x = 0$

Newton's second law gives us then:

$$m\frac{\mathrm{d}^2 r_i}{\mathrm{d}t^2} = F_{\mathrm{c}i} + F_{\mathrm{E}i} + F_{\mathrm{VE}i} + F_{\mathrm{sf}i} \tag{13.7}$$

And (13.5) projected on x, y, and z leads to the equations of motion for each bead

$$\begin{aligned} \frac{\mathrm{d}^2 x_i}{\mathrm{d}t^2} = \frac{1}{m} * \Bigg(& \sum_{\substack{j=1 \\ j \neq i}}^{N} \frac{e^2}{l_{i,j}^3}(x_i - x_j) + \frac{\pi a_{i,i+1}^2 \sigma_{i,i+1}}{l_{i,i+1}}(x_{i+1} - x_i) \\ & - \frac{\pi a_{i-1,i}^2 \sigma_{i-1,i}}{l_{i-1,i}}(x_i - x_{i-1}) - \frac{\alpha\pi a_{av}^2}{\sqrt{x_i^2 + y_i^2}} K_i |x_i| \mathrm{sign}(x_i) \boldsymbol{i} \Bigg) \end{aligned} \tag{13.8}$$

$$\frac{\mathrm{d}^2 y_i}{\mathrm{d}t^2} = \frac{1}{m} * \left(\sum_{\substack{j=1 \\ j \neq i}}^{N} \frac{e^2}{l_{i,j}^3}\left(y_i - y_j\right) + \frac{\pi a_{i,i+1}^2 \sigma_{i,i+1}}{l_{i,i+1}}(y_{i+1} - y_i) \right. \\ \left. - \frac{\pi a_{i-1,i}^2 \sigma_{i-1,i}}{l_{i-1,i}}(y_i - y_{i-1}) - \frac{\alpha \pi a_{av}^2}{\sqrt{x_i^2 + y_i^2}} K_i |y_i| \mathrm{sign}(y_i) \boldsymbol{j} \right) \tag{13.9}$$

$$\frac{\mathrm{d}^2 z_i}{\mathrm{d}t^2} = \frac{1}{m} * \left(\sum_{\substack{j=1 \\ j \neq i}}^{N} \frac{e^2}{l_{i,j}^3}\left(z_i - z_j\right) + \frac{\pi a_{i,i+1}^2 \sigma_{i,i+1}}{l_{i,i+1}}(z_{i+1} - z_i) \right. \\ \left. - \frac{\pi a_{i-1,i}^2 \sigma_{i-1,i}}{l_{i-1,i}}(z_i - z_{i-1}) - \frac{e V_0}{h} \right) \tag{13.10}$$

Jet's trajectory is simulated using the software MATLAB R2017b. Differential equations of motion are transformed into a state-space notation which consists of first order differential equations that are used as input for MATLAB's ode45 solver. All position velocity and tension coordinates of the system are transformed to a new state vector. The size of this state vector depends on the number of beads present in the system.

Another example of using mathematical modeling in electrospinning optimization is the use of **neural networks**. With the use of a series of biocompatible polymers and solvents (polycaprolactone–PCL and polyethylene glycol–PEG), solutions were tested in various electrospinning settings in order to produce microscale fibers. The production of these fibers is directly related to the electrospinning parameters in use, hence the importance of optimizing this process. Upon simple fiber optimization, the electrospinning setup was progressed to include rotational collection for scaffold development. A series of scaffold samples were built from various solutions in different parameter conditions. The scaffolds were analyzed with scanning electron microscope images for fiber diameter measurement. Furthermore, dynamic loading, creep, and stress relaxation tests were conducted in order to interpret the material properties of each sample. Of these samples, those with least permanent deformation after loading were recreated for cell seeding attempts, which is the current state of our project.

Commercially available Matlab R2017a was used in the study for the creation of the neural network. For prediction electrospinning diameter, supervised multi-layer perceptrons network (MLP) and Back Propagation algorithm with linear activational functions were used. The data set was split into training (13 samples) and test (3 samples) subset. Ratio of PCL and PEG, flow rate (ml/h), voltage (kV), time (min),

rpm were selected as input factors affecting the diameter of the electrospinning fibers. The output data was the mean diameter of the collected electrospinning fibers (μm). Trial and error approach, throughout varied the number of layers and number of nodes in hidden layer(s), was used to train neural network. Number of layers were varied from 2 to 10 and number of neurons from 4 to 10 per layer. The criteria to choose the "best MLP model" were minimal test error and maximum R2. After the training process, prediction ability of the developed network was examined by external validation with the unseen three test samples. Due to the small number of experiments available, all 13 samples were used as the training dataset, and the test data set was used by interpolating the experimental dataset.

13.3 Results of Experimental and Numerical Modeling

The model is validated firstly by comparing obtained results with those of Vught (Vught 2010). Since in this report the influence of each parameter on the lateral displacement of the beads on the collector is examined, we have performed the same examination. The parameters investigated were number of beads (10–100), viscosity, surface tension coefficient, elastic modulus, initial jet radius, and applied voltage. We compared then the results to those of Dasri (Table 13.1) (Dasri 2011), due to the similar modeling idea. The model is also partially compared with (Zeng et al. 2009). For fiber diameter and density analyses the use of scanning electron microscopy (SEM) is required. ImageJ uses image processing techniques to convert SEM fiber images into a purely black and white image. The given image scale in micrometers is used to alert the program of the conversion distance in pixels. The plugin, DiameterJ runs algorithm to determine the average diameter of the fibers in the image. Diameter results can be linked back to the type of solution used and the parameters in place (Figs. 13.3 and 13.4).

Optimal neural network with the minimum root mean square (RMS) was achieved using five layers with six neurons per layer and only in the sixth layer sigmoid function was used (Fig. 13.5). One hidden layer is normally adequate to provide an accurate prediction and more than one hidden layer can be used for modeling complex problems. MLP was tested with a set of test data. Description of the inputs for the training data set is given in Table 13.2.

Regression coefficients for the training dataset and test data set were 1 and 0.99962, respectively, (Fig. 13.6), which indicated that we are on a good tract to find optimal model of electrospinning diameter prediction. The results showed that the predicted and target values of the fiber diameter were very highly correlated both in the training and the test dataset. Individual values for both datasets are given in Table 13.3.

Table 13.1 Comparison of our results and investigated similar models

References	Investigated parameter	Results from the reference	Our results
Vught (2010)	Number of beads (10–100)	Displacement increased with the number of beads	Displacement increased with the number of beads
	Effect of the viscosity	Viscosity increased by 10^5, the lateral displacement of the beads does not seem to vary a lot	Viscosity increased by 10^5, the lateral displacement of the beads does not seem to vary a lot
	Surface tension coefficient	No remarkable difference in lateral displacement	Increase of coefficient from 700 g/s^2 to from 900 g/s^2, and also the decrease to 300 g/s^2, shows no significant differences in displacement
	Elastic modulus	We observe that the beads tend to form clusters when the elastic modulus is high but that when the elastic modulus is smaller, the beads tend to spread	Vught's paper shows that if G is increased to 107 g/cms^2 (from 106) the lateral displacement is smaller when if it is decreased to 105 g/cms^2 the beads will be more dispersed on the collector. In turn we obtain the following results
	Initial jet radius	Radius is increased to 200e^{-4} cm (from 150e^{-4}) resulting in larger lateral displacement and when decreased to 100e^{-4} cm, displacement is smaller	Small number of beads does not allow us to observe a tremendous difference. However, we can say that the beads are a bit more dispersed on the collector when the initial radius is higher
	Voltage applied between the needle and the collector	If the voltage is drastically increased (from 10,000 to 50,000 V, meaning 5 times bigger) the displacement of the beads on the collector becomes slightly larger when it undergoes a small decreasing (from 10,000 to 5000 V) the displacement stays approximately the same	We observe that increase in voltage affects the jet to get straight and have a smaller lateral displacement, but according to other studies (Reneker 2000), the length of the straight part of the jet increases when the applied voltage is increased

(continued)

Table 13.1 (continued)

References	Investigated parameter	Results from the reference	Our results
Dasri (2011)	Voltage applied between the needle and the collector	Increased from 500 to 1000 V then 1500 V and finally to 2 kV. He observed the increasing of the applied voltage accelerates the beads and make them achieve the collector faster	We observed the same effect: when we kept the same parameters and varied the voltage we observed that our system of 50 beads (same as in literature) tend to form a loop that is, at the same time, closer to the collector when the voltage is higher
	Impact of initial radius	Lateral displacement of the beads on the collector was larger when the initial radius is increased	The program met a problem of integration
	Elastic modulus	Difference is slight when modulus is increased up to 100 times	Difference is slight when modulus is increased up to 100 times
	Surface tension	Did not have a significant influence on the beads' trajectory	The trajectory remained approximately the same if we change the surface tension coefficient from 300 to 900 g/s^2
	Initial radius of the jet	Increasing the jet radius to 500e^{-4} cm leads to a larger area of incoming beads on the collector plate, while decreasing the radius to 200e^{-4} cm obtains a smaller area	Met some problems of integration when we tried to put an initial radius of 500e^{-4} cm
	Viscosity	Using $\mu = 104$ g/cm or $\mu = 107$ g/cm, the trajectory does not affect the trajectory	Obtained trajectory is approximately the same
Zeng et al. (2009)	Voltage applied between the needle and the collector	7.5 to 17.5 kV resulted in a slight increase from 101 to 138 nm and to a slight decrease from 138 to 123 nm when the voltage was increased from 17.5 to 25 kV	Similar results, the applied voltage did not affect a lot the value of the fiber's diameter
	Distance between the electrodes	Distance increased from 10 to 30 cm, resulting in decreased diameter of the fibers from 134 to 10 nm	Same tendency is observed

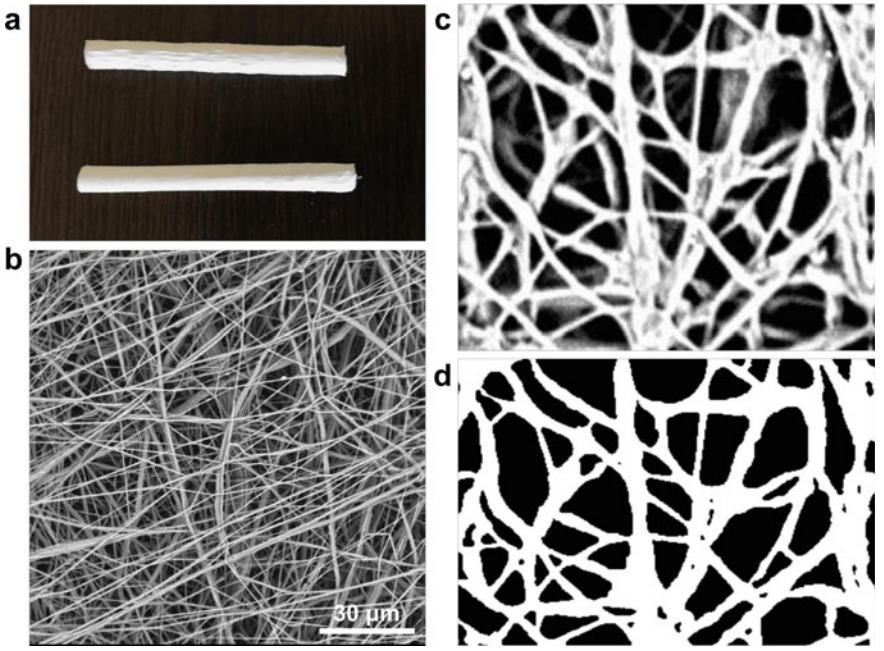

Fig. 13.3 **a** Scaffold obtained on rotational collector; **b** SEM image, 2150X magnification of scaffold; **c** Section of SEM image of 2150X magnification; **d** Binary image of SEM section

13.4 Summary and Outlooks

The electrospinning methodology for the creation of drug-eluting biodegradable scaffolds is not sufficiently developed, and the assumption is that it will be exploited significantly in the future. The advantage of electrospinning lies in the simplicity, cost effectiveness, and technical variability in the production of scaffolds. The development of electrospun scaffolds for antimicrobial action may be of particular importance. Specifically, in situations where skin areas that are significantly affected by microorganisms are treated, electrospun developed scaffolds carrying antibiotics can be used for very local administration. Also, anti-aging agents focusing on wrinkles and skin imperfections can rely heavily on hyaluronic and chitosan prepared electrospun scaffolds. This type of scaffolds is biodegradable, significantly absorbs water, and helps in re-hydration of the skin. The disturbance of the water balance of the skin causes a significant number of pathological conditions. Burned skin requires special care during regeneration. Biodegradable scaffolds can make a significant contribution through three mechanisms: physical protection of burned skin, continuous hydration, and elution of specific drugs in the treatment of the burned skin. Treatment of the skin, the largest human organ, using electrospun fabricated scaffolds may also include support for affected tensile strength, elasticity, and problems with collagen production. In addition to skin treatment, scaffolds prepared in this

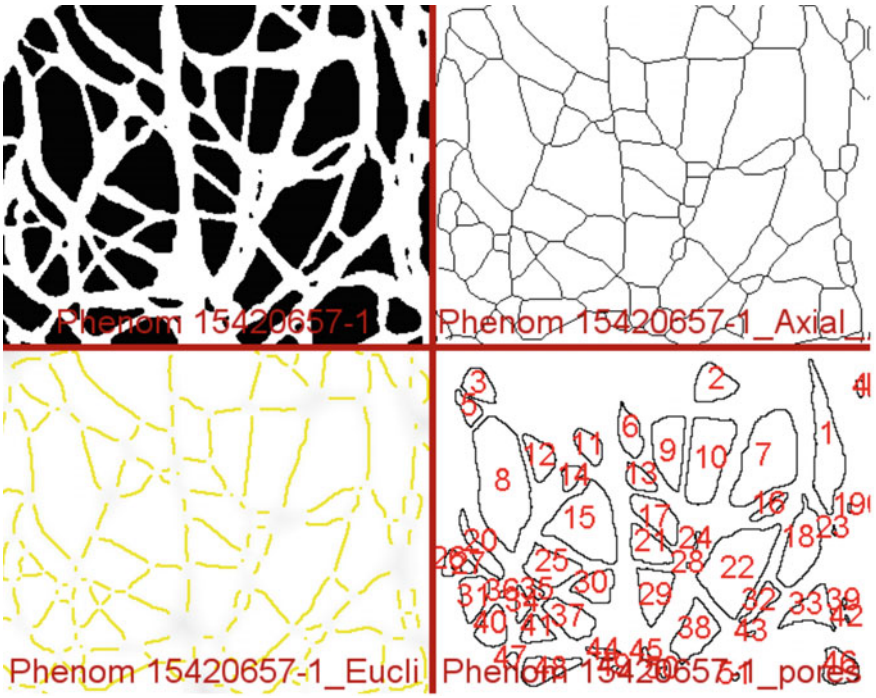

Fig. 13.4 Diameter J tool processing images of sample

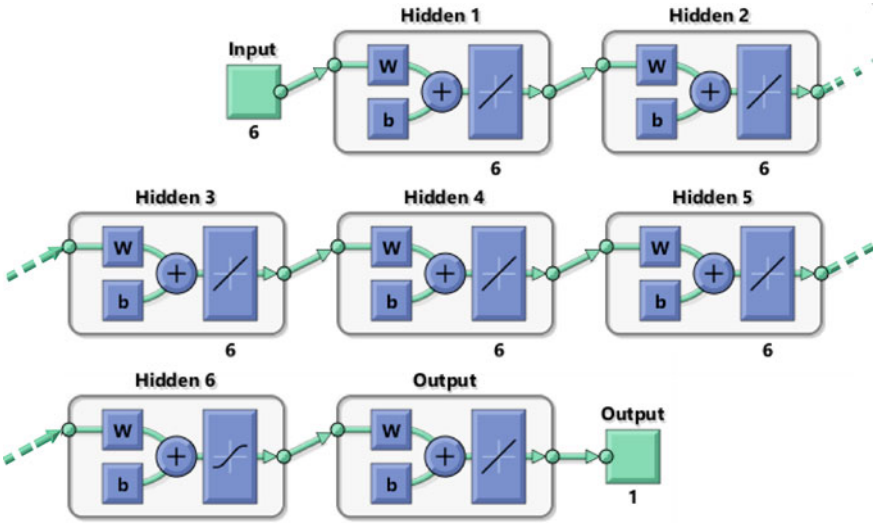

Fig. 13.5 Constructed optimal neural network

Table 13.2 Experimental training dataset description

Sample number	PCL	PEG	Flow rate (mL/h)	Voltage (kV)	Time (minutes)	Rpm
1	1	0	0.6	15	30	140
2	1	0	1.4	10	15	120
3	1	0.2	0.5	16.5	10	140
4	1	0.2	0.5	16.5	15	160
5	1	0.2	0.5	16.5	120	140
6	1	1	0.6	12	60	140
7	1	1	0.6	12	90	140
8	1	1	0.8	16	120	140
9	1	1.2	0.2	12	150	100
10	1	1.2	0.4	12	150	100
11	1.2	1	0.4	12	60	160
12	1.5	1	0.6	12	150	100
13	1.5	1	0.6	12	40	60

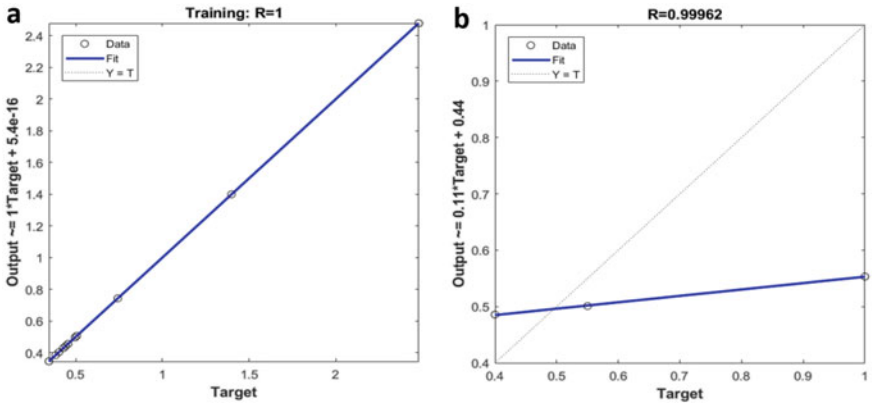

Fig. 13.6 **a** Regression for the training dataset; **b** Regression for the test dataset

way can be used to coat parts or whole organs. Namely, when a physically induced organ trauma occurs, coating with a biodegradable drug-carrying film can be of great support in assisting organ regeneration in the required period. Implantation of the electrospun structures into the bone, blood vessels, and other organs can significantly promote tissue regeneration and healing.

Regarding the electrospinning simulations, developed models will help in understanding the fundamental physical phenomena that govern the process of electrospinning and the same concepts of simulations in the service of biomaterials could be

Table 13.3 Predicted and experimental values of fiber diameters for the training and test dataset

Dataset	Sample number	Experimental diameter mean (μm)	Predicted diameter (μm)
Training	1	1.3981	1.3981
	2	0.3833	0.3833
	3	0.742	0.7420
	4	0.4279	0.4279
	5	2.4775	2.4775
	6	1.3981	1.3981
	7	0.4414	0.4414
	8	0.4556	0.4556
	9	0.3451	0.3451
	10	0.5054	0.5054
	11	0.4279	0.4279
	12	0.4023	0.4023
	13	0.4953	0.4953
Test	14	1.000	0.5531
	15	0.400	0.4856
	16	0.555	0.5007

extended to other experimental setups (electrospraying, dry spinning, melt electrospinning, etc.). Used model allowed us to evaluate the influence of some parameters (viscosity, initial radius, surface tension, elastic modulus…) on the trajectory and the displacement of the beads on the collector. Except some problems of integration, most of our results show good agreement with reports from literature. This will allow us in further studies to determine the optimal parameters to obtain optimal fibers without needing to repeat costly experiences. Further studies will also consist of modifying the script in order to obtain a more realistic jet that goes from straight to whipping jet.

A good understanding of the whole process of nanofiber production is necessary for further application in biomedicine. Parameter optimization enables us to adequately combine all the necessary factors on which fiber composition, mechanical properties, the ability to incorporate the drug into fibers and desorption into the affected tissue at exactly the right place depend. In this regard, a multi-disciplinary approach is needed to have a good understanding of the chemical properties of polymers and applied drug, biology to understand biocompatibility, mechanics, medicine, and other branches of science.

Numerical simulations will open the door to the faster delivery of results in biomaterials field, reducing the time gap between the idea and clinical application of pharmacokinetics and pharmacodynamics in drug delivery.

Important Notes

- Electrospinning is a method that is used for production of microfibers derived from various types of electroconductive polymers based on applying of the high electrostatic field.
- Electrospinning derived fibers found their application in many fields of science and industry, e.g., biotechnology and biomedical, tissue and environmental engineering, defense, controlled drug release, and others.
- As electrospinning process is driven by many variable parameters, such as polymer parameters, process parameters, and the ambient parameters, and it is very important to optimize this process for achieving the stabilized production of fibers.
- Electrospun created fibers could serve as promising drug carriers with very wide application in treatment of various human affected areas.
- The variety of possible applied polymers, procedures of electrospinning, and applied drugs gives us a huge number of possible combinations to create electrospun fibers on demand.

Questions for Future Research

- **How fibers can be optimized for effective loading with exogenous molecules and even cells for anti-aging medicine?** In the last decade, electrospinning has been widely developed in the field of drug delivery application. Fiber optimization is necessary to obtain the exactly desired morphologies. The incorporation of biomacromolecules such as enzymes, antibodies, and DNA is an important segment in this effort. Specifically, the future challenge will be based on the optimization of electrospinning in order to incorporate biomacromolecules with the correct orientation. For example, the solid incorporation of antibodies into microfibers also implies their orientation so that the surface of the fibers has antibodies oriented with their active sites toward the outside. In this way, it will be possible to prepare microfibers with significantly increased affinity to the target analyte, e.g., pollutants, microorganisms, viruses, even cancer cells.
- **How could we effectively modify fibers for enhancing the versatility of drug carrier fabrication and engineering?** Fiber production is a cost effective and fast process. In a situation where we will be able to specifically modify them, it will be possible to create electrospun fibers capable of containing so-called nanoparticle drug bearing gaps, filters to isolate circulation tumor cells directly into the bloodstream, inexpensive real-time

biosensing systems for detecting viruses and bacteria. Development of techniques to modify fibers would, therefore, be desired so that the versatility of drug carrier fabrication and engineering can be enhanced in the future.

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Glossary

Discrete models Discrete analogue of continuous modeling.

Electrohydrodynamics The study of the dynamics of electrically conducting fluids.

Electrospinning A technology used for production of continuous nano/microscale fibers using a very high-voltage power supply.

Finite element method Numerical approach/simulation used to achieve finite element analysis of physical phenomena in a wide range of use.

Neural networks Series of algorithms that strive to recognize possible relationships in a big data set via usage of process that functions in the similar manner as the human brain functions.

PAK software A BioIRC in-house produced software for graphical pre- and post-processing, linear and geometrically and materially nonlinear structural analysis, linear and nonlinear heat conduction, laminar flow of incompressible fluid and heat transfer, and other similar purposes.

Polycaprolactone A biodegradable polyester with a low melting point of around 60 °C and a glass transition temperature of about −60 °C.

Polyethylene glycol A polyether substance with numerous applications, from engineering to medicine. PEG is also known as polyethylene oxide (PEO) or polyoxyethylene (POE), depending on its molecular weight.

Scaffold An artificial structure used for the support of the formation of new viable tissue for a medical purpose. The main aim of scaffold production is the mimicking the extracellular matrix, so the cells can proliferate and communicate in the most optimal fashion.

Tissue engineering An engineering discipline which involves the use of a combination of cells, engineering materials, and suitable biochemical factors to improve or replace biological functions.

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Chapter 14
Surface Modification Strategies in Enhancing Systemic Delivery Performance

Sudip K. Das and Nandita G. Das

Abstract In Chap. 13, electrospinning has been introduced as a strategy to manipulate the physical properties of a therapeutics-loaded system and to enhance the versatility of drug delivery. In the remaining chapters in Section V, the use of chemical means to manipulate the surface properties of a carrier for more effective systemic drug delivery will be discussed. As a matter of fact, a therapeutic agent must stay in the body as long as it is needed to reach the intended site of action in order to elicit a therapeutic response. To extend the blood retention time of an agent, one commonly used strategy is surface modification. In this chapter, we will first discuss the principles of clearance of particulate drug delivery systems from the body by the mononuclear phagocyte system (MPS), followed by a discussion of the possible use of polymers as modifiers of the surface properties of the particulate systems for enhanced performance in systemic drug delivery. Finally, the applications of surface-modified particulates in non-MPS targeting, including cancer chemotherapy and delivery across the blood–brain barrier, will also be discussed.

Keywords Particle uptake · Mononuclear phagocyte system (MPS) · Opsonization · Surface modification · Multidrug resistance · Blood–brain barrier · Non-MPS targeting

14.1 Introduction

Particulate drug delivery systems occupy a key role in present day pharmaceutical and cosmetic products (Al Jamal and Kostarelos 2011; Lai and Shum 2016; Lai 2016). Despite their presence in pharmaceutical practice for quite some time, particulate systems are not popular as injectables, except for a few types of insulins. **Liposomes** are the first particulate system to be used as parenteral products (Al Jamal and Kostarelos 2011). It took about 30 years of research for liposomes to become commercially available. Surface-modified particulate systems are particulates with

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altered surface properties to specifically act for a definite purpose; for the realm of this chapter, it is primarily the modification of the mononuclear phagocyte system (MPS) recognition and thereby provides a modified disposition in the body.

Surface-modified particles are mainly used for: (1) chemical technology to modify the **adsorption** characteristics of particulate systems toward gases and liquids, (2) diagnostic tests and immunological assays, and (3) delivery of drugs modifying uptake by the MPS. In this chapter we will focus on drug delivery systems that modify the normal uptake of particulates by the MPS. The benefits of anti-aging research have evolved in the last decade because of the significant relationship between the aging process and age-related diseases (Fernandes 2016). Therefore, the potential of surface-modified particulate systems has immense potential in age-related diseases, like cardiovascular diseases, cancer, and neuro-degenerative diseases (Magalhães et al. 2017).

14.2 Rationale of Using Particulate System and Concept of Biorecognition

There are several factors that should be considered to study the uptake of particulate systems by the mononuclear phagocyte system.

14.2.1 The Mononuclear Phagocyte System

Macrophages, commonly defined as mononuclear phagocytes (previously known as the reticuloendothelial system), were the first cell type identified as protecting the host from antigenic invasion. During the last 100 plus years since Metchnikoff (1891) discovered macrophages, they have been identified as the major mediators of humoral and cell-mediated immunity. Macrophages develop from a myelomonocytic **stem cell** in the bone marrow through monoblast, promonocyte, and monocyte stages until they form structurally heterogeneous tissue macrophages. In their early lifecycle the macrophages exist briefly in the bloodstream as adolescent cells called monocytes and constitute 3–7% of peripheral blood leukocytes. On reaching the tissues, monocytes undergo transformation to tissue macrophages and they are named based on a residency of 2–3 months as (Roitt et al. 1996): circulating blood monocytes, Kupffer cells in liver, various macrophages and microglia in brain. Macrophages can be of a wide variety of shapes depending on their residency, normally measuring 10–40 μm in diameter. The cells contain vacuoles and an approximately ovoid nucleus of 6–12 μm in diameter. The perinuclear cytoplasm contains mitochondria, many lysosomes, endoplasmic reticulum, and a Golgi apparatus, therefore, can synthesize and secrete proteins. The membrane proteins of macrophages have a rapid turnover half-life of 7 h that varies with receptor engagement (Elgert 1996).

The predominant role of macrophages is to remove particulate antigens by **phagocytosis**. They recognize, remove, and respond to foreign bodies and macromolecular ligands (Gordon 1987) by virtue of their expression of a variety of plasma membrane receptors for opsonized targets and also by direct recognition by the so-called pattern recognition receptors (Moretti and Blander 2014; Medzhitov and Janeway 1997). Macrophages internalize particulate antigens either by phagocytosis or receptor-mediated endocytosis, and digest most of them by lysosomal proteolysis. Some of the antigens are re-expressed as a fragment on the macrophage surface in relation to class II **major histocompatibility complex** (MHC) molecules that are presented to the **T cells**. Macrophages carry many proteins on their surfaces, some of which are receptors for antibodies or complements. Clearance of particles from the bloodstream is greatly enhanced if specific antibodies are present on the surface. Complexes between positively and negatively charged components (Law and Levine 1977) and opsonization increases the trapping efficiency of neutrophils and macrophages. It is possible to blockade the mononuclear phagocyte system by injecting a very large dose of colloidal carbon intravenously. Thereupon, the animal might be infected with any type of pathogenic microorganism.

14.2.2 Adsorption of Proteins to Surfaces

Proteins are complex biological molecules that contain a range of components that may all or partially interact with surfaces. Because of the surface-active properties of proteins, they tend to aggregate at interfaces and readily adsorb, especially if the surface is hydrophobic (Kawaguchi 1985). The cell interactions are mediated through layers of adsorbed proteins (Lai 2018). Andrade (1987) presented a summary of the hypothesis (Table 14.1) related to protein adsorption on the surfaces, the predominant factors being surface charge, surface energy, interfacial free energy, and surface motion (Sawyer 1984; Andrade 1976; Neumann 1979; Chin et al. 1978; Andrade and Halady 1986; Leininger 1972; Kim 1974; Nyilas and Ward 1977). The fate of a particulate system in contact with blood depends on the composition and conformation of the protein that covers the surface of the particles. The interaction of the protein with the surface of the particles depends on the concentration as well as the size of the protein and leads to adsorption of the protein on the surface of the particulates (Vroman 1982; Wojcieowski and Brash 1993). Protein adsorption on any surface depends on the nature of the surface, whether it is hydrophilic or hydrophobic, and follows a sequence of events involving interaction of proteins, reversible attachment followed by conformational change of the surface and ending in a quasi-irreversible fixation (Lundstrom and Elwing 1990; Soderquist and Walton 1980). Van Oss et al. (1975) and Neumann et al. (1979) demonstrated that phagocytic engulfment occurred more readily as the material became more hydrophobic, while platelets adhered more readily to hydrophilic than hydrophobic surfaces. Random coil proteins such as caseins adsorb in thick layers (10–15.5 nm) while compact globular proteins like β-lactoglobulin produce much thinner layers. Due to the highly

Table 14.1 Classical hypotheses of surfaces and blood compatibility

Property	Biological response	References
Negative surface charge	Plasma protein and cell repulsion	Sawyer (1984)
Interfacial free energy (IFE)	Decrease in IFE decreases protein adsorption	Andrade (1976)
	Difference between interfacial tension of adsorbate and cell, and adsorbate and vapor, determines protein adsorption	Neumann (1979)
	High interfacial free energy provides heat of adsorption that drives conformational change and contact activation	Chin et al. (1978)
Surface energy	Decreased surface energy provides optimum compatibility with blood	Andrade and Halady (1986)
Adsorption	Albumin adsorption or immobilization results in decreased thrombosis	Leininger (1972)
	Surface-adsorbed glycoproteins especially fibrinogen results in platelet adhesion	Kim (1974)
Surface motion	Increased motion leads to decreased adsorption and denaturation	Nyilas and (Ward 1977)

folded structure, globular proteins can potentially undergo extensive conformational changes when adsorbed to surfaces while the random coil protein lies close to the surface, with part of the polypeptide chains extending in the aqueous phase (Mackie et al. 1991). The more stable globular proteins such as lysozyme and ribonuclease retain most of their native structure when adsorbed onto surface and are expected to desorb more readily on dilution, while the proteins such as IgG, serum albumin, and hemoglobin appear to lose most of their secondary structures (Haynes and Norde 1995). The actual thickness of the adsorbed layer is dependent on a number of factors, including pH, surface charge on the particles, charge on the protein, and extent of denaturation of the proteins. Moreover, the electrostatic interaction (at a specific pH) among the surface, the protein and the adjacent adsorbed protein molecules is important to determine stable adsorption. Van Oss et al. also presented a correlation between the contact angle on the surface of the bacteria and the average number of bacteria phagocytosed per neutrophil. Experimentation with liposomes show that accumulation of liposomes at the MPS is related to the interaction of liposomes with plasma proteins which is dependent on the phospholipid type (Allen 1991), surface charge and size (Monkkonen 1994), and membrane rigidity (Patel et al. 1983) that result in different nature of liposome clearance from the circulation (Senior 1987).

14.2.3 Phagocytosis (Opsonization) as a Surface Phenomenon

Proteins in the serum that adsorb on the surface of foreign materials are called opsonin—means a relish or a sauce in Greek, and thereby prepare the material to be recognized by the MPS as foreign bodies and are to be phagocytosed. Thus, opsonization is a process that prepares the surface of a material for phago cytosis by adsorbing certain compounds (Lai and Rogach 2017a, b). Van Oss et al. (1975) in their extensive treatise showed that addition of complement and antiserum to non-phagocytosed bacteria resulted in significant increase of contact angle, and the same concept could be applied to particulate systems. Normally, hydrophobic nanoparticles adsorb more proteins than the hydrophilic nanoparticles leading to higher opsonization and shorter blood circulation compared to hydrophilic nanoparticles (Nguyen 2017).

Normally, intravenous administration of most non-modified particulate systems leads to rapid uptake in the liver and spleen within 5–10 min as has been reported with cyanoacrylate (Gipps 1988), polystyrene (Illum et al. 1986), polyacryl starch (Laakso et al. 1986), polyhydroxybutyrate (Koosha 1989), and albumin particles (Sugibayashi 1977). To localize the drugs in tissues or even to keep the particulates circulating in blood for longer periods of time is a challenge. The adsorption of proteins (opsonization) dictates the organ distribution of particulate systems, while not only the amount and type of the protein but also the protein conformation can determine the in vivo distribution (Patel and Moghimi 1990). Recently, Cao et al. demonstrated that protein binding affinity (equilibrium association constant) of polymeric nanoparticles can be correlated with in vivo fate of nanoparticles (Cao 2020). Therefore, if targeting is desired in the liver and spleen tissues, we would want quick opsonization and if we want the opposite, i.e., longer circulation time in the blood, we would like to have delayed or minimized opsonization.

14.2.4 Biopharmaceutical Factors Affecting the Uptake of Particulate Systems

There are three basic physical factors that affect the surface properties of the particulate systems leading to promotion of phagocytosis; hydrophobicity of the surface, surface charge, and the size of the particulate system. The major problem of the current carriers used in particulate drug delivery that includes biodegradable and non-biodegradable polymers and lipids is their hydrophobic nature. The hydrophobicity of the surface determines the interaction of the particulate systems with blood leading to opsonization and successive removal by phagocytosis. In case of non-biodegradable polymers, the contact angle may be close to 90°, while in case of biodegradable polymers the contact angle may be around 60–75°, which could be related to their chemical structures. It has been mentioned earlier that hydrophobic particles get opsonized quickly. Increase in surface hydrophobicity leads to enhanced adherence

and ingestion by phagocytes. Surface-modified hydrophilic albumin microparticles showed a reduced uptake in cultures of mouse peritoneal macrophages compared to hydrophobic non-modified particles (Artursson et al. 1983). The particles rapidly phagocytosed in the cell culture mimicking fast clearance from the circulation in vivo. Although a relationship between the reduction in surface hydrophobicity of coating films and the uptake by MPS using contact angle measurement has been described, prediction of hydrophobicity using contact angle measurement may not be the best technique, and the details are described later under the methods to determine hydrophobicity.

For intravenous delivery of particulate systems, it is not desirable to have any particles above 5 μm that could clog the vessels. The concept of filtration of drugs by the lung macrophages was exploited to deliver drugs to lungs using albumin microspheres (Illum and Davis 1982). Normally, the particles that bypass the lung capillary are taken up by the macrophages in the liver or the spleen. Zilversmit et al. documented the effect of size of gold particles on the rate of clearance from the blood compartment (Zilversmit et al. 1952). Macrophages phagocytose large vesicles more rapidly than the small vesicles. The size dependency of liposomes on organ distribution demonstrated (Diederichs 1996) small unilameller systems being less rapidly cleared from the system than the large multilamellar vesicles (Devine 1994). In vivo studies on the clearance of colloidal particles from the circulation and their uptake by liver macrophages have demonstrated the particle size effects (Yokoyama 1975; Scott et al. 1967; Juliano and Stemp 1975). In fact, it has been shown that reduction of the particle size alone could improve cellular uptake, irrespective of the other surface properties of nanoparticles (Cho 2010). While clathrin-mediated endocytosis is the predominant mechanism for less than 200 nm particles, caveolae-mediated processes occur up to about 500 nm (Rejman 2004). A number of studies showed that around 50 nm is the optimum size range for cellular uptake in the in vitro experiments (Elias 2013; Jiang 2008; Lu 2009). Recent studies revealed that phagocytosis by macrophages depends on the particle shape (Champion and Mitragotri 2006). Particles with aspect ratio of about three were internalized four times faster than spherical particles of the same volume. In addition, it was also shown the interplay of size and shape in the process of phagocytosis (Champion and Mitragotri 2009).

Particulate colloidal systems could possess anionic or cationic charge, or they could be neutral. The electrostatic stabilization of particulate colloids in dispersion is essential to avoid particle growth causing embolism. Normally, the anionic particles are eliminated faster from blood than positively charged or neutral ones (Petrak 1993; Tabata and Ikada 1988). However, the exclusive effect of surface charge of particles and their clearance from the blood compartment are very difficult to estimate and thus literature reports contradictory results. Van Oss (1978) stated that the zeta potential does not appear to be directly linked to the facility with which cells become phagocytosed, and that the connection between phagocytosis and zeta potential (if any) is far from simple. In contrast, Stossel et al. (1972) concluded that particles with large surface charge (negative or positive) are cleared rapidly by macrophages in vitro, while systems with low surface charge are cleared slowly. In

most instances, the surface charge is intimately related to surface hydrophobicity and size. According to Van Oss, colloidal particles presenting hydrophilic surfaces with a low contact angle will be almost ignored by phagocytic systems. No difference in blood circulation times and organ accumulation between different nanoparticle preparations with positive, neutral, and negative surface charges was observed in rats, suggesting that the in vivo fate of albumin nanoparticles is significantly influenced by factors not reflected in the in vitro cell culture models (Roser et al. 1998). Within 30 min after injection into a rat, anionic Aco-HSA liposomes (human serum albumin derivatized with cis-aconitic anhydride covalently coupled to liposomes with a size of approximately 100 nm) were completely cleared from the blood and almost exclusively taken up by the liver, whereas in control liposomes 80% was present in the blood at the time point. Endothelial cells were shown to account for almost two-thirds of the hepatic uptake of the Aco-HSA liposomes, the remainder being recovered mainly in the liver macrophages (Kupffer cells) (Kamps 1994). The surface charge affects the particle–cell interaction at short distances. Involvement of immunoglobulin in the particle–cell interaction (as in receptor mediated endocytosis) reduces the effect of surface charge (Schwendner et al. 1984), indicating that the effect of surface charge of colloids is negligible in receptor-mediated phagocytosis of the particles. Roerdink et al. concluded that opsonization of liposomes with complement greatly stimulates uptake of liposomes by murine macrophages. However, most of the opsonization conferred by complement can be prevented by the presence of negatively charged membrane lipids (Roerdink 1983).

14.3 Surface Modification or Steric Protection with Polymers

In order to protect particulate systems from being engulfed by the phagocytes, normally they are attached to, or coated with a protective polymer. There are two major methods to protect the particulate systems: (1) by grafting a polymer chain covalently on to the surface of a particle, and (2) by adsorption or incorporation of protective polymers on the surface of the particulate systems. In both situations, the hydrophilic parts protrude toward the bulk solution and protect the particles from interaction with the blood proteins (Blunk 1993). The term “steric stabilization” was introduced by Naper in 1983 (Naper 1983). The major work in this area has been done with liposomes. One of the most popular methods is to coat liposomes with some hydrophilic protective polymer, like polyethylene glycol (PEG) (Kilbanov 1990). Coating of the liposomes with PEG sterically hinders the interaction of the blood components with the liposome surface (Allen 1994; Woodle 1993), thereby preventing opsonization of the liposome surface and the engulfment of the liposomes by the MPS (Senior 1987). Steric stabilization occurs as a result of repulsion between two overlapping polymer layers (Klein 1986). There are basically three parameters that should be fulfilled for the criteria of steric repulsion (Amiji et al. 1994):

1. Surface-modifying polymer should be tightly bound to the surface and not replaced by plasma components.
2. A portion of the polymer should extend in the bulk aqueous medium, with optimal extensions and flexibility of the polymer segments that determines the dominance of steric repulsion over the **van der Waals attractive forces**. Therefore, block copolymers with hydrophilic and hydrophobic chains are more effective than homopolymers (Naper 1983).
3. The surface-modifying polymer should coat the surface completely; any incomplete coverage of the surface may leave it prone to opsonization.

The bypassing of MPS by particulate systems mainly depends on the polymer structure and characteristics and determines the kinetics of disposition of the polymers in the body. Factors that affect the disposition of intravascularly administered polymers are the polymer size and interaction with the blood components. Normally, large polymers are subject to more opsonization depending on the interactive moieties s on the polymer chains and the opsonins available in the immunocomplex (Ehlenberger and Nussenzweig 1977), rendering the large polymers more prone to phagocytosis. Ross et al. showed that it was not a single opsonin interaction but multiple opsonin interactions with polymers being involved in the process (Ross et al. 1985). Immunocomplex half-lives depend on the opsonin molecules attached to it and could vary depending on whether they are immediately phagocytosed (Brown et al. 1970) and if not, they could even circulate in the body for a few days (Atkinson and Frank 1974). In case of small polymers, opsonization followed by phagocytosis is rather less plausible than the more obvious renal clearance (Delgado et al. 1992).

A varied number of functional groups may be responsible for surface recognition. A number of receptors can recognize carbohydrates, especially receptors in the liver (Rice and Lee 1990). Various receptors can bind to peptide side chains. In fact, most of the functional groups interact with the immunocomplexes, to a varied extent, and the polymers with several positively charged groups or highly hydrophobic groups interact, especially in vivo. Hydrophilic polymers with low nucleophilicity appear to have the best circulating properties. **Amphiphilic polymers** used for surface modification consist of a hydrophobic part that adsorbs on the particle surface and a hydrophilic part that protrudes in the solution and protects the particles from interactions with blood components. Torchilin et al. reported that the surface-grafted chains of flexible and hydrophilic polymers form dense "conformational clouds" preventing other macromolecules from interaction with the surface, both in the form of dense particle coating as well as in the form of a relatively loose brush (Torchilin and Trubetskoy 1995). Jeon et al. studied the interactions of surface-modifying polymers with blood proteins and established relationships between steric repulsion, van der Waals attraction and hydrophobic attraction, and the surface density and chain length of polyethylene oxide (PEO). High surface density and long chain length of PEO are desirable for protein resistance. Also, the surface density has a greater effect than chain length on steric repulsion (Jeon 1991). The biodistribution of surface-modified particles is dependent on the competing processes of reduction in the adsorption of opsonins and the selective adsorption of certain plasma proteins

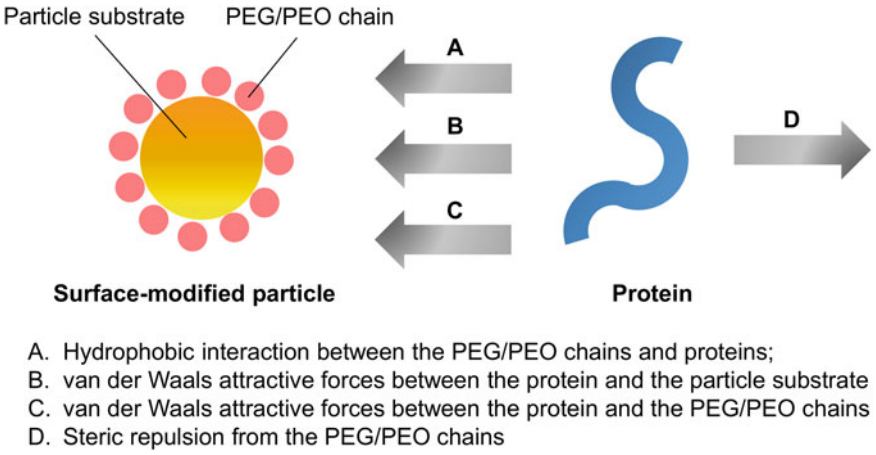

Fig. 14.1 Interactions between a surface-modified particle and the opsonins

(dysopsonins) (Moghimi 1993). As the PEO chains are compressed in solution, the surface density of PEO increases gradually. This might make the coated surface hydrophobic,therefore, an optimum chain length of coating polymers is necessary to impart hydrophilic properties (Jeon and Andrade 1991). Another interesting aspect is that hydrophilicity is not enough to protect the particles. Dextran-coated liposomes were cleared faster from the circulation than PEG-coated liposomes, although dextran is more hydrophilic than PEG (Pain 1984). Also, the chain flexibility that contributes to providing the long-circulating feature could have a varied number of chain conformations (Torchilin and Papisov 1994; Blume and Cevc 1993). Figure 14.1 illustrates the interactions of a surface-modified particle and the blood proteins.

14.3.1 Polyethylene Glycol (PEG)

Minimization of polymer interactions with biological systems is the prime requirement in the design of long-circulating particulates. Major groundbreaking work was initiated with the reporting of surface-modified long-circulating liposomes such as “stealth liposomes” (Allen 1989) or sterically stabilized liposomes (Papahdjopoulos 1991). The effect of PEG in surface modification to decrease the phagocytosis of particulates has been widely studied in liposomes (Senior 1991; Filion and Philips 1997) and polymeric particles (Allemann 1995). An advantage of using PEG in surface modification is that it is non-toxic and is approved by the FDA for internal use (Harris 1985). In recent years, amphiphilic molecules containing both hydrophobic and hydrophilic parts have been employed to modify the surface of particulate systems. The longer action of liposomal incorporated ganglioside (GM1) was achieved with a coating of PEG (Allen and Chonn 1987; Mori 1991). The

phenomenon is explained by involvement of surface charge and the hydrophilicity of PEG-coated liposomes (Gabizon and Papahadjopoulos 1992) and reduced opsonization of plasma proteins on the surface of particulates (Lasic 1991). The easiest method to coat particle surfaces is to adsorb amphiphilic polymers like PEG, provided the surface is hydrophobic. These polymers undergo hydrophobic interactions with the particulate surface, while their hydrophilic parts get exposed to the plasma proteins on intravenous administration (Blunk 1993). However, there are limited literature reports on the surface modification of nanoparticles using adsorption or incorporation of PEG within the polymer matrix. The other way to protect the surface is to graft a polymer chain onto the surface by covalent bonding to induce "steric stabilization" (Naper 1983).

In the adsorption method, the matrix polymer and PEG or substituted PEG is dissolved in a common solvent like dichloromethane and the particles are formed by W/O/W solvent evaporation method or spray drying process. It was demonstrated that PEG was homogeneously dispersed on the microspheres and decreased phagocytosis to a significant extent (Lacasse 1988). X-ray photoelectron spectroscopy confirmed that the greater the amount of PEG-distearate in the formulation, the higher is its concentration on the surface (Lacasse et al. 1998). Also, a range of polylactide-PEG block copolymers either self-disperse in aqueous medium to form micelles or produce stable particles, where the drug is entrapped in the hydrophobic matrix of polylactide coated with hydrophilic PEG or its derivative. A number of studies have been conducted with polystyrene nanoparticles with surface-adsorbed PEG and blocks of poly(propylene glycol), and it was reported that the adsorption was kinetically controlled and decreased with an increase in surface roughness and polarity (Illum and Davis 1983). High molecular weight PEG has been shown to reduce surface roughness of the polymer particles which could be due to reduction of surface tension between the particles and aqueous phase (Monnier 2016). Although non-biodegradable polystyrene particles are not a realistic therapeutic system, this model was later applied to PLGA microparticles coated by amphiphilic diblock PEG-PLA copolymers (Dunn 1994). The major problem with adsorbed polymers is that they might efficiently desorb in vivo, especially in the presence of blood components with high affinity for the particle surface (Petrak 1993).

It has now been well documented that PEG covalently bonded to proteins forms macromolecular conjugates with longer blood circulation time than native proteins, with less immunogenic reactions (Abuchowski 1977). A review thoroughly covers the details of PEGylation reactions and biochemical and biological characterization (Bailon and Berthold 1998). On attaching the PEG or PEG derivatives, it has been reported that the **graft polymer** of PEG on to poly(lactide-co-glycolide) or poly(D,L-lactide) can be synthesized by ring opening polymerization of the respective polymer in the presence of a catalyst, stannous octoate (Churchill and Hutchinson 1986; Deng 1990). This catalyst is very effective and widely used to synthesize PLA-PEG copolymers (Kricheldorf and Meier-Haack 1993) and is approved by FDA for use as a food stabilizer (Gilding and Reed 1979). In case of liposomes, the PEG-grafted polymers appear to be better than using other polymers for grafting due to better stability of the coating, and increased blood residence time (Allen 1994). Also, the blood half-life

of PEG increases from 18 min to 1 day as the molecular weight increases from 6,000 to 190,000 (Yamaoka et al. 1994). An important factor that should be kept in mind while formulating particulates with PEG is that PEG given intravenously in large doses over a prolonged period of time may cause nephrotoxicity (Erickson 1996). Although a large therapeutic window (approximately 600-fold) exists between the maximum PEG burden from a currently marketed biological agent and the doses of PEG associated with human toxicity, molecular weight of PEG is known to have significant effect acute toxicity (LD_{50}) in rats and mouse (Webster et al. 2009). It has also been shown that pegylation of particulate systems could cause generation of anti-PEG-IgM (Wang et al. 2007; Ishida 2007). Densities and chain-length of PEG on the surface of particles often cause steric shielding (Hatakeyama et al. 2013; Fang 2017), thereby preventing their interaction with cellular receptors (Wang and Thanou 2010; Pelaz 2015; Schöttler et al. 2016; Barui 2020). Therefore, very careful optimization of PEG orientation on the surface of nanoparticles is desirable to effect maximum cellular uptake of particles. A list of more representative examples demonstrating the use of PEG in surface modification of polymer nanoparticles has been shown in Table 14.2 (Lacasse et al. 1998; Hagan 1995; Gref 1994; Stolnik 1994; Dunn 1994; Peracchia 1994; Verrecchia 1995; Yeh 1995; Piskin 1995; Li 2000; Pean 1999; Lacasse 1998; Hawley et al. 1997). They illustrate the versatility and wide use of PEG in the design and development of polymeric nanoparticle systems in drug delivery.

14.3.2 Block Copolymers of Ethylene Oxide and/or Propylene Oxide

The amphiphilic surface modifiers, polyethylene oxide (PEO)/polypropylene oxide (PPO), have been most successful in extending the circulation half-life of particulate systems (Abuchowski 1977). Particulate carriers are either grafted to PEO (Harper 1991; Maste 1994) or adsorb amphiphilic PEO copolymers (Moghimi 1993). A number of amphiphilic PEO and PPO copolymers of different compositions and molecular weights are available as poloxamers and poloxamines. Poloxamers are ABA block copolymers represented as EO–PO–EO, while poloxamines consist of four PEO and PPO blocks joined together by an ethylene diamine bridge represented as $[EO–PO]_2–N–CH_2–CH_2–N–[PO–EO]_2$. Poloxamers and poloxamines are marketed by BASF Performance Chemicals, NJ, under the trade names of Pluronic® and Tetronic®, respectively. In addition, BASF also markets Pluronic® R as PO-EO-PO and Tetronic® R as $[PO\text{-}EO]_2–N–CH_2–CH_2–N–[EO–PO]_2$. Reversing the hydrophobic and hydrophilic blocks creates surfactants similar to Pluronic surfactants, but with some important differences. While the Pluronic surfactants are better emulsifiers and dispersants and cover a broader range of molecular weights, Pluronic® R surfactants have lower foaming, greater defoaming, and reduced gelling tendencies. In addition, Pluronic® R surfactants are terminated by secondary hydroxyl groups, which have lower reactivity and acidity than the primary hydroxyl

Table 14.2 Polyethylene glycol-modified polymer particulates

Objective	Surface-modifying polymer	Particulate matrix polymer	Method of preparation	Active ingredient	References
Study of surface morphology of spray-dried particulate	PEG 400 distearate	Poly(D,L-lactic acid)	Blend of polymers in DCM and spray drying	–	Hagan (1995)
PLA-PEG micelles	PEG	Poly(D,L-lactic acid) (PLA)	Graft copolymer of PLA-PEG in ratio of 1.5:2 and 2:5	Testosterone	Gref (1994)
PLGA grafted with PEG	PEG	Poly(lactide-co-glycolide)	Graft copolymer of PLGA and PEG	Lidocaine	Stolnik (1994)
PLA-PEG adsorbed onto the surface of PLGA nanospheres	PLA-methoxy-PEG	Poly(lactide-co-glycolide)	PLA-PEG adsorbed after preparation of PLGA nanospheres by precipitation solvent evaporation	–	Dunn (1994)
In vivo biodistribution	PLA-PEG	Poly(lactide-co-glycolide)	PLA-PEG adsorbed after preparation of PLGA nanospheres	–	Peracchia (1994)
Physicochemical characterization	PEG	Polyesters and polyanhydrides	Emulsification solvent evaporation—grafted PEG	Lidocaine, prednisolone	Verrecchia (1995)
Comparison of surface and non-surface modified particles	Methoxy-PEG	Poly(D,L-lactide)	Solvent diffusion of PLA-methoxy-PEG solution	Albumin	Yeh (1995)
Delivery of proteins using PLGA microparticles	PEG	PLGA	Blend of PLGA and PEG using multiple emulsion solvent evaporation method	Ovalbumin	Piskin (1995)
PLA-PEG micelles	PEG	PLA	Copolymer made by transesterification	–	Li (2000)

(continued)

Table 14.2 (continued)

Objective	Surface-modifying polymer	Particulate matrix polymer	Method of preparation	Active ingredient	References
Protein stability	PEG	PLA	PLA-PEG bulk ring opening polymerization—emulsion solvent evaporation	Glucose oxidase	Pean (1999)
Protein stability on co-encapsulation	PEG 400	PLGA	Blend of PEG and PLGA	Nerve growth factor	Lacasse (1998)
Effect of plasma protein adsorption and phagocytosis	PEG	PLGA, PLA and polyanhydride	Blend of methoxy PEG with PLGA, PLA and polyanhydride	–	Hawley et al. (1997)
Comparison of PEG coated nanoparticles on lymph node uptake	PEG	PLGA and PEG-PLGA-PLA	Coating by adsorption and coprecipitation—separation of unadsorbed polymer	^{125}Iodine and ^{111}Indium oxine	Abuchowski (1977)

groups terminating Pluronic® surfactants and the same applies for Tetronic® R and Tetronic® (Performance Chemicals and Pluronic and Tetronic Surfactants 2020). Only a few members among the poloxamer and poloxamine series are able to modify the surface characteristics of the particulate systems. Normally, poloxamers with high molecular weights such as 338 provide an effective steric barrier (Illum and Davis 1984), whereas poloxamers with lower molecular weight such as 235 do not (Illum 1987). In recent years, the most promising copolymers used for surface modification of particulate systems appear to be PEO grafted to a biodegradable polymer, such as poly(D,L-Lactide)-PEO (Stevels 1995) and poly(caprolactone)-PEO (Peracchia 1994), by ring-opening polymerization in the presence of methoxy-PEO and stannous octoate as the catalyst. It is postulated that, in contact with water, highly hydrated and flexible PEO forms dense "conformational clouds" over the particulate surface, inhibiting the interaction with opsonins and the phagocytic cells (Torchilin and Papisov 1994). The adsorption of PEO-based surfactants as well as PLA-PEO block copolymers on PLGA or PLA proceeds via hydrophobic interactions between the hydrophobic particle surface and the hydrophobic moieties, while the hydrophilic PEO parts form a "hair-like" coat around the particle. However, due to the partial reversible nature of the adsorption process, it is difficult to predict the stability of the adsorbed steric barrier. In order to solve this problem, PEO-grafted nanoparticles have been developed by direct grafting of PEO moieties on the preformed particles (Muller and Kissel 1993) or by in situ polymerization in the presence of PEO moieties (Peracchia 1997).

Most of the earlier studies on surface modification with poloxamers reported the coating of non-biodegradable particles of poly(methyl methacrylate) or polystyrene. PEO adsorbed surfaces have been reported using PEO homopolymers of high molecular weight that adsorb effectively (Kato 1981). It has been reported that 30 PO residue Pluronics adsorbed on hydrophobic surfaces did not decrease adsorption of albumin significantly while 56 PO residues of Pluronics were effective in the prevention of protein adsorption (Amiji and Park 1992). Labeled polystyrene microparticles of specific size range were incubated with poloxamer 338 for 24 h, and disposition was recorded in experimental animals after intravenous administration. It was reported that poloxamer 338 and poloxamine 908 were effective in reducing the liver uptake of the labeled poly(methyl methacrylate) nanoparticles, while the uptake of nanoparticles in non-MPS organs like heart, GI-tract, ovary, muscles, and brain were significantly increased (Troster et al. 1990). Alyautdin et al. demonstrated that the polysorbate 80 coating of analgesic dalargin adsorbed poly(butyl cyanoacrylate) nanoparticles delivers the drug across the blood–brain barrier compared to dalargin solution, dalargin bound to nanoparticles without coating with polysorbate 80, and a simple mixture of dalargin, nanoparticle, and polysorbate 80 mixed directly before IV injection (Alyautdin 1995). It was reported by Muller et al. that ethoxylated polymers and surfactants can be easily adsorbed onto polystyrene particle surfaces,however, it was difficult to coat the polyester (polylactide, polylactide/glycolide nanoparticle surface with the same ethoxylated polymers (Muller and Wallis 1993). This is possibly due to the inherent hydrophilic surface of polyester particles produced by W/O/W solvent evaporation in aqueous polyvinyl alcohol solution. Solid lipid

nanoparticles (SLN) produced by dispersing melted lipids in an aqueous solution of poloxamine 908 or poloxamer 407 proved more efficient in avoiding phagocytic uptake than polystyrene particles surface modified with the same polymers (Muller 1996). Considering the fact that SLNs are tenfold less toxic than the polylactide nanoparticles (Maaben 1993) and 100-fold less toxic than the cyanoacrylate particles (Lherm 1992), they are one of the most viable carrier systems for long circulation of drugs. Blending of Pluronic® copolymers with PLG or PLGA is a logical approach toward the development of the W/O/O microencapsulation technique. Yeh et al. studied the physicochemical characteristics of PLG blended with hydrophilic Pluronic® F127 and more hydrophobic Pluronic® L121 (Yeh et al. 1996). Secondary ion-mass spectroscopy indicated the presence of PEO-PPO on the surface of PLG microspheres produced by W/O/W solvent evaporation in the solution of PEO-PPO surfactants (Coombes 1994).

Thus, biodegradable triblock copolymers are potential carrier systems for surface modification of particulate systems. Moreover, if a protein drug is encapsulated in the particles, its stability is protected as the higher hydrophilicity of PEO block that leads to a higher water content in the matrix, possibly preventing aggregation of proteins (Kissel 1994). ABA triblock copolymers, consisting of poly(L-Lactic acid) or poly(L-Lactic-co-glycolic acid) and poly(ethylene oxide) were synthesized by bulk polymerization, using aluminum tri-isopropoxide as catalyst (Youxin and Kissel 1993). Normally nanoparticles are prepared by emulsion solvent evaporation of PLA-PEO block copolymer, in which PLA is racemic (M = 30,000) and linked to PEO (M = 2,000). After solubilizing it in acetone (25–50 g/L) the polymers are precipitated in pure water (Labarre 1994). In a study, Gurny et al. reported various blends of fluorescent-labeled PLA and PLA-PEO diblock copolymers, produced by a salting out process (Leroux 1995), and a clear relationship between the PEO contents, and a decrease in the in vitro cell uptake was demonstrated (Jaeghere 1999). An interesting process for synthesis of triblock poly(L-Lactide)-block-poly(oxyethylene)-block-poly(L-Lactide) copolymer in the absence of any added catalyst has been suggested. This method may be advantageous as it does not leave any trace of non-biodegradable catalysts, but it is not competitive against the catalyzed methods since very long reaction times (few days) are necessary to have 70–90% yield (Cerrai and Tricoli 1993). Ronneberger et al. (1997) studied the biocompatibility of microparticles made from ABA triblock copolymers and observed that implanted microparticles caused an initial acute but localized inflammatory response. Muscle tissue surrounding the injection sites did not show irreversible changes such as necrosis and degeneration. The implantation sites were gradually replaced by collagenous tissue during the course of degradation. The results show that the microparticles prepared from ABA-triblock copolymers can be considered as a biocompatible carrier system with properties similar to PLGA or PLA.

14.4 Surface-Modified Particulates in Sequestering Multidrug Resistance Proteins

Success in the treatment of some disseminated cancers with chemotherapy has led to understand why many other cancers are naturally resistant to anti-cancer drugs or become resistant to chemotherapy after several rounds of treatment. Although over 50 anti-neoplastic small-molecule drugs are in use, only a few are effective for a specific tumor type because of intrinsic or primary drug resistance. The initial and subsequent chemotherapy treatments allow a tumor to develop acquired or secondary resistance because malignant cells that survive the drug are resistant to that drug. The broad-spectrum resistance to structurally and mechanistically diverse anti-cancer agents constitutes the multidrug resistance (MDR) phenotype. Multidrug resistance of cancer cells is a serious challenge in the treatment of neoplastic diseases and is the leading cause of the failure of chemotherapy. Cancer cell chemoresistance is based on the development of several mechanisms among which one of the most important concerns is the overexpression of membrane proteins that remove cytotoxic compounds from the cytoplasm as an **efflux pump**. The leading archetype of these proteins is the P-glycoprotein, a member of the ATP-binding cassette (ABC) superfamily of transporters, or traffic ATPases. Tumor cells carrying this phenotype are characterized by the overexpression of an energy-dependent drug transport protein, P-glycoprotein (P-gp) (Ling 1987; Ban 1992), belonging to the superfamily of the ATP-binding cassette (ABC) (Ling 1997; Krishnan et al. 1997). The overexpression results in a decreased accumulation of the drug within the cancer cell because the cell can efficiently pump out the hydrophobic anti-cancer drug molecules (Ford and Hait 1990; Gottesman and Pastan 1993). It has been shown that a major mechanism of resistance of cancer cells to natural product anti-cancer drugs such as adriamycin, etoposide, vinblastine, actinomycin D, and taxol is expression P-glycoprotein (P-gp) or the multidrug transporter (Gottesman 1995). This pump system contributes to drug resistance in about 50% of human cancers by preventing adequate exposure of anti-cancer drugs in cancer cells.

In the recent years, a number of other drug efflux transport proteins, 190–210 kDa multidrug resistance associated protein (MRP) (Barrand et al. 1997; Hollo 1996), 110 kDa lung resistance associated protein (LRP) (Izquierdo 1996), and new genes, have been discovered in cancer cells with a multiple drug resistance phenotype (Baggetto 1997). While P-gp is thought to have a major role in acquired drug resistance (Abe 1996), the widespread expression of MRP in many solid organ and untreated tumor cells (Kruh 1995) suggests MRP may have a more important role in intrinsic drug resistance. Like P-gp, MRP is also an ATP-dependent efflux pump that actively transports selected chemotherapeutic agents. MRP has different substrate characteristics and different inhibitor specificity from P-gp and has a broad specificity for amphiphilic anions (Jedlitschky 1996). The ABC transporter breast cancer resistance protein (BCRP) has been cloned, sequenced and developed into a diagnostic tool (Ross 2000; Natarajan et al. 2015).

In order to decrease the toxicity, to improve bioavailability to target tissue and to enhance the selectivity of existing drugs, many drug-delivery systems for chemotherapeutic agents have been proposed in recent years (Gupta 1990). Biodegradable nanoparticles and lipid nanoemulsions have received a growing interest for drug targeting, because they can be easily prepared with well-defined biodegradable polymers (Allemann et al. 1993) and lipids. The reason of targeting tumors with nanoparticles is because certain neoplastic cells have been found to exhibit an enhanced endocytotic activity (Kreuter 1983) which could be due to cell surface expression of critical molecules (Elkin et al. 2015). In addition, since capillaries having an increased vascular permeability supply some particular tumors, one can anticipate that nanoparticles will gain access to extravascular tumoral cells (Jain 1987). Another interesting property of anti-cancer drug-loaded nanoparticles is their ability to overcome pleiotropic resistance. In vitro studies evaluating the efficacy of nanoparticulate delivery systems have been used to evaluate the efficacy of anti-neoplastic agents. In vitro studies evaluating the efficacy of nanoparticles on MDR cells have been demonstrated with doxorubicin (Tokes et al. 1982; Kubiak 1989; Nemati et al. 1994). By coupling doxorubicin to nanospheres, DXR efflux from the tumoral U-937 cells was considerably reduced (Astier et al. 1988). Two mechanisms have been proposed to explain how the nanoparticles counteract the MDR: (i) inability of P-glycoprotein to reject nanospheres-bound DXR outside the cell, (ii) DXR bound to nanospheres can efficiently interact with the membrane of the resistant cells, inducing at the final stage a perforation of the cytoplasmic membrane (Rogers et al. 1983) and is dependent on the nanospheres density (Astier et al. 1988). Although the reduction in the general toxicity of anti-cancer drugs bound to nanoparticles has been demonstrated in a number of cases (Couvreur et al. 1982), in certain situations anti-cancer drugs bound to nanoparticles have often been associated with a higher acute toxicity (Kreuter and Hartmann 1983). The increase of toxicity could be attributed to the accumulation of the carrier in the organs of the mononuclear phagocyte system, such as spleen (Brasseur et al. 1980). This confirms the importance of developing drug delivery systems that can avoid the MPS (when it is not the target site) and of designing nanoparticulate systems having appropriate physicochemical properties.

One of the major applications of long-circulating liposomes has been in cancer chemotherapy. Cytosine arabinoside, vincristine, epirubicin, and doxorubicin are among the drugs that have been developed (Gabizon 1994). The doxorubicin containing liposome based on Stealth® technology, Doxil® (Janssen) is commercially available for use in AIDS-related Kaposi's sarcoma. Because of the small particle size of 50 nm, Daunosome® (Gilead Sciences) is also considered a long-circulating formulation. In addition, Genexol-PM® (Samyang Biopharm), Naulasta® (Amgen), Oncaspar® (Takeda) are also long-circulation formulations based on polymeric platforms.

14.5 Surface Modification as a Strategy to Target Non-MPS Organs

Surface modification of particulate systems not only reduces the clearance of the particles by MPS, but it also improves the availability of the drug, as the particles can increase the disposition of drugs in organs that do not belong to the MPS. Borchard et al. (1994) observed an increased uptake of surfactant-coated poly(methyl methacrylate) nanoparticles by bovine brain microvessel endothelial cell monolayers in vitro. Using fluorescent polysorbate 80 coated fluorescence isothiocyanate dextran labeled poly(butyl cyanoacrylate) nanoparticles, Kreuter et al. (1995, 1997) showed that surface-modified particulates can be taken up by the endothelial cells lining the brain blood vessels and demonstrated transport of fluorescence into the Purkinje cells of the brain shortly after intravenous injection.

Besides endocytosis, the other possibility by which the above drugs exhibit increased brain uptake may be inactivation of the P-glycoprotein efflux pump that is present in the brain endothelial cells. In another interesting observation, poloxamer 338 and poloxamine 908, non-ionic surfactants that are widely used as surface-modifying agent to bypass MPS, were found to be ineffective in transporting drugs across the blood–brain barrier while polysorbate 80 was found to be effective (Troster et al. 1990). A recent review described delivery of small molecule drugs as well as macromolecules across blood-brain barrier using surface modified nanoparticles (Tosi et al. 2020).

14.6 Summary and Outlooks

Effective systemic delivery is vital for the development of biogerontological interventions (Lai 2020; Lai 2013). Over the years, different strategies have been reported for tackling aging. For instance, whole-body knockout of *Pla2r1* in a murine model of premature aging has been found to decrease the reduction in the bone content (Griveau 2018). In addition, by manipulating isoprenylcysteine methylation in a premature aging mouse model via whole-body reduction of the expression and activity of isoprenylcysteine carboxyl methyltransferase (ICMT), body weight of the mouse is increased, the grip strength is normalized, and bone fractures and death of the mouse are prevented (Ibrahim 2013). These advances in anti-aging medicine, however, fail to be practiced in reality, partly because of the failure of systemic delivery for whole-body genetic and pharmacological treatment. Such failure is partly attributed to the rapid removal of the therapeutic agent from the blood circulation before the arrival of that agent to tissues. To enhance the efficiency of systemic delivery, prolonging the blood circulation time is one of the prerequisites. In this chapter, we have presented advances in surface modification strategies for optimization and modification of particulate systems. Based on the promising potential as evidenced by the literature, we anticipate that surface-modified particulate systems

will continue with their enormous promise in the delivery of a number of drugs not only by virtue of bypassing the mononuclear phagocyte system, but also in the treatment of multidrug resistant cancers and drug delivery across the blood–brain barrier. By taking the criteria discussed in this chapter into consideration during the design of the nanocarrier, it is possible to develop delivery systems with prolonged circulation half-life, avoiding the phagocytic uptake by the mononuclear phagocyte system, thus releasing drugs for a prolonged period of time, with better therapeutic efficacy and reduced toxicity.

Important Notes

- The electrostatic interaction (at a specific pH) between the surface and the protein and between adjacent adsorbed protein molecules is important to determine stable adsorption.
- Protein adsorption on any surface depends on the nature of the surface, whether it is hydrophilic or hydrophobic, and follows a sequence of events involving interaction of proteins.
- There are three basic physical factors that affect the surface properties of the particulate systems leading to promotion of phagocytosis: hydrophobicity of the surface, surface charge, and the size of the particulate system.
- Stability of the dispersed particulate systems in blood could be improved by manipulation of hydrophilicity and surface charge.
- Surface-modified particulates could be beneficial for drug delivery across blood–brain barrier and also in multidrug-resistant malignant tumors.

Questions for Future Research

- **How to optimize hydrophilicity, surface properties, and particle size for maximum circulation time in the blood?** It has been widely understood that the blood retention time of a carrier can be manipulated by changing the hydrophilicity, size, and surface properties. However, currently manipulation of these factors during carrier optimization is by trial-and-error method. Methods to more rationally optimize the properties of a carrier are in dire need.
- **Which PEGs would be the most suitable and effective one for surface modification?** Over the years, many PEG derivatives have been proposed based on the immunological considerations; however, right now it is poorly understood how different derivatives affect the ultimate performance of a carrier. Solving this problem may increase our understanding of the mechanisms and structure–activity relationships involved in the PEGylation of nanocarriers.

- **What would be the optimum PEG or PEO coating that will not cause steric hinderance for particle uptake by the target cells?** PEG or PEO coating has been extensively used for surface modification of nanoparticulate systems; however, they may cause steric hindrance and may ultimately reduce the efficiency of cellular uptake, leading to a reduction in the performance of the carrier in drug delivery. Strategies to help optimize the structure, composition, and thickness of the coating can enhance the efficiency in structural modification using PEG and PEO.
- **What would be the best approach for targeting of particulate systems to cancer tissues using surface-modified particles and active targeting moieties?** Cancer is an age-associated disease. Over the years, different agents (ranging from folic acid to transferrin) have been adopted to modify the surface of carriers so as to render the delivery systems into tumor-targeting agents. Differences in the targeting efficiency mediated by different ligands, however, have not been seriously compared till now. Studies in this aspect may help identify the best approach for targeting of particulate systems to cancer tissues.

Glossary

Adsorption Adhesion of molecules from a gas, liquid, or dissolved solid to a surface.

Amphiphilic polymers Polymers possessing both hydrophilic and lipophilic properties.

Efflux pump A proteinaceous transporter localized in the cytoplasmic membrane cells.

Graft polymer A branched copolymer with one or more side chains of a homopolymer attached to the backbone of the main chain.

Liposomes Spherical vesicles having at least one lipid bilayer. Liposomes can be used as a vehicle for administration of bioactive agents.

Macrophages White blood cells that surround and kill microorganisms, remove dead cells, and stimulate the action of other immune system cells.

Major histocompatibility complex Proteins that are essential for adaptive immunity.

Phagocytosis The process by which a cell uses its plasma membrane to engulf a large particle, giving rise to an internal compartment called the phagosome.

Stem cell A cell that has the ability to develop into many different cell types, from muscle cells to brain cells.

T cells Lymphocyte immune cells that protect the body from pathogens and responsible for immunity against cancer cells.

van der Waals attractive forces Weak non-covalent, non-ionic forces between atoms or molecules.

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Chapter 15
Layer-by-Layer Functionalization for Oral Liposomal Formulations in Anti-aging Medicine

Yi Wang and Wing-Fu Lai

Abstract In the previous chapter, an overview of the use of polymers in modifying the surface properties of nanoparticulate systems has been presented. In this chapter, we will continue to talk about surface modification, but will focus on its applications to lipid-based systems, particularly liposomes which are one of the non-viral vectors that have reached clinical trials and hence have shown high future potential for transposition to humans for anti-aging purposes. Till now, administration of liposomes is performed mainly via injection due to their low oral bioavailability; however, compared to parenteral administration, oral administration is highly desirable in the practical sense for most treatment modalities because it avoids the risk associated with injection and gives higher patient compliance. In this chapter, we will present an overview of the roles of Layer-by-Layer (LbL) technology, which is one of the most frequently used technologies for surface modification of various nanoparticulate systems in the realm of biomedical applications and in the development of oral liposomal formulations for systemic delivery, with a focus on the major principles of molecular design and engineering strategies of LbL functionalized liposomes.

Keywords Drug delivery · Layer-by-Layer assembly · Surface functionalization · Liposomes · Oral delivery

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15.1 Introduction

Liposomes are a commonly used drug carrier composed mainly of phospholipids and cholesterol. They are in the form of spherical vesicles and can be classified into various types based on the size and the lipid bilayer structure, including small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV) and multivesicular vesicles (MVV) (Daraee et al. 2016). Among these types, LUV, SUV and MLV have been widely studied for oral drug delivery, while MVV has only been used for parenteral administration (Tantisripreecha et al. 2012; Tahara et al. 2018; Catalan Latorre et al. 2018, 2016). Over the years, clinical trials have been performed for various liposomal formulations and shown promising therapeutic results. For example, liposomal doxorubicin was reported to be well tolerated at myelosuppressive doses and to produce less venous sclerosis in cancer treatments as compared to its free form (Rahman et al. 1990; Owen et al. 1992). Other examples of clinically tested liposomal formulations including paclitaxel liposome (Wang et al. 2010; Zhang et al. 2009) and liposome-encapsulated all-trans retinoic acid (Boorjian et al. 2007) have also been extensively studied. In light of the enormous clinical success, liposomal formulations draw particular interests in drug delivery research, and have high potential for transposition to humans for the future development of biogerontological interventions.

Despite numerous laboratory and clinical success that have been achieved in therapeutics delivery by liposomes, their applications in oral drug administration are still limited, mainly by the action of gastric acid, bile salts and pancreatic lipases in the gastrointestinal (GI) tract, which not only significantly reduce the number of intact liposomes reaching intestinal epithelia (Liu et al. 2015) but also cause undesired payload leakage during the delivery process (Hu et al. 2013). Additionally, liposomes are often trapped by GI **mucus** due to enhanced **hydrophobic interactions derived from** their surface tensions (Ensign et al. 2012), resulting in a further reduction in absorption. In order to facilitate trans-epithelial absorption, various strategies have been developed to enhance the physicochemical stability of liposomes in the GI tract (Zhang et al. 2010; Ng et al. 2001; Kazakov 2016; Katayama et al. 2003; Pantze et al. 2014; Zhao et al. 2018; Minato et al. 2003; Mohanraj et al. 2010; Dwivedi et al. 2010; Richards and Gardner 1978; Parmentier et al. 2011; Kato et al. 1993). Surface coating such as Layer-by-Layer (LbL) technology is one of the strategies that draw particular research interest for its outstanding tunability, ease of operation and superb applicability (Such et al. 2011; Ok et al. 2003).

LbL technology is a self-assembly surface coating technique that involves a sequential assembly of functional polymeric materials onto a core (such as liposomes) and develops hierarchically organized drug delivery vehicles (Fig. 15.1) (Such et al. 2011; Ok et al. 2003; Correa et al. 2019; Mizrahy and Peer 2012). LbL modification grants the liposome core a multilayered **polyelectrolyte** film coating resulting in added functionality beyond that of the core template, such as the introduction of stimuli-responsive drug release characteristics (Li et al. 2012; Yang et al. 2012; Ye et al. 2012), extended systemic circulation time (Morton et al. 2013; Ramasamy et al. 2014) and active targeting functionality (Dreaden et al. 2014). The idea of LbL assembly was first proposed in 1966 by Iler (1966), who generated multilayered films

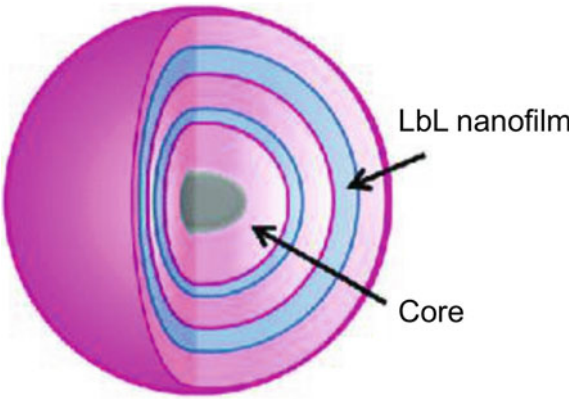

Fig. 15.1 Hierarchical structure of LbL functionalized nanoparticle. Reproduced from (Mizrahy and Peer 2012) with permission from royal society of chemistry

by alternating deposition of counter-charged colloidal particles (Fig. 15.2a) (Akiba et al. 2017). This technique has then been widely employed for the fabrication of multilayered polymer films. In the 1990s, the LbL technology was further developed to generate microcapsules with a hollow structure by removing the core template after LbL deposition of the multilayered coating (Fig. 15.2b) (Akiba et al. 2017; Caruso et al. 1998; Donath et al. 1998), which extended the application of LbL technology to three-dimensional systems and in turn dramatically expanded research on LbL techniques. Over the years, researchers have revealed the application potential of LbL technology in diverse areas, including the fabrication of multilayered reactors (Sun et al. 2015), conducting electrodes (Cho et al. 2020) and the development of stimuli-responsive drug release systems (Huang et al. 2015). In cases of liposomes as an extensively used drug vehicle, their versatility and functionalities have been greatly enhanced by the application of LbL technology. The present chapter aims at illustrating the molecular design and engineering of LbL functionalized liposomes as advanced systemic delivery systems for future design and development of biogerontological interventions. In fact, besides liposomes, there are other types of nanocarriers that have been exploited for treatment development (Fig. 15.3) (Senapati et al. 2018). Their advances in systemic delivery for biogerontological interventions have already been discussed in Sect. 2 of this book. Although this chapter focuses only on liposomes, regarding the fact that LbL functionalization is largely a physical process, it is expected that the strategies and principles presented in this chapter could also provide insights into functionalization of other nanocarriers for the development and execution of anti-aging therapies.

15.2 Preparation of LbL-Functionalized Liposomes

LbL-functionalized liposomes are prepared in three steps: fabrication of the liposome core, LbL coating and further surface modifications when necessary. The core can be prepared by several methods, among which sonication has been the most frequently used one (Caddeo et al. 2019). A drug-loaded liposome core is often prepared by

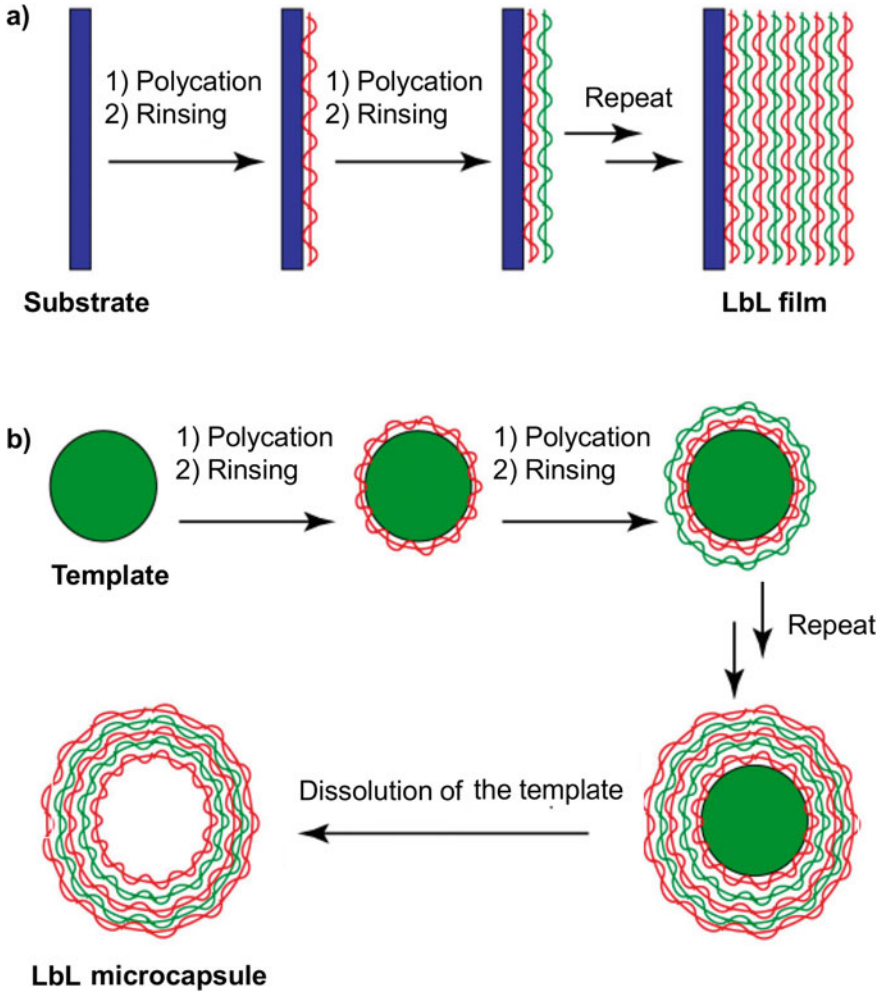

Fig. 15.2 Preparation of **a** LbL films and **b** microcapsules. Reproduced from (Akiba et al. 2017) with permission from MDPI

sonicating a dispersion containing phospholipon 90G, stearylamine and the drug molecules. The generated cores have a diameter of approximately 80 nm with a positive zeta potential due to stearylamine that is positively charged (Caddeo et al. 2019). Moreover, sonication is often used in conjunction with thin film hydration. This combination was used to fabricate quercetin-loaded liposomes by hydrating a lipid thin film with a quercetin-containing PEG solution prior to sonication and extrusion (Priprem et al. 2008). In addition, other optional methods such as detergent **dialysis** (Zumbuehl and Weder 1981), reversed phase evaporation (Szoka and Papahadjopoulos 1978), solvent-injection techniques (Deamer 1978), high pressure

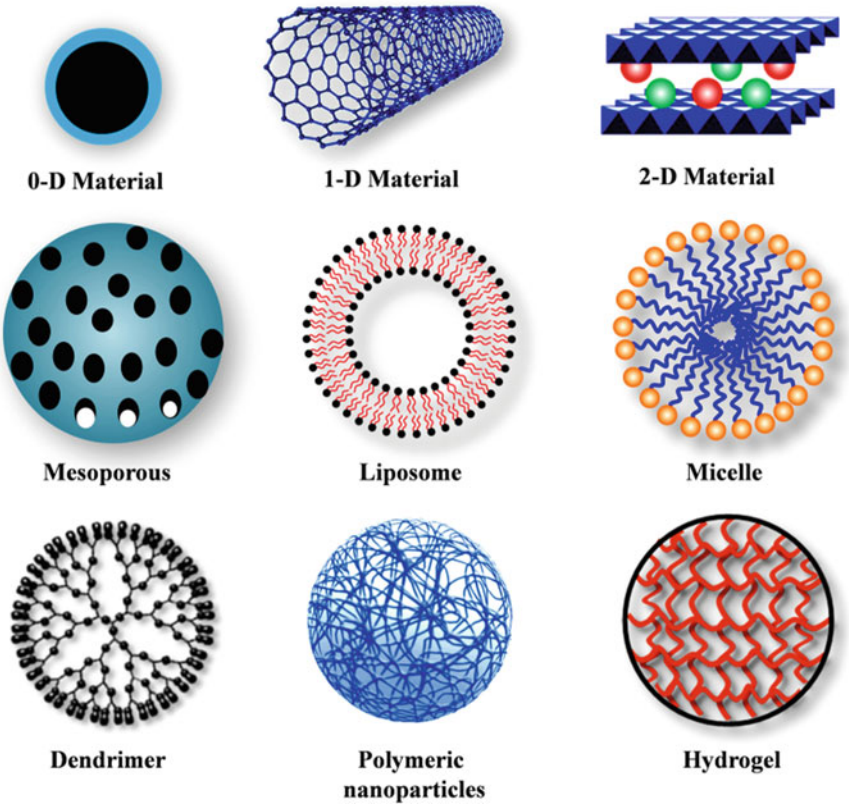

Fig. 15.3 Some of the nanocarriers studied in the literature for treatment development. Reproduced from with permission Senapati et al. (2018) from nature

extrusion (Hope et al. 1985), microfluidization (Pradhan et al. 2008), supercritical **anti-solvent** method (Lesoin et al. 2011), **dual asymmetric centrifugation** (Massing et al. 2008) and membrane contactor technology (Laouini et al. 2011) have been developed and employed for the preparation of liposome cores.

LbL functionalization is performed after the fabrication of the core by various coating methods (Ciobanu et al. 2007). The heat-up strategy, for instance, generates nanoparticles in a multishelled structure by incorporating multiple identical or different epitaxial shells onto the same metal core, which, however, is unpractical for "soft" cores (Wang et al. 2011). In cases of liposome cores, **electrostatic interactions** have been frequently employed to deposit counter-charged polyelectrolytes onto the core (Such et al. 2011; Ok et al. 2003; Correa et al. 2019). In contrast, other interfacial interactions such as hydrogen bonds (Wang et al. 1997), hydrophobic interactions (Lojou and Bianco 2004) and van der Waals forces (Sato and Sano 2005) have only been used in limited cases fundamentally due to the vast availability of polyelectrolytes for biomedical use, such as chitosan, xanthan, alginate, dextran sulfate and hyaluronic acid. Using polyelectrolytes for LbL coating provides a better control

over the biocompatibility of the end product and a straightforward design of the LbL coating protocol and operation procedures (Correa et al. 2019).

Despite the LbL coating that protects the surface phospholipids in the liposome membrane from the outer GI environment, the stability of the LbL layers would be compromised by media with a high polarity or high salt concentrations due to the nature of electrostatic interactions (Li et al. 2015). In order to overcome this limitation, additional covalent crosslinking has been incorporated to LbL systems in a recent study (Li et al. 2015) by crosslinking 4, 4′-diazostilbene-2, 2′-disulfonic acid disodium salt (DAS) into the shell via UV irradiation. The resultant product was reported to have an enhanced stability and release sustainability of the payload (Li et al. 2015). This demonstrated a success in employing a combination of multiple types of physical/chemical interactions for enhancing the stability and performance of the LbL layer.

Another exciting aspect of LbL coated drug vehicles is the capability of delivering a combination of therapies in various approaches. For instance, nucleic acids can be incorporated directly into the LbL film independent of the drugs loaded into the core (Gu et al. 2017), allowing the co-delivery of small interfering RNAs (siRNAs) and in turn an additional RNA inference therapy. The incorporated siRNAs are protected by the LbL film from premature degradation (Yao et al. 2015). In addition, a selected anionic film that targets cancer cells allows LbL siRNA carriers to deliver cargo intracellularly (Deng et al. 2013).

15.3 Principles of Molecular Design of LbL-Functionalized Liposomes

With the aid of physicochemical interactions, LbL functionalization is technically easy to perform, which, however, does not guarantee an improved delivery performance via the oral route. Proper optimization of the functionalization process is necessary to achieve a desired efficacy. For instance, LbL-functionalized liposomes have an increased particle size, which may result in an influence on cellular uptake as **M cells** can only efficiently absorb mucoadhesive particles with a size below 1 μm (Ensign et al. 2012). This has already been demonstrated by Kaminski who reported an increase in the particle size of dioctadecyldimethyl ammonium bromide (DODAB) liposomes to approximately 60–130 nm after coated with xanthan and a further increase to 165 nm after incorporation of a galactomannan layer (Kaminski et al. 2016). In order to control the increase in size, it is practical to reduce the size of the liposome core by increasing the duration of the sonication procedure and hence the energy input (Jain et al. 2012). This, however, can only decrease the diameter of the liposomes to a limited extent due to the consequent increase in surface tensions of liposome particles (Guan et al. 2011).

Surface charge of LbL-functionalized liposomes is another factor that affects the delivery performance as it determines the efficacy of cellular uptake by **Peyer's**

patches (Tomizawa et al. 1993). For instance, a negatively charged particle will be preferentially absorbed by intestinal epithelium (Tomizawa et al. 1993) due to the M cell pathway (Ling et al. 2006; Shukla et al. 2016). Proper selection of charged LbL or core materials is an essential approach to control the overall charge of the final product. To form positively charged liposomes, cationic lipids are often selected to be incorporated into the liposome membrane, such as (2,3-dioleoyl-propyl)-trimethylamine (DOTAP), dimethyl dioctadecyl ammonium bromide (DDAB), DODAB and trimethylhexadecyl ammonium bromide (CTAB) (Kaminski et al. 2016). Similarly, anionic lipids such as 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), dihexadecyl phosphate and 1,2-dipalmitoyl-sn-glycero-3-phospho-l-serine (DPPS) may be selected for the generation of liposome cores with a negative charge (Benne et al. 2018).

Conditions of the solution in which an LbL system is synthesized would significantly impact the stability, loading efficacy and delivery performance of the end product (Correa et al. 2019). However, very limited works have been carried out on the influences of solution conditions on LbL systems partially due to the sensitivity of electrostatically stabilized structures to pH, ionic strength and salt compositions (Correa et al. 2019). A few studies in this regard have been reported, including the work by Gittins et al. (2001) who demonstrated that colloidal gold particles layered more efficiently in the presence of sodium chloride but flocculated when the concentration of sodium chloride reached 30 mM or higher due to a consequent electrostatic shielding that compromises the stability of the colloidal suspension (Schneider and Decher 2008). Huang et al. (2005) reported that carbon nanotubes could only template LbL assembly at a relatively higher salt concentration (0.4–1.0 M sodium chloride). In the case of liposomes, Correa et al. (2019) illustrated that solution conditions such as pH, ionic strength, salt composition and valency had significant impacts on the synthesis efficiency of LbL liposomes, their loading capacity for nucleic acids, stability of the end product and systemic circulation duration of the loaded therapeutics.

In addition to the aforementioned parameters, the design of the composition of the liposome core can also affect the performance of the LbL product by altering particle sizes, polydispersity index and drug encapsulation efficiency (Jain et al. 2012). This has been confirmed by an earlier study (Priprem et al. 2008) which showed that a change in the molar ratio of egg phosphatidylcholine (EPC) to cholesterol in liposomes from 1:1 to 9:1 has resulted in an increase in the mean size of the liposome particles from approximately 210 to over 600 nm and a change in surface charge from −32 to −13 mV. The influences of lipid composition on the properties of the liposome core are, however, complex and relatively stochastic, and can only be determined mainly on a trial-and-error basis.

Last but not least, the chemical structures of the loaded drug are often taken into account in regards of the encapsulation efficacy, especially when structural modifications are needed for therapeutic purposes. For instance, the encapsulation of flavonoids by EPC liposomes can be affected by the position of hydroxyl groups and the presence of a sugar moiety on the flavonoid structure, concerning the strong interactions between aglycones and EPC acyl chains which result in a higher affinity for

the lipophilic region of liposomes, and comparatively weak interactions of flavonoid glycosides with the liposome membrane (Goniotaki et al. 2004). Vice versa, the liposomal formulation per se may affect the payload in return in terms of its biological activities. For example, liposomal quercetin exhibited lower cytotoxicity as compared to free quercetin in SF268, MCF7 and H460 cells, while liposomal isoscutellarein showed higher cytotoxic effects to those cancer cell lines as compared to its free form (Goniotaki et al. 2004). Therefore, the design of LbL-functionalized liposomes has to be tailored and optimized, when necessary, specifically for the properties and therapeutic purposes of the loaded cargo prior to attaining an ideal liposome prototype for oral drug delivery.

15.4 Engineering LbL Coatings to Enhance Intestinal Absorption

Liposomes subjected to oral administration will undergo destruction by gastric acid in the stomach and by intestinal surfactants and enzymes in small intestines, resulting in undesired release of the payload (Liu et al. 2015; Hu et al. 2013; Cohn et al. 2010; Kokkona et al. 2000; Tian et al. 2016). The remaining intact liposomes will penetrate the mucus layer and be absorbed by the intestinal epithelia via the M cell-to-lymph pathway as integral vesicles (Guan et al. 2015; Niu et al. 2012). Due to the complexity of the processes of digestion and intestinal absorption, specific strategies need to be employed to optimize the engineering of the LbL coating in order to enhance the performance of the functionalized liposomes for intestinal absorption.

One strategy aims to extend the elimination half-life of liposomes in small intestines by enhancing the **mucoadhesion** of liposomes to intestinal epithelium, which can be achieved by ionic interactions between positively charged LbL coatings and negatively charged constituents of mucus such as the sialic and sulfonic acid residues (Han et al. 2012; Chen et al. 2012). In this regard, polycationic materials such as chitosan and PLL are suitable candidates for the outer layer of LbL coatings (Tomizawa et al. 1993). On the other hand, exhibition of mucoadhesive properties alone is insufficient for effective oral drug delivery because the intestinal permeability of the LbL functionalized liposomes is still restricted by the turnover time of the mucus layers (Jones et al. 2009). In addition, negative charged materials were reported to facilitate subsequent intestinal absorption (Tomizawa et al. 1993). Hence, liposomes with the capacity of penetrating mucus (which increases the chance of direct contact with epithelium and thereby the uptake by clathrin- or caveolae-mediated endocytosis) are more preferable than those undergo mucus entrapment for intestinal absorption (Li et al. 2011; Zhu et al. 2013). However, commonly used polymers that show mucus penetrating ability, including Pluronic F127 (Li et al. 2011; Zhu et al. 2013) and PEG (Yuan et al. 2013), are often neutral and therefore can hardly be used directly for LbL assembly mediated by ionic interactions. A feasible way to overcome this limitation is to modify one of the polycationic or polyanionic

layers in the LbL system by incorporating these mucus penetrating moieties into the LbL coating.

Another approach to facilitate intestinal absorption is to incorporate specific ligands such as biotin (Zhang et al. 2014a, b), folic acid (Anderson et al. 1999, 2001) and mannose (Wang et al. 2015) into the LbL coating. Although evidence of this approach is still limited, the feasibility of this concept has been supported by an earlier study (Verma et al. 2016) where vitamin B_{12} was conjugated to chitosan via carbodiimide chemistry for the preparation of LbL liposomes in order to facilitate receptor-mediated endocytosis. By applying this concept to incorporate the surface of the coated liposomes with targeting ligands, it is expected that the liposomes generated can exhibit enhanced adhesion and accumulation at the absorption sites via ligand–receptor interaction and can undergo pinocytosis/phagocytosis by both the antigen-presenting cells in the GI tract and the M cells in the follicle-associated epithelia of Peyer's patches. Despite the fact that M cells only take approximately 1% of the total intestinal epithelial cell population (Giannasca et al. 1999; Lopes et al. 2014), the M cell pathway is one of the preferable absorption mechanisms for liposomes as it involves less membrane hydrolases, fewer lysosomes and less glycocalyx (Agrawal et al. 2014). Additionally, M cells are exposed to chime without secreting mucus, which thereby provides an outstanding accessibility for liposomal absorption via endocytosis and phagocytosis (Buda et al. 2005). Therefore, the M cell pathway is one of the most important routes of liposomal absorption upon oral administration and should be accounted for the engineering of LbL delivery systems in high priority.

15.5 Summary and Outlook

The limitations of conventional liposomes, including poor stability, poor intestinal absorption and low resistance to GI destabilizing factors, have inevitably hindered their applications in oral drug delivery for an extended period of time. The emergence of LbL technology may have the potential to take liposomal drug delivery systems to the next level by overcoming these limitations. The increasingly rapid pace of research in the field of electrostatically assembled drug delivery systems is revealing the promise of LbL liposomes for therapeutic applications (Correa et al. 2016). The LbL technology has proven to be ideal for generating new drug formulations for a broad variety of therapeutic agents, including phytochemicals, peptides, nucleic acids etc. (Gu et al. 2017; Yao et al. 2015; Goniotaki et al. 2004; Correa et al. 2016). However, development of LbL technology for oral drug delivery is still at an early stage in regards to numerous technical challenges such as toxicity concerns, high polydispersity and particle aggregation issues (Correa et al. 2019, 2016; Goniotaki et al. 2004), which need to be carefully addressed prior to extensive clinical applications. Moreover, in consideration of the various molecular forces and physicochemical processes that mediate the preparation of LbL products and affect the

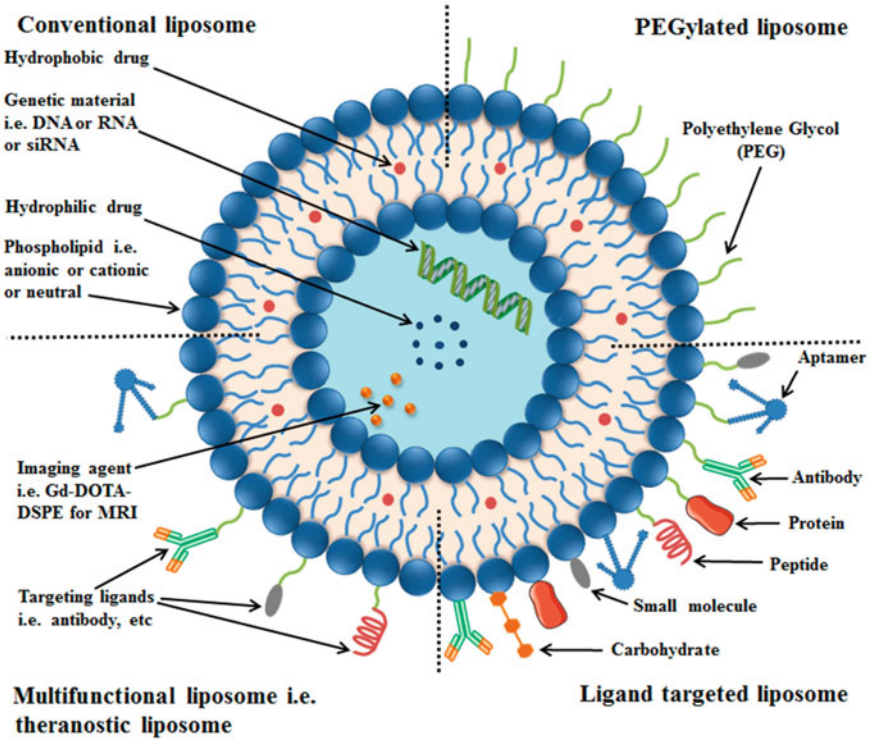

Fig. 15.4 Some ligands commonly used to modify the surface of liposomes. Reproduced from Riaz et al. (2018) with permission from MDPI

encapsulation efficacy, stability and bioactivities of the drug delivery system, optimization of the conditions and specific engineering strategies need to be designed accordingly.

Future research on LbL-functionalized drug delivery systems should further focus on improving the stepwise assembly process which allows spatial separation of the encapsulated drugs and functional constituents in or exterior to the core. This will enable the design of delivery systems that are capable of releasing a series of therapeutic agents in a controlled and pre-programmed manner with high efficacy and precision. Also, ligand conjugation may be incorporated into the outermost layer of the LbL coating to enhance the versatility and performance of the liposomes during intervention execution in future anti-aging medicine (Fig. 15.4) (Riaz et al. 2018). Finally, dedications should be taken for development of rapid assembly and mass manufacture of LbL systems, as well as the stability during long-term storage. These will facilitate the translation of LbL drug delivery systems from laboratory research to clinical practice.

Important Notes

- Conventional liposomes as a popular drug delivery system have significant limitations, of which the most notable ones are the instability in the gastrointestinal tract and poor intestinal absorption.
- LbL-functionalized drug delivery systems are prepared by coating a multilayer of polymeric LbL films onto a liposome core using interfacial physicochemical interactions such as, in the majority of cases, electrostatic interactions.
- The design of LbL coating is based on several molecular principles, including particle size, surface charge, conditions of the solution in which the system is synthesized, lipid composition of the liposome core and chemical structure of the loaded drug.
- Commonly used strategies for engineering LbL coatings for enhancing intestinal absorption include using mucoadhesive/mucus penetrating materials for the LbL films and incorporating specific ligands into the LbL coating. These strategies aim to improve the cellular targeting function and cellular uptake.
- Technical challenges of the LbL technology need to be addressed prior to extensive clinical applications, such as toxicity concerns, high polydispersity and particle aggregation issues.

Questions for Future Research

- **How to control the particle size of LbL functionalized liposomes**? Increasing the duration and energy input of the sonication process would theoretically reduce the size of liposomes. The resulted reduction in size, however, has a limit due to the gradually increased surface tensions (hydrophobic interaction energy) during the process of sonication. In order to breakthrough this threshold, certain parameters need to be adjusted according to the surface thermodynamic theory to control the increase in hydrophobic interactions, such as selection of the solvent, temperature control and adjusting suspension density.
- **How to select materials for the LbL layers considering the surface charge of the end product**? The surface charge of a drug carrier should be designed according to the intended pathway of the therapeutic agent. A negatively charged particle will be preferentially absorbed by intestinal epithelium, while a positive charged particle possesses mucoadhesion property in small intestines. Therefore, the surface charge of the end product should be selected based on the targeted cells and the absorption pathways.
- **How to select the conditions of the solution in which the LbL system is synthesized**? Solution conditions such as pH, ionic strength, temperature

and salt composition can significantly influence the physical properties and bioactivities of the end product. A careful determination of the conditions is necessary and can be performed on a trial-and-error basis or by statistical modeling.

- **In cases of delivering of a combination of different therapeutic agents with the same vehicle, how can the release order and rates be controlled**? Liposomes can carry different drugs with their bilayers structure. This advantage allows co-delivery of multiple drugs. Also, each layer of the LbL coating has the capacity to carry drugs, which further enhances the co-delivery efficacy and provides spatial separation to different loaded drugs. Therefore, controlled and pre-programmed release of a combination of drugs can be achieved by engineering the drug carrier system specifically for the intended purpose.

Glossary

Anti-solvent A solvent in which the agent-of-interest shows poor solubility and precipitates.

Dialysis A method of separating chemical entities based on diffusion across a semi-permeable membrane.

Dual asymmetric centrifugation A centrifugation technique in which the container spans around the axis in the middle of the centrifuge and also around the axis in the middle of the container.

Electrostatic interactions The net result of attractive forces between co-charged molecules or the repulsive forces between counter-charged molecules.

Hydrophobic interactions The interactions among apolar or weakly polar molecules in an aqueous environment.

M cells Specialized epithelial cells of the mucosa-associated lymphoid tissues.

Mucoadhesion The adhesion of a foreign material to mucosal surfaces.

Mucus The viscous liquid consisting predominately of glycoproteins, namely mucins.

Pever's patches Small masses of lymphatic tissue found throughout the ileum region of the small intestine.

Polyelectrolyte A polymer having ionizable groups along the polymer chain.

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Chapter 16
Use of Cell-Penetrating Peptides to Enhance Delivery Performance

Toru Miwa and Kazuhito Tomizawa

Abstract Surface modification, as presented in Chaps. 14 and 15, is one of the commonly used strategies in enhancing the chemical and biological properties of a carrier for systemic therapeutics delivery. Apart from enhancing the stability of the carrier or extending the residence time of the carrier in plasma, once the carrier reaches a target cell, surface modification can enhance the internalization of the carrier into the cell for action. One method of achieving this is to decorate the carrier surface with the cell-penetrating peptide (CPP), which is a peptide that can import large cargoes across nuclear membrane. In this chapter, we will use hearing loss as an example to illustrate the possible use of the CPP, namely poly-arginine (6–12 residues), to enhance cellular internalization of an exogenous agent. Though the short half-life and non-specific transduction of CPP are some drawbacks of using CPP in carrier design, recent studies have already provided some clues for overcoming these problems, and have shed light on the use of CPPs for design of systemic delivery systems in the future.

Keywords Sensorineural hearing loss (SNHL) · Cell-penetrating peptides (CPPs) · Embryonic inner ear · Adult inner ear

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16.1 Introduction

Over the years, different methods have been proposed for enhancing the efficiency of cellular uptake. One of the methods is the use of the **cell-penetrating peptide** (CPP), which is a peptide that can import large cargoes across nuclear membrane. To demonstrate the possible use of CPP in carrier optimization for enhanced delivery performance, hearing loss will be used as a model. There are two reasons for this. The first one is because the model of hearing loss is highly accessible for local intervention administration. This facilitates the monitoring of the intervention process. Another reason is because hearing loss is not only an age-related symptom, it may also affect adults and even children. In fact, according to the most recent estimate by the World Health Organization, about 360 million people (approximately 5.3% global population) suffer from disabling hearing loss (World Health Organization 2019). Hearing loss in children has been repeatedly demonstrated to affect academic, behavioral, and cognitive development and decrease overall quality of life (Wake et al. 2004). Deleterious effects of hearing loss in adults and elders also generate morbidity as it has been linked to poor overall physical functioning, social interaction, and overall decreased quality of life (Crowson et al. 2017). Medical therapies for hearing loss, including age-related hearing loss (ARHL), are not well-developed despite the number of people suffering from disabling hearing loss worldwide and multi-dimensional burden of hearing loss.

Sensorineural hearing loss (SNHL), including congenital hearing loss and ARHL, is the most common form of hearing loss globally, which is caused by inner ear dysfunction (Vos et al. 2015). The development of drugs to treat or prevent SNHL has proven to be challenging. Many investigators have sought to characterize the biochemical, molecular, and intra-cellular mechanisms in both normal state and pathological process-impaired hearing function. Physiologically, the inner ear has two basic functions: hearing, which occurs in the cochlea, and balancing, which occurs in the semicircular canals and vestibule. The cochlea is divided into three compartments: scala vestibule, scala tympani, and scala media. The scala media, a part of the endolymphatic space, contains the **organ of Corti**. The organ of Corti contains three cell population: inner **hair cells**, outer hair cells, and supporting cells (Fig. 16.1). Hair cells have stereocilia that emerge from their apical surface. Receptor potentials, generated by deflection of the stereocilia within the inner hair cells, induce neurotransmitter release at the synaptic ends (Jia et al. 2007). Therefore, sound waves are transmitted via the outer and middle ear to the inner ear fluid in the cochlea before being transduced to electrical signals via inner hair cells. These signals are subsequently transmitted to the brain via efferent neurons and perceived as sound.

The mammalian inner ear and its sensory neurons develop from the otic placode, a thickened patch of head ectoderm (Fritzsch et al. 1998). Subsequently, one **otocyst** per side is formed by invagination of the otic placode at the level of the hindbrain at 4 weeks gestation in humans and embryonic day 9.5 (E9.5) in mice. Soon after, the otocyst forms and neuroblasts delaminate from the ventral region of the otocyst. These neuroblasts coalesce adjacent to the developing inner ear and begin to form

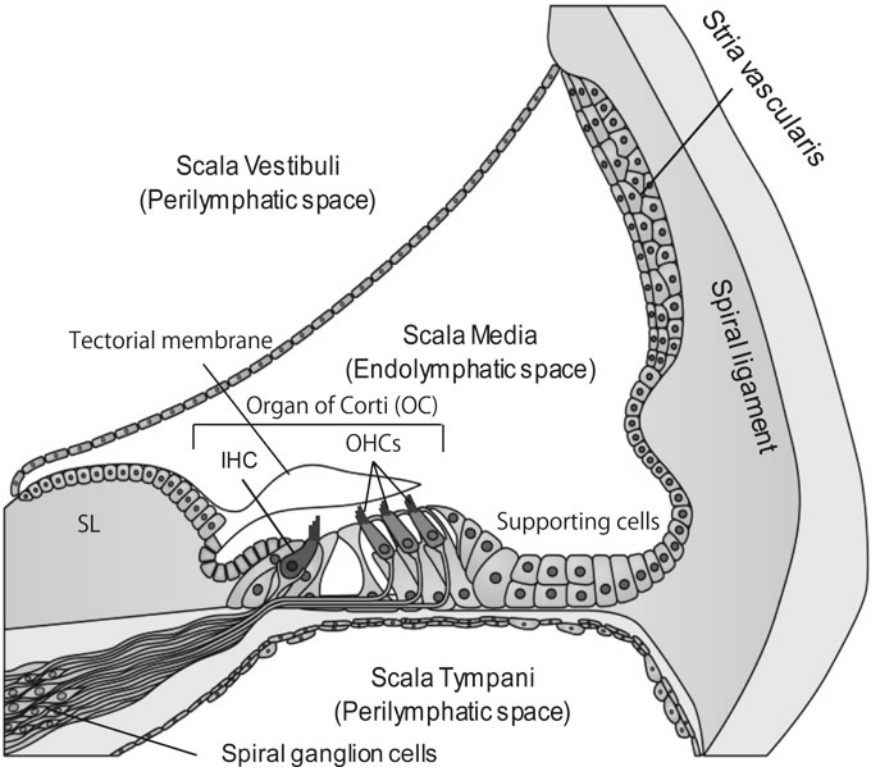

Fig. 16.1 Transverse section image of the cochlea. The adult mammalian cochlea is divided into three compartments: the scala vestibule, scala tympani, and scala media. This image represents a cross-section of the scala media, which contains the OC. The OC contains three cell populations: IHCs, OHCs, and SCs. The two types of auditory hair cells (IHCs and OHCs) have critical roles as mechanoelectrical transducers for hearing. Auditory hair cells are covered by the tectorial membrane. The stria vascularis, located in the lateral wall of the scala media, is responsible for K^+ secretion into the endolymph and endocochlear potential production. Reproduced from Minoda et al. (2015) with permission from Springer Nature

the statoacoustic ganglion (Morsli et al. 1998). By E12.5 in mice, the positions of the developing sensory patches, which form from a single common patch in the otocyst, can be identified (Kelley 2006). The cochlear part of the otocyst begins to elongate into a spiral structure. The two and one-half turns of the coiled cochlea are not completed until 25 weeks gestation in humans, while the mouse cochlear duct completes three-quarters of one turn around E13.5. The appropriate spatiotemporal control of gene expression is necessary for normal development of the inner ear. Inappropriate spatiotemporal control or cochlear damage by acoustic trauma, aging, drugs, or other sources can lead to SNHL. Once the auditory pathway is damaged, non-regenerative and irreversible conditions can occur after a sufficient timespan. Therefore, the major obstacles for SNHL therapies are limited treatment time window

and the complicated cochlear anatomy (Crowson et al. 2017; Ahmadzai et al. 2019; Sabbagh et al. 2017).

There are three important time points in hearing loss treatments: (1) before hearing loss, when the auditory pathway is not yet damaged, (2) immediately after hearing loss, when the auditory pathway has been damaged but can still be restored, and (3) after hearing loss, when the auditory pathway is already damaged in a non-regenerative/irreversible condition (Fig. 16.2). In short, therapies administered before or immediately after hearing loss aim to prevent irreversible damage to the auditory pathway. In contrast, treatments after hearing loss are more complicated as they must regenerate the damaged cochlea. Therefore, current hearing loss treatment modalities have focused on the first two time points. An anatomical problem is also involved in treating hearing loss. The unique and complex anatomic structure of the mammalian inner ear presents an obstacle that contributes to the lack of effective and safe delivery methods for therapeutic drugs or molecules into the cochlea (Figs. 16.1 and 16.2). In this chapter, we will exploit the use of cell-penetrating peptide (CPP)-mediated protein transduction as a technique for SNHL therapy. Our objective is to critically discuss the use of CPP-mediated protein transduction in the mammalian inner ear for SNHL treatment, including congenital hearing loss and ARHL, and based on the findings, we hope to exploit the possible routes for future design of carriers for systemic delivery that show enhanced performance also at the level of cellular internalization.

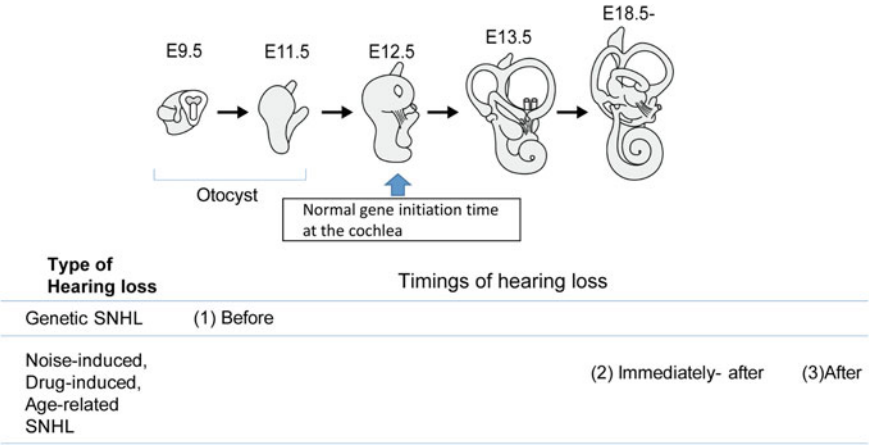

Fig. 16.2 Types and timing of hearing loss. There are three important time points to recognize while treating hearing loss: (1) before the hearing loss, when the auditory pathway has not yet been damaged, (2) immediately after the hearing loss, when the auditory pathway has been damaging but normal function still can be restored, and (3) after the hearing loss, when the auditory pathway has already been damaged and is in a non-regenerative/irreversible condition. In short, therapies administered before or immediately after hearing loss aim to prevent irreversible damage to the auditory pathway. In contrast, treatments after hearing loss are more complicated because they must regenerate the damaged cochlea. Therefore, therapies targeting the time points (1) and (2) have previously been attempted. Reproduced from Minoda et al. (2015) with permission from Springer Nature, and from Miwa et al. (2019) with permission from Biology Open

16.2 Experimental Section

16.2.1 Ethical Approval

All animal experiments were approved by the Committee on the Use and Care of Animals at Kumamoto University and the National Defense Medical College. They were performed according to accepted veterinary standards.

16.2.2 Recombinant Proteins

Enhanced green fluorescent protein (EGFP) fused to a nine-arginine peptide (EGFP-9R) and recombinant EGFP-9R were expressed and purified. In brief, the constructed plasmids were transfected into BL21-DE3 *Escherichia coli* cells. Protein expression was induced by 0.1 mM isopropyl 1-thio-b-D-galactopyranoside. The expressed proteins were purified using a nickelnitrilotriacetic acid-agarose column (Invitrogen, Carlsbad, CA, USA). The proteins were stored after dialysis against phosphate-buffered saline (PBS). For recombinant **X-linked inhibitor of apoptosis protein** (XIAP) and XIAP fused to 9R (XIAP-9R) purification, mouse full-length Xiap cDNA was amplified through polymerase chain reaction using appropriate linker primers before subcloning into the EcoRI–XhoI or BamHI–EcoRI sites of pET21a(+) (Novagen, Madison, WI, USA). Subcloning facilitated fusion with the 9R sequence at the carboxyl terminus. The proteins were similarly generated and stored.

16.2.3 Otocystic Inoculation of Recombinant Proteins into Mouse Embryos

Pregnant CD-1 mice at 11.5 days postcoitum were deeply anesthetized with intraperitoneal administration of 4 mg/kg xylazine (Bayer, Shawnee Mission, KC, USA) and 120 mg/kg ketamine–HCl (Daiichisankyo, Tokyo, Japan) in 0.9% NaCl. The abdominal wall was sterilized with 70% alcohol and 10% povidone–iodine. The uterus was exposed using low midline laparotomy before being placed on a transparent surgical stage and illuminated from beneath with a fiber optic beam to visualize the rostral and caudal branches of the primary head vein, between which the otocyst resides. EGFP-9R or EGFP supplemented with the tracking dye 0.1% fast green (Sigma-Aldrich, St. Louis, MI, USA) was injected via oral pressure into the lumen of the left-side otocyst (Fig. 16.3, arrow) using a heat-pulled glass micropipette connected to a Silastic tube with an outer diameter of 5 mm. During surgery, the locations of the embryos that had undergone inoculation were recorded so that they could

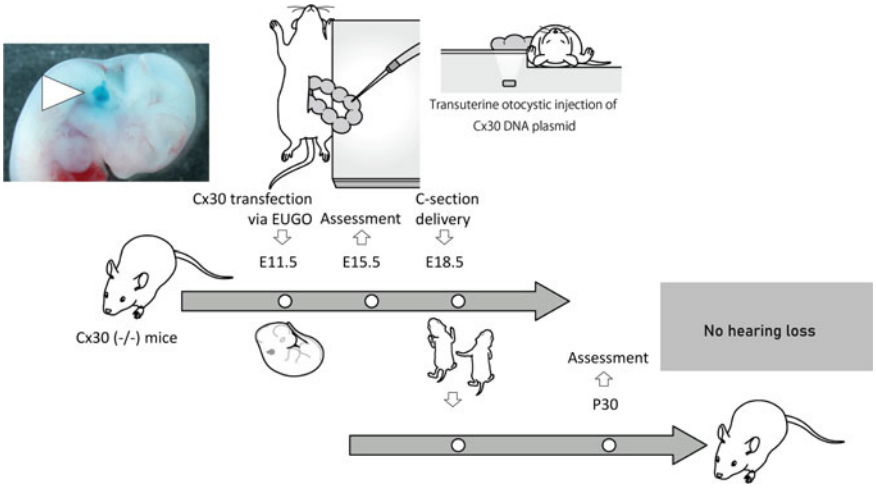

Fig. 16.3 The embryonic stage is the ideal period for genetic hearing loss treatment. We report that transuterine gene transfer into the embryonic inner ear in Cx30-knockout mice successfully treated hearing loss. Gene transfer was performed by embryonic gene transfer in the developing inner ear in Cx30-deficient mice, which successfully prevented the subsequent manifestation of the hearing loss phenotype. Electroporation-mediated transuterine gene transfer was performed in otocysts (EUGO) in Cx30-deficient mice at E11.5. Embryos were delivered via C-section at E18.5 and the pups that underwent gene transfer at E11.5 were passed to surrogate dams to raise the embryos. These pups did not demonstrate hearing loss at P30. Reproduced from Minoda et al. (2015) with permission from Springer Nature, and from Miwa et al. (2019) with permission from Biology Open

be identified later. The abdominal skin was closed with a stapler (MikRon Precision, Inc., Gardena, CA, USA), and 30 mg/kg chloramphenicol (Daiichisankyo) was administered intraperitoneally.

Pregnant mice whose embryos had undergone otocystic inoculation were euthanized under deep anesthesia at 3, 6, 12, 18, and 24 h after inoculation. The embryos were harvested and fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C. The tissues were aligned for sectioning, frozen in a dry ice/alcohol mixture, and stored at −80 °C until sectioning. For cryostat sections, the entire head was embedded in OCT compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan) and 10 mm thin sections were made serially. The cryostat sections were washed three times with PBS (5 min per wash). The nuclei were stained with Hoechst 33,258 dye (Molecular Probes, Eugene, Oregon, USA) for 30 s. The specimens were mounted on glass slides with Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA) and examined under a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

16.2.4 Treatment Via the Round Window Membrane in Adult Guinea Pigs

Hartley guinea pigs weighing 250–300 g each (Kyudo, Co., Tosu, Saga, Japan) were used for the experiments to verify CPP-mediated protein transduction treatment in noise-induced hearing loss. The EGFP and EGFP-9R experiments involved three groups: single EGFP application (s-EGFP group), single EGFP-9R application (s-EGFP-9R group), and double EGFP-9R application (d-EGFP-9R group) in animals. In the third group animals, EGFP-9R was re-applied 24 h after the initial application in the following manner. Guinea pigs were anesthetized via intraperitoneal administration of 10 mg/kg xylazine (Bayer) and 40 mg/kg ketamine-HCl (Daiichisankyo) in 0.9% NaCl. The animals underwent a postauricular incision and the mastoid bullae were opened. Subsequently, a 5–10 mm^3 gelatin sponge (Astellas Pharma Inc., Tokyo, Japan) soaked in 5 μL EGFP (24.4 mg/mL) or EGFP-9R (4.0 mg/mL) was placed on the round window niche of the left ear before intraperitoneally administering 20 mg/kg chloramphenicol. The holes in the mastoid bullae were sealed immediately with muscle tissue and the skin was closed. Cochleae were extracted 12, 24, 48, and 72 h after protein transduction from the s-EGFP-9R group animals under deep anesthesia using an overdose of xylazine-HCl and ketamine-HCl. The cochleae were extracted from the s-EGFP group animals 12 and 24 h after the treatment and 48 and 42 h after the first treatment from the d-EGFP-9R group animals. Normal untreated cochleae were used as controls and the control data were expressed as the 0 h data. Auditory functions of the s-EGFP and s-EGFP-9R group animals were analyzed 28 days after treatment and they were subsequently euthanized for further morphological analyses.

The XIAP and XIAP-9R experiments involved three groups of 4-week-old Hartley guinea pigs (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan): a single XIAP application group (s-XIAP), a single XIAP-9R application group (s-XIAP-9R), and a saline application group (saline) in animals. Under general anesthesia with ketamine and xylazine, a 5–10 mm^3 gelatin sponge soaked in 0.1 mg/mL XIAP or XIAP-9R was placed on the RW niche in the right ear of the animals 12 h before noise exposure. A gelatin sponge immersed in saline was used as the control. Subsequently, auditory functional analyses and morphological analyses were performed.

16.2.5 Noise Exposure

Guinea pigs were anesthetized by intraperitoneally administering 50 mg/kg ketamine and 1.0 mg/kg medetomidine (ChemScene, Monmouth junction, NJ, USA) and exposed to an octave band noise centered at 4 kHz at 116 dB sound pressure level (SPL) for 2 h in a ventilated sound exposure chamber. The sound chamber was fitted with speakers (Model 2380A; JBL, Northridge, CA, USA) driven by a noise generator (DANAC-31; Dana Japan, Tokyo, Japan) and a power amplifier (D-45; Crown

International, Elkhart, IN, USA). Sound levels were calibrated (Type 6224 precision sound level meter; Aco Instruments, Tokyo, Japan) at multiple locations within the sound chamber to ensure stimulus uniformity.

16.2.6 Auditory Thresholds

Auditory thresholds were measured using the auditory brainstem response (ABR, System 3; Tucker-Davis Technologies, Alachua, FL, USA). The animals were anesthetized by intraperitoneally administering xylazine and ketamine. Electrodes were placed beneath the pinna of the treated ear and at the vertex just below the surface of the skin. The ground electrode was placed under the contralateral ear. An average of 1024 sweeps were calculated for 4, 8, and 12 kHz in the s-EGFP and s-EGFP-9R groups and for 4, 8, 16, and 32 kHz in the s-XIAP and s-XIAP-9R groups. The stimulus levels near the threshold were varied in 5-dB increments, and the threshold was defined as the lowest level at which waves in the ABR could be clearly detected via visual inspection. The hearing thresholds were measured 28 days after protein transduction in the s-EGFP and s-EGFP-9R groups. In the s-XIAP and s-XIAP-9R groups, the ABR was measured 1 and 14 days before and after noise exposure, respectively.

16.3 Results and Discussion

16.3.1 Protein Transduction into Embryonic Inner Ear in Mus musculus

To demonstrate the effect of CPP in enhancing delivery performance, we use a hearing loss mouse model, in which we targeted the developing inner ear of the model that had a loss-of-function mutation leading to congenital hearing loss. One important issue regarding embryonic treatments is the need to treat embryonic inner ears in the maternal uterus, which is technically feasible but not facile. The otocyst, which is formed by invagination of the otic placode on each side of the head at the level of the hindbrain at E9.5 in mice, may be the best target because it is a closed and isolated epithelial vesicle that is the origin of the anatomical structure of the adult inner ear (Miwa et al. 2011, 2013, 2017, 2019; Minoda et al. 2015). Various approaches like the use of viral vectors, **electroporation**, microinjection, and liposome encapsulation have been used to introduce target genes and molecules into otocysts to manipulate the cells (Miwa et al. 2013, 2017, 2019; Minoda et al. 2015; Brigande et al. 2009; Bedrosian et al. 2006). However, there have been few reports on direct manipulation of otocysts in mammals in vivo because mammal otocyst inoculation requires an intricate surgical technique. The otocysts cannot be visualized directly and it is necessary to manipulate the embryos via the uterine wall.

Previous studies have demonstrated successful otocystic inoculation of exogenous genes in mice using viral vectors (Bedrosian et al. 2006). However, these strategies have several limitations like low transfection efficiency, cell type restrictions, cellular toxicity, and the requirement for optimization for each tissue (Bedrosian et al. 2006). We reported successful treatments via electroporation-mediated transuterine gene transfer to the embryonic inner ear in Cx30-knockout mice (Miwa et al. 2013). **Connexins** (Cx), which are proteins that assemble to form vertebrate gap junctions, are crucial for auditory function (Wang et al. 2011a) and a large deletion within the Cx30 gene is the second most frequent cause of non-syndromic SNHL (Castillo et al. 2002). Homozygous Cx30 deleted-mice have severe hearing impairment and demonstrate a complete loss of endocochlear potential, which represents the transepithelial difference in electric potential between the endolymphatic and perilymphatic compartments and is crucial for normal hearing function (Teubner et al. 2003). We used electroporation-mediated transuterine gene transfer to otocysts at E11.5, and induced robust transgene expression in the cochleae of developing inner ears.

Consequently, we showed that gene supplementation to insert the wild-type Cx30 gene into the otocysts of E11.5 Cx30-knockout mice prevented postnatal hearing loss (Fig. 16.3). These results demonstrated that the induction of a target gene before the "normal gene initiation time" can prevent postnatal hearing loss. Electroporation-mediated gene transfer is effective for transfecting genes into a wide range of cell types, including the lining cells and its vicinity of the otocysts. However, it has several drawbacks as the technique has two major steps: inoculation of a plasmid and electroporation. The long exposure time of the embryos required for these two-step manipulations increases the risk of embryo survival after manipulation. In addition, the non-optimized electroporation conditions may cause cell toxicity or less efficient gene transfection. Therefore, we used CPP to enhance the efficiency of protein transduction to reduce the steps. CPPs are short cationic peptides with the ability to traverse the cell membranes of several mammalian cell types (Schmidt et al. 2010). Protein transduction does not require manipulation of multiple steps, and in vivo protein transduction using octa-arginine or nona-arginine gave high transfection efficiency with no cell specificity and minimal cytotoxicity (Guidotti et al. 2017). Attachment of polyarginine peptides to high molecular weight therapeutic molecules should prove to be useful for facilitating their penetration to therapeutic targets in the inner ear. We showed that a peptide comprised 9R residues effectively delivered EGFP into the developing inner ear cells and adjacent cells when EGFP-9R was inoculated into mouse otocysts. Furthermore, the EGFP remained active for 12–18 h after otocystic inoculation without causing any deterioration of auditory or vestibular function (Miwa et al. 2011) (Fig. 16.4). CPP-mediated protein transduction into otocysts is a simpler method compared to electroporation-mediated gene transfer and may be an effective and safe method of delivering target proteins into and around the lining cells of otocysts. However, CPP-mediated otocystic transduction has several drawbacks, including a short half-life and non-specific targeting of the CPP-transduced proteins. EGFP-9R levels in otocysts were not maintained for 24 h. The short half-life may limit its usefulness. Repeated application of the proteins may resolve this problem. However, it is technically difficult to repeatedly inoculate a

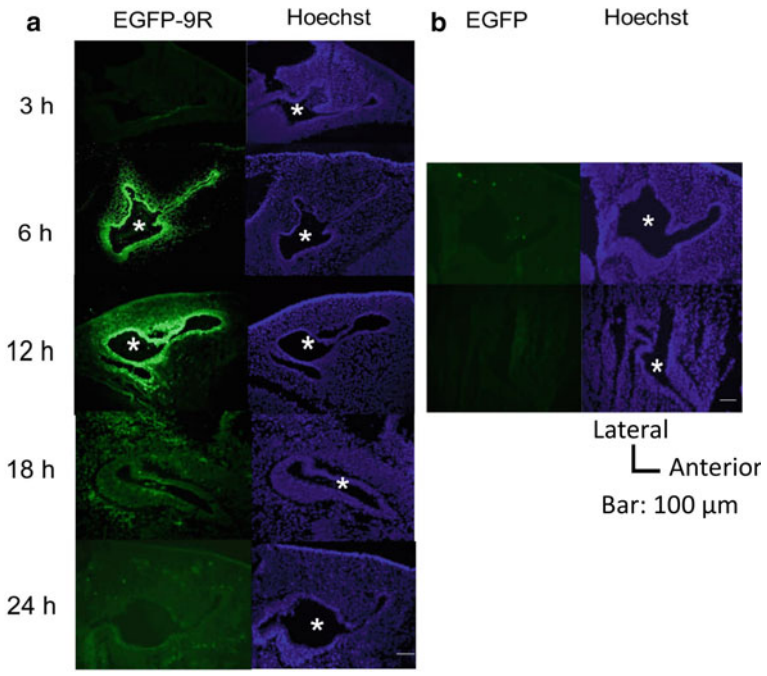

Fig. 16.4 Sequential images of EGFP fused to a nine-arginine peptide (EGFP-9R)-inoculated otocysts fluorescently labeled with EGFP-9R (green) and Hoechst stain (blue). **a** EGFP expression was clearly detected at 6 and 12 h, but it was undetected at 18 and 24 h. **b** EGFP non-fused to a nine-arginine peptide (EGFP)-inoculated otocysts fluorescently labeled with EGFP (green) and Hoechst stain (blue). EGFP expression was not detected at any stage. The scale bar indicates 100 µm. Reproduced from Miwa et al. (2011) with permission from Springer Nature

substance into the developing inner ear. Moreover, repeated application may disturb the normal tissue development (and in this case, the development of the inner ear) (Miwa et al. 2011).

16.3.2 Protein Transduction into Adult Inner Ear in Guinea Pigs

In the preceding section, we use mice as a model to demonstrate the possibility of using CPP in enhancing therapeutics transfer. In fact, we have already reported the use of CPP-mediated protein transduction therapy against noise-induced hearing loss in mice in the literature (Takeda et al. 2016). Generally, systemic application and intratympanic administration of dexamethasone are adapted as clinical therapies for recent hearing loss (Crowson et al. 2017; Sabbagh et al. 2017). However, the inner ear is isolated from the systemic circulation and middle ear by the "blood–labyrinth

barrier" (BLB) (Liu et al. 2013) and the **round window membrane** (RWM), respectively. These complex structures make drug delivery into the inner ear difficult (Liu et al. 2013; Borkholder et al. 2014). The RWM is the membranous septum between the middle ear and the perilymphatic space in the cochleae in humans, monkeys, felines, and rodents. The development of treatment delivery strategies may be challenging due to the limited permeability of the RWM. Permeability through the RWM can be affected by factors like molecule size, configuration, concentration, liposolubility, electrical charge level, and membrane thickness (Borkholder et al. 2014). In vivo experiments have revealed that 1-μm microspheres can traverse chinchilla RWMs, but 3-μm spheres cannot (Goycoolea and Lundman 1997). Lundman et al. (1992) demonstrated that the passage of endotoxins with a molecular weight >100,000 kDa through a normal RWM was limited. Thus, low molecular weight molecules pass through the RWM, and high molecular weight molecules do not readily pass through the RWM. Indeed, intratympanic drug therapy utilizing low molecular weight molecules has been performed successfully for recent hearing loss in the clinic and hospital. Two examples include aminoglycoside antibiotics (e.g., gentamicin: molecular weight is 478) for the treatment of Meniere's disease and glucocorticoids (e.g., dexamethasone: molecular weight is 392) for the treatment of SNHL (Crowson et al. 2017; Borkholder et al. 2014). Although the impenetrable nature of the RWM for high molecular weight molecules may be part of the inner ear defense system, the lack of penetration by high molecular weight molecules could simultaneously constitute a barrier to good therapeutic outcomes when intratympanic drug therapy is applied via the RWM for inner ear dysfunction.

To tackle the problems mentioned above and also to further illustrate the possible use of CPP in enhancing therapeutics delivery, we examined the safety and efficacy of protein transduction using a CPP consisting of a 9R peptide (EGFP-9R) via the RW niche (Takeda et al. 2016) (Fig. 16.5). To show the feasibility of CPP-mediated treatment via the RW, we used XIAP-9R treatment prior to acoustic trauma. XIAP, with a molecular weight of 84,000, is the most potent member of the inhibitors of apoptosis (IAP) family and physically interacts with caspase-9 at its BIR3 domain and

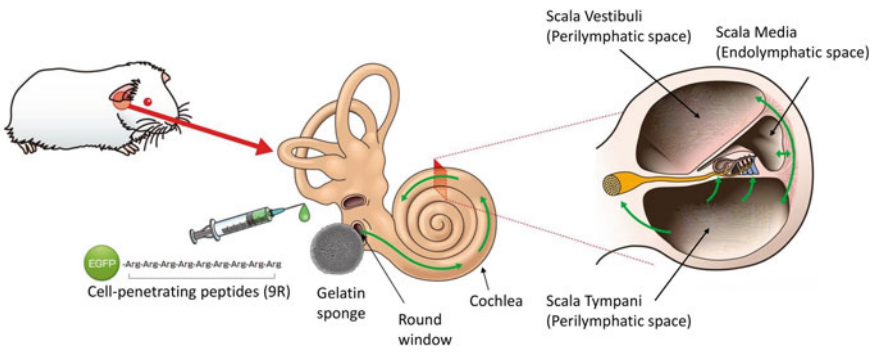

Fig. 16.5 Graphical abstract of poly-arginine-mediated treatment via RWM

with caspase-3 and caspase-7 at their BIR2 domains, thus interfering with apoptosis signaling pathways (Bratton et al. 2002; Obexer and Ausserlechner 2014). Apoptosis is a programmed cell death process that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells (McIlwain et al. 2013). This process is implicated in several inner ear disorders including drug-induced, noise-induced, age-related, and genetic hearing loss conditions (Op de Beeck et al. 2011). Caspases, a family of cysteine proteases, are essential effector molecules that initiate apoptosis (Wang and Lenardo 1997). XIAP has a protective effect against several types of inner ear damage. Tabuchi et al. (2007) have reported the protective effect of XIAP against gentamicin-induced hair cell damage in an in vitro study utilizing rat cochleae and Wang et al. (2010, 2011b) have reported that XIAP overexpressed in mice exerts a protective effect against noise-induced and age-related hearing loss in cochlear hair cells and spiral ganglion cells. Thus, XIAP appears to have protective effects against drug- and noise-induced hearing loss. However, only one report has examined the treatment efficacy of XIAP in vivo. Cooper et al. (2006) have successfully demonstrated the protective effect of XIAP against cisplatin-mediated ototoxicity using an adeno-associated viral vector injected in rat scala tympani. When considering the possible clinical applications of adeno-associated viral vector-based XIAP treatment in humans, the direct injection of viral vectors in the scala tympani, a part of the perilymphatic space, may cause inner ear damage. Moreover, the direct injection of therapeutic molecules into either the perilymphatic or the endolymphatic spaces of the cochleae have not been performed clinically because of the damage risk. Thus, there is a need for a simpler and safer strategy to administer XIAP into the cochleae than directly injecting them into the scala tympani. CPP-based XIAP treatment may be the necessary treatment modality. Additionally, this strategy may be an important treatment modality for administering other therapeutic molecules for the treatment of various inner ear diseases. We could detect XIAP-9R in the cochlea for at least 24 and 48 h after single and double administration, respectively, which significantly reduced putative hearing loss and the number of apoptotic hair cells lost in the cochleae (Fig. 16.6). Therefore, we believe that CPP-mediated protein transduction via the RWM is a proven, valid, and reliable strategy for protein transduction into the cochleae and may be a simple and promising treatment modality for decreasing cochlear damage caused by apoptotic mechanisms in SNHL. Although single CPP-mediated protein transduction via the RW niche exerts more efficient protein transduction compared to protein transduction without CPP for at least 24 h after treatment, the short duration of single CPP-mediated protein transduction may limit the usefulness of this method. Repeated applications may solve this issue. Following an additional application at 24 h after the first, protein transduction levels at 48 h after the first application were significantly higher compared to controls.

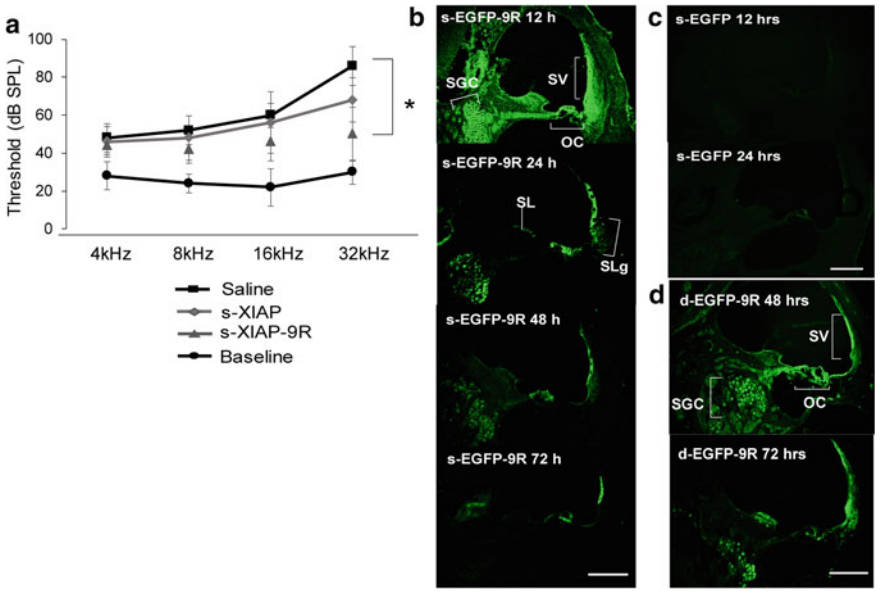

Fig. 16.6 ABR testing results 14 days after noise exposure and sectional images after enhanced green fluorescent protein (EGFP) or EGFP-9R transduction. **a** A significant difference was found in the threshold value between the s-XIAP-9R and saline groups at 32 kHz. $*P < 0.05$. **b** The sections of the middle turns of the cochleae. In the s-EGFP-9R group, EGFP was strongly detected in the SV, OC, and SGC at 12 h. EGFP was strongly detected in the SV, OC, and SGC and slightly detected in the spiral limbus (SL) and spiral ligament (SLg) at 24 h. EGFP levels were moderately detected in the SV and SG at 48 h and only slightly in the SV at 72 h. **c** At the middle turn of the cochleae in the s-EGFP group, EGFP was slightly detected in the SGC and SV at 12 and 24 h, respectively. **d** At the middle turn of the cochleae in the d-EGFP-9R group, EGFP was significantly detected in the SV and SGC at 48 h. The scale bars indicate 50 μm. Reproduced from Takeda et al. (2016) with permission from Elsevier B.V.

16.4 Summary and Outlooks

As described above by using SNHL as an example, the important element that should be carefully considered for the use of CPP in intervention execution is the short blood plasma half-life of CPPs administered in vivo. Indeed, extracellular proteases may degrade the delivery carriers before they reach the target site, reducing the efficacy of the conjugated drugs. Greater stability can be achieved using non-natural amino acids or D enantiomers that are less sensitive to enzyme degradation than the L enantiomers (Järver et al. 2010). CPP–cargo complexes internalized by endocytosis can also be subjected to degradation by acidic pH when they are trafficked into late endosomes or lysosomes. Thus, endosomal escape must be highly efficient to facilitate the early release of therapeutic cargoes from endosomes into the cytosol (Varkouhi et al. 2011). Mäe M et al. have reported strategies based on the use of PepFects (PFs), a series of peptides originally derived from the amphipathic CPP Transportan 10, to

overcome CPP–cargo entrapment in the endosomal compartments. PFs significantly promote endosomal escape and improved delivery efficiency (Mäe et al. 2009). Thus, protein transduction using CPPs with non-natural amino acids, D enantiomers, PFs, and adjusted acidic pH may be useful for protein transduction into the adult and embryonic inner ear.

Here it is worth noting the limitation in systemic administration. While systemic delivery can help to tackle age-associated symptoms (e.g., genetic mutations and mitochondrial dysfunction) bodywide, it might not be effective when tacking some local age-associated symptoms (e.g., hearing loss). The non-specific nature of CPP-mediated delivery upon systemic administration may, therefore, be a drawback when considering clinical applications to tackle local age-associated symptoms. In this case, site-targeted or tissue-targeted delivery would, thus, be more preferable. CPP internalization can be limited to certain cell types and may depend on cell-specific membrane constituents or lipid composition (Koppelhus et al. 2002). In addition, it has been reported that only moderate CPP translocation can occur through tight junction-forming epithelial cell layers under physiological conditions (Violini et al. 2002; Simon et al. 2011; Lindgren et al. 2004), but CPP uptake can be enhanced under inflammatory circumstances, in which tight junctions are compromised (Foerg et al. 2007). Thus, it is reasonable to hypothesize that a greater understanding of the internalization mechanisms exploited by these peptides may facilitate the design of CPPs capable of targeting specific cell types or tissues. The direct consequence of this achievement would be the optimization of the therapeutic efficacy of CPPs and a possible decrease in the toxic side effects caused by their non-specific actions. Since each tissue expresses specific markers like proteins or receptors on its vasculature (called "vascular bed-specific zip codes"), a proposed method to improve the specificity of CPP-derived therapeutics was the insertion of homing peptide ligands into CPP–cargo complexes. Homing peptide ligands are meant to target tissue- or cell-specific receptors, enabling selective drug delivery. A similar approach can also be exploited to target CPP-derived therapeutics toward intracellular organelles like mitochondria, lysosomes, the Golgi apparatus, or the nucleus (Svensen et al. 2012). A groundbreaking advancement toward improving CPP specificity was achieved by the recent development of activatable CPPs (ACPPs), which comprise peptides whose adsorption and cellular uptake is minimized by a covalently attached inhibitory domain. Cleavage of the linker connecting the inhibitory and CPP moieties by tissue-specific proteases dissociates the inhibitory domain, enabling the cleaved ACPP to enter cells. This strategy has been mainly used in tumor-affected tissues, which are characterized by a specific microenvironment with upregulated proteases, acidic pH, lower transmembrane potential, and hypoxia. These elements can be exploited to selectively activate ACPPs (Reissmann 2014). Tsien RY's group developed the first protease ACPP able to target many xenograft tumor models from various cancer sites

and a transgenic spontaneous breast cancer model. Membrane-bound and secreted proteases of cancer cells, mostly matrix metalloproteases, can cleave the linker between the polycationic CPP and the polyanionic neutralizer, thereby activating the CPP. After activation, the released peptide can transport cargoes into tumors and metastatic cells (Aguilera et al. 2009; Olson et al. 2009). Thus, the attachment of homing peptides or other targeting motifs to CPPs increase their retention in specific tissues, cell types, and intracellular locations (Matsushita et al. 2001).

The therapeutic molecules and methods of administration should be modified before future clinical applications, too. In other words, CPP can bond with other molecules in addition to proteins and peptides. Although the most common application of CPPs is the transduction of proteins and peptides as presented in this chapter, other applications including CPP-mediated oligonucleotide delivery are also being studied (Aartsma Rus 2003; Lu et al. 2003). Systemic CPP administration should be investigated to find alternative administration methods. The surface of particles was functionalized using a cholesterol moiety to improve the bioavailability and stability of the therapeutic agents and to render them more suitable for systemic administration. The functionalized compound fused MPG-8/siRNA particles were injected intravenously in mice with xenograft tumors, yielding a significant reduction in tumor size (Crombez et al. 2009). An alternative approach for efficiently delivering therapeutic siRNAs in cancer models was developed, which was a TAT fusion protein with a double-stranded RNA-binding domain (TAT–DRBD) that binds to siRNAs with high avidity and serves as an excellent vehicle for siRNA delivery (Eguchi et al. 2009). The TAT–DRBD system has been used to deliver epidermal growth factor receptor (EGFR) and AKT serine/threonine kinase 2 (Akt2) siRNAs in intracranial glioblastoma cancer mouse models to induce synthetic lethal RNAi responses that significantly enhance longevity (Michiue et al. 2009).

In summary, the use of CPP in carrier design is one of the feasible strategies to enhance the efficiency of therapeutics delivery. This has already been demonstrated in this chapter by using the hearing loss model. For translation of the work into further development of interventions to tackle aging, CPP-mediated systemic administration should be further investigated in premature aging models or models of other age-associated diseases. Recently, molecular therapies for hearing loss and age-associated diseases such as gene replacement, antisense oligonucleotides, RNA interference, and CRISPR-based gene editing have been tested in the literature (Omichi et al. 2019). Anti-aging therapies utilizing TAT–DRBD system-mediated gene transfer, antisense oligonucleotides, RNA interference, or CRISPR system should also be examined in further studies.

Important Notes

- The CPP is a peptide that can import large cargoes across nuclear membrane.
- CPP can facilitate the delivery of protein, peptide, or oligonucleotide into target tissues and cells for execution of a treatment in anti-aging medicine.

- The attachment of homing peptides or other targeting motifs to CPPs may increase the retention of CPPs in specific tissues, cell types, and intracellular locations.

Questions for Future Research

- **What are the feasible methods to increase CPP stability in the extracellular environment?** While CPP can enhance the cellular internalization efficiency of a carrier, it may be denatured or degraded during circulation in blood after systemic administration. Methods to enhance the stability of CPP would help to facilitate the use of CPPs in the design of systemic drug carriers in practice.
- **What methodological advances will increase selective cell and tissue drug delivery by CPPs?** Site-targeted or tissue-targeted delivery would be more preferable. Since each tissue expresses specific markers like proteins or receptors on its vasculature, a proposed method to improve the specificity of CPP-derived therapeutics was the insertion of homing peptide ligands into CPP–cargo complexes. A similar approach can also be exploited to target CPP-derived therapeutics toward intracellular organelles, like mitochondria, lysosomes, the Golgi apparatus, or the nucleus. A groundbreaking advancement toward improving CPP specificity was achieved by the recent development of activatable CPPs (ACPPs), which comprise peptides whose adsorption and cellular uptake is minimized by a covalently attached inhibitory domain. Cleavage of the linker connecting the inhibitory and CPP moieties by tissue-specific proteases dissociates the inhibitory domain, enabling the cleaved ACPP to enter cells. The attachment of homing peptides or other targeting motifs to CPPs increase their retention in specific tissues, cell types, and intracellular locations.
- **What are the best linker strategies to permit cargo release from CPP after cellular internalization?** Greater stability can be achieved using non-natural amino acids or D enantiomers that are less sensitive to enzyme degradation than the L enantiomers. CPP–cargo complexes internalized by endocytosis can also be subjected to degradation by acidic pH when they are trafficked into late endosomes or lysosomes. Strategies are based on the use of PepFects (PFs), a series of peptides originally derived from the amphipathic CPP Transportan 10, to overcome CPP–cargo entrapment in the endosomal compartments. Thus, protein transduction using CPPs with non-natural amino acids, D enantiomers, PFs, and adjusted acidic pH may be useful for protein transduction into tissues in future preclinical and clinical trials.

Glossary

Auditory thresholds The minimum sound pressure level which can hear.

Cell-penetrating peptide A peptide that can import large cargoes across nuclear membrane.

Connexins (Cx) Proteins which assemble to form vertebrate gap junctions are crucial for auditory function and a large deletion within the Cx30 gene is the second most frequent cause of non-syndromic SNHL.

Electroporation A microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell.

Hair cells Cells which have stereocilia that emerge from their apical surface. They induce neurotransmitter release at the synaptic ends. Therefore, sound waves are transmitted via the outer and middle ear to the inner ear fluid in the cochlea before being transduced to electrical signals via inner hair cells. These signals are subsequently transmitted to the brain via efferent neurons and perceived as sound. Once they are damaged, non-regenerative and irreversible conditions can occur after a sufficient timespan.

Organ of Corti An organ which contains three cell population: inner hair cells, outer hair cells, and supporting cells.

Otocyst An embryonic structure in vertebrates that develops into the inner ear in the adult.

Round window membrane A 1–3 μm membranous septum between the middle ear and the perilymphatic space in the cochleae.

Sensorineural hearing loss The most common form of hearing loss globally, which is caused by inner ear dysfunction.

X-linked inhibitor of apoptosis protein A protein which is a member of the inhibitor of apoptosis family of proteins.

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Part VI
Optimization of Delivery Technologies for Intervention Development

Chapter 17
Use of Numerical Simulation in Carrier Characterization and Optimization

Nenad Filipović and Marko N. Živanović

Abstract In Section V, different strategies in modifying the properties of a carrier to enhance the efficiency and versatility of systemic drug delivery have been delineated. As the last section in this book, from this chapter onwards, different parameters and techniques to streamline the translation of the developed carrier from concept to reality in anti-aging medicine will be presented. As the first chapter in this section, we will introduce the possible roles played by computational techniques in characterization and optimization of a drug delivery system. In particular, we will use iontophoresis as an example to illustrate the possibilities to couple experiments with simulations in the field of pharmacokinetics and pharmacodynamics in drug delivery. Drug flux based on its concentration in dexamethasone (Dex) will be experimentally examined, after which flux through homogeneous media will be simulated. It is expected that, once a comprehensive in silico platform, which includes appropriate numerical tools for fitting could contribute to drug-delivery makers to perform faster in silico experiments, can be achieved, more effective determination of the performance criteria of a drug delivery system will be made technically viable. This will enhance the optimization of different delivery technologies for intervention development in the future.

Keywords Iontophoresis · Drug delivery · Numerical simulation · Transdermal administration

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17.1 Introduction

Compared to other delivery methods which involve intravenous administration, methods that enable administration in a comparatively non-invasive route (e.g., **transdermal** administration and oral administration) are more attractive, particularly when anti-aging medicine (which might involve long-term and regular administration of therapeutics) is concerned. **Iontophoresis** is one of these comparatively non-invasive techniques. It is used to enhance the transport of drug ions across a tissue barrier. This technique involves the application of a low potential across the skin in order to drive ions into and across the membrane (Kalia et al. 2004). Transdermal molecular transport may be facilitated by iontophoresis-activated systems. In these devices, the applied current density in iontophoresis can be modulated to produce a programmed and controlled release of the drug (Dixit et al. 2007; Fatouros and Bouwstra (2004); Chang et al. 2000). The transport mechanism involves electromigration and electroosmosis in addition to diffusion, and the drug concentration in the donor solution can also exert a significant influence on the process (Dixit et al. 2007; Nugroho et al. 2005). There is a direct correlation among the delivery rate, the electric field across the membrane, and the amount of medicine in the patch (Keister and Kasting 1986; Simon et al. 2006). Other important factors are the molecular size and formulation pH. The rate of drug penetration through the skin is improved by the application of a direct current.

The effectiveness of iontophoresis is influenced by several factors, such as skin structure and texture, physicochemical composition of the driven substance, and the applied iontophoresis parameters. Skin texture has been shown to be a very important parameter. The number of hair follicles, skin fat composition, and the existence of imperfections in the skin significantly affect iontophoresis efficacy. In other words, the conditions of iontophoresis need to be adjusted for each skin type. The main physical mechanisms are needed to be defined: the type of applied charged particle; current flow (strength and density) increases skin permeability; and potential difference between electrodes. The skin is made up of three distinct and related physiologically regions: external exposed **stratum corneum**, **epidermis** (upper inactive layer), and lower bloodstream networked active layer of **dermis**. In order for the drug to reach the dermis, that is, the bloodstream, it needs to first pass through the stratum corneum layer and the epidermis. The dermis is 1–2 mm thick and contains a large network of fibers which also represent a special type of barrier. In addition to these physiological parameters, the process iontophoresis itself and the type of administered drug need to be optimized. For example, insulin iontophoresis showed that efficacy depended on insulin dose and current density.

The outermost layer of the skin is the stratum corneum. Major features of the stratum corneum include a negative background charge and a high electrical resistance (Kalia et al. 2004). Also, important characteristic of the stratum corneum is that the electrical resistance drops dramatically when the applied voltage is higher than 0.1–2 V. The pathways for transdermal drug delivery are schematically presented

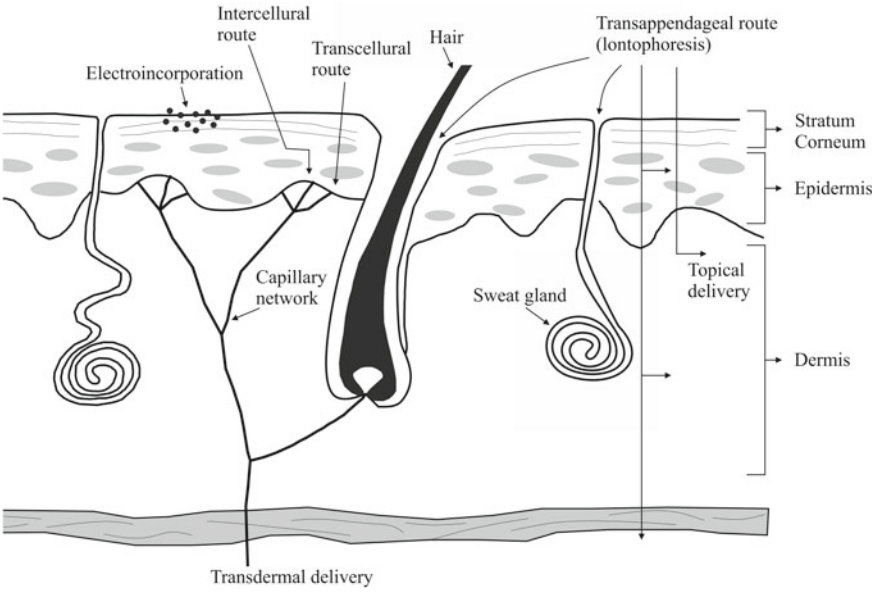

Fig. 17.1 Description of transport of therapeutic compounds during transdermal iontophoresis

in Fig. 17.1. Transdermal iontophoresis of **vasoactive drugs** has become one of the experimental in vivo human models to study microvascular function in the skin non-invasively under various conditions (Tesselaar and Sjoberg 2011).

17.2 Experimental and Numerical Modeling

In the paper by Filipovic et al. (2017), they first described experimental protocols for the crosslinked poly(acrylic acid) hydrogels synthesized via thermal polymerization. A solution of 0.04 g of crosslinker (**N,N′-methylenebisacrylamide**) in 4 ml of water was mixed with 3 g of acrylic acid on a magnetic stirrer. Polymerization was performed in an oven, at 70 °C for 45 min. The formed hydrogels were then cooled and cut into disk-shaped samples with diameter of 4 cm. Drug loading was performed via hydrogel incubation in dexamethasone phosphate (DEX-P) solutions. The concentrations and volumes of DEX-P solutions were defined in preliminary trial experiments, which ensured control of the incubation process and achievement of the desired final amounts of drug (2, 4, and 8 mg) in hydrogel samples. Porcine full-graft skin samples of 0.5 cm thickness were obtained and used following OECD 428 (2004a + 2004b), OECD GD 428 (2004c), WHO/IPCS (WHO 2006) recommendation and stored at −20 °C. For a single iontophoretic experiment the setup that was used is a drug-loaded hydrogel disk (2.1 cm) and a ring-shaped hydrogel sample (without drug), which were placed on a 5 × 5 cm skin sample and positioned

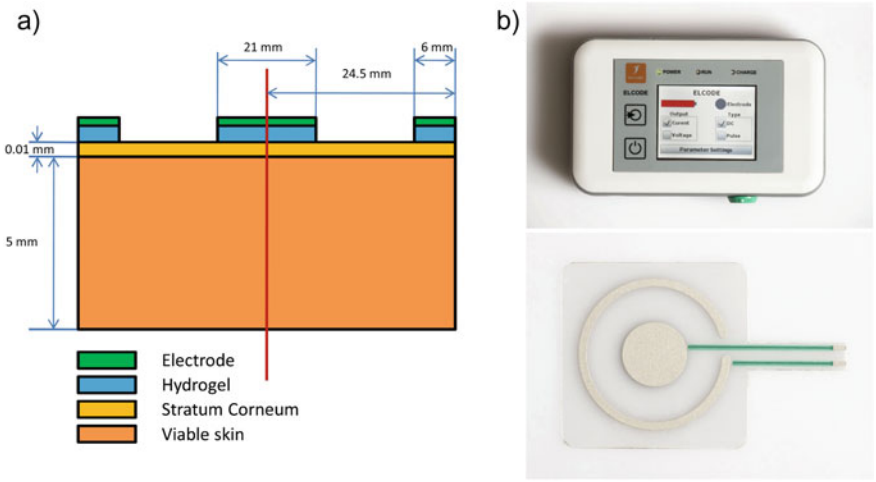

Fig. 17.2 Geometry used in simulation **a**, electrode and stimulator **b**

to ensure contact with the electrodes placed on top, as presented in Fig. 17.2a. The power source was a custom-made iontophoretic stimulator, capable of working in both current and voltage-controlled modes, as well as providing direct or pulsating current (Fig. 17.2b). Experiments were performed in current-controlled conditions and the following parameters were varied: current density (0.3, 0.4, and 0.5 mA/cm^2), duration (1, 2, and 4 h), and initial drug loading (2, 4, and 8 mg). Drug extraction from skin samples was performed using hot water, ultrasound-assisted extraction, as described in (REF). The procedure was done in three subsequent repetitions for each skin sample. The obtained extracts were analyzed using HPLC (Bischoff, UV/VIS Lambda 1010, C18 ProntoSil column). More detailed description of experiment could be found in (Filipovic et al. 2016).

Regarding the modeling of transdermal iontophoresis, the authors (Filipovic et al. 2016) refer to Nernst–Planck definition of species flux through homogeneous media (in the absence of the convective solvent flow). The flux resulting from the electrochemical potential gradient is typically decoupled into a diffusion term corresponding to activity or concentration gradient-driven flow and an electromigration term that accounts for the force of the electric field on charged molecules. The **Nernst–Planck flux** is generally represented with:

$$J_i = -\left[D_i \frac{\partial c_i}{\partial x} + u_i c_i \frac{\partial \varphi}{\partial x} \right] \tag{17.1}$$

where D_i, u_i, c_i, and ∂_φ are diffusion coefficient, electrical mobility, concentration, and kinetic potential gradient, respectively. The diffusion coefficient and electric mobility are constants of proportionality between flux and the concentration and potential gradients, respectively. They are related by the Nernst–Einstein equation:

$$u_i = \frac{z_i D_i F}{RT} \tag{17.2}$$

We want to show here the effect of iontophoresis on diffusion of drug in *stratum corneum* using finite element method (Filipovic et al. 2011), against passive diffusion represented by Fick's law:

$$J_i = -D_i \frac{\partial c_i}{\partial x} \tag{17.3}$$

The geometry used in simulation is represented in Fig. 17.2. Mesh for the model consisted of 16,816 3D finite elements, which resulted in good convergence (Filipovic et al. 2016). The equations that are governing the process are Nernst–Planck without electroneutrality and the voltage equation. The former is shown as follows:

$$\delta_{\text{ts}} \frac{\delta c}{\delta t} + \nabla \cdot (-D\nabla c - z u_m F c \nabla V) = 0 \tag{17.4}$$

where δ_{ts} is time scaling coefficient, D is diffusion coefficient, c is drug concentration, z is charge number, u_m is mobility, F is Faraday constant, and V is potential. For the voltage equation, it is shown as follows:

$$-\nabla \cdot \left(\sigma \nabla V - J^e\right) = Q_j \tag{17.5}$$

where σ is electric conductivity, V is potential, J^e is external current source, and Q_j is current source. The coupling of Eqs. (17.4) and (17.5) is performed in a standard way where nonlinear terms are described in iterative-incremental form (Filipovic et al. 2011). Fitted parameters for simulation based on the experimental results are the following:

$$\begin{aligned}
\delta_{\text{ts}} = 1,\ D_{\text{stratum}} = 4.45\text{e}^{-16}\left[\frac{\text{m}^2}{\text{s}}\right],\ D_{\text{skin}} = 5.2\text{e}^{-12}\left[\frac{\text{m}^2}{\text{s}}\right],\\
c_{\text{patch}} = 2\left[\frac{\text{mol}}{\text{m}^3}\right],\ c_{\text{effective}} = 1\left[\frac{\text{mol}}{\text{m}^3}\right],\\
z = 2,\ u_m = 2577.34 \cdot D\left[\text{s}\frac{\text{mol}}{\text{kg}}\right]\\
F = 96485\left[\frac{\text{c}}{\text{mol}}\right],\ \sigma_{\text{stratum}-\text{skin}} = 0.0000125\left[\frac{s}{m}\right],\ \sigma_{\text{dermis}} = 0.227\left[\frac{\text{s}}{\text{m}}\right].
\end{aligned} \tag{17.6}$$

17.3 Results of Experimental and Numerical Modeling

In vitro measurement of the drug flux was based on its concentration in DEX-P retained within the membrane (Cázares-Delgadillo et al. 2010). The flux for iontophoresis was prescribed from drug loading boundary condition from experiments. Different current densities, different time for simulation, and different drug loading were prescribed. For example, to simulate 2 mA we set the potential at 2 V, where voltage distribution has been shown in Fig. 17.3.

When a voltage is applied for some time on the skin, all of the drug loads (Dex-P) in the patch is transported into the skin (Fig. 17.4). This is significantly faster than the diffusion-based transport where the majority of the drug load (Dex-P) remained in the patch after several hours. The results obtained by varying current density, initial amount of DEX-P in donor hydrogel and duration of drug delivery experiments are presented in Figs. 17.5, 17.6 and 17.7, together with the corresponding cumulative values of drug concentrations calculated using the described theoretical model. Experimental amount of DEX-P in each skin sample is a sum of the values measured after three subsequent extractions. Generally, the first extraction yielded some 70–75% of the total drug extracted, the second extraction 20–25%, and the third less than 5%. These percentages were observed for both passive and active delivery experiments. As can be seen in Fig. 17.5, increasing current density, while maintaining all other parameters fixed, resulted in an almost linear dependency of the drug delivered to the skin.

Similar conclusions can be drawn from Figs. 17.6 and 17.7, which present the influence of the duration of delivery and the initial drug load in the gel, respectively. However, for passive delivery, the relative increase of DEX-P amount is somewhat larger during the first two hours of experiment (Fig. 17.6), compared to the 2–4 h period, which is less pronounced in the case for current-assisted delivery. This

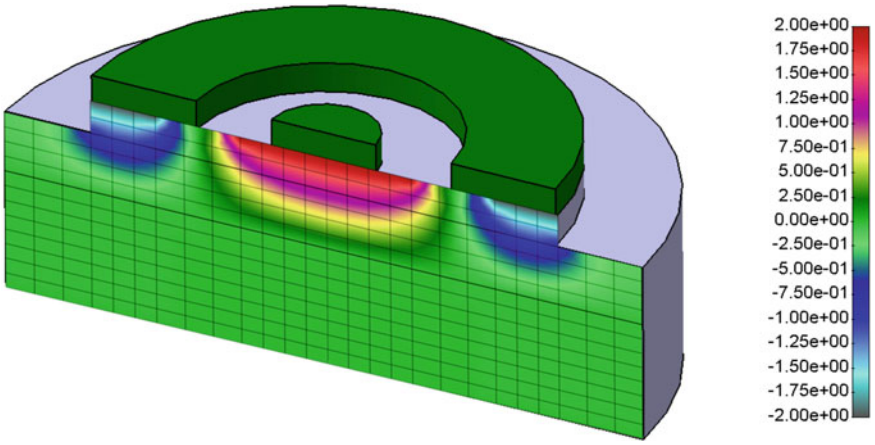

Fig. 17.3 Voltage distribution after applying the current source of 2 mA

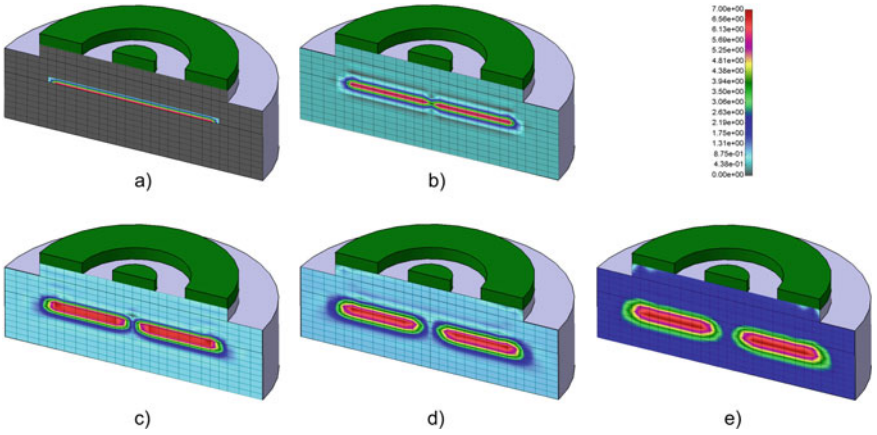

Fig. 17.4 Concentration profile of Dex during iontophoresis for the current 1.5 mA and different duration after: **a** 0.1 h; **b** 0.3 h; **c** 0.5 h; **d** 1 h; **e** 2 h

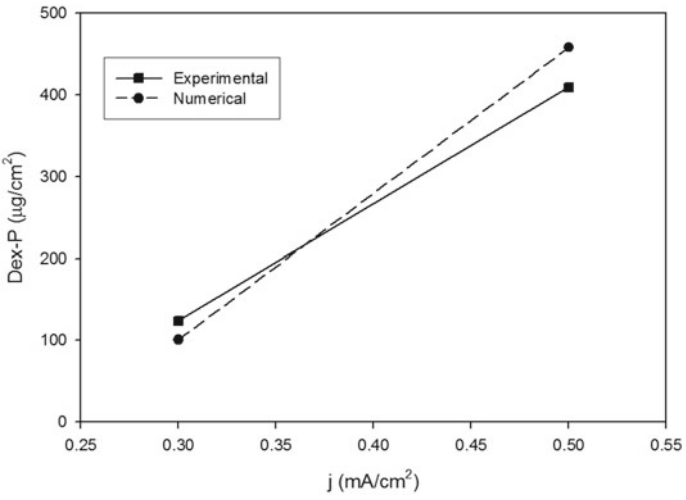

Fig. 17.5 Amount of Dex-P in skin after active delivery (2 h, initial drug load 4 mg) for different current densities

can probably be explained by the decrease of the concentration gradient (between hydrogel and skin) during the later stages of the experiment which, unlike in active delivery, is the only driving force for passive delivery. It is important to point out that similar trends are predicted by the theoretical model. Good correlation between experimental and numerical data was also observed for the results obtained for different initial drug loadings (Fig. 17.7). As expected, higher drug loadings in hydrogel resulted in higher DEX-P amounts delivered to skin. However, a larger relative increase was measured in the case of active delivery (compared for 4 and

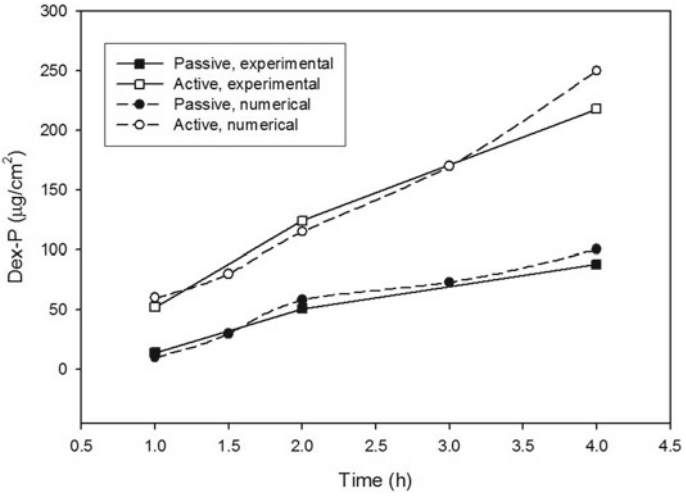

Fig. 17.6 Amount of Dex-P in skin after passive and active delivery (0.3 mA/cm^2) for different delivery durations, initial drug load 4 mg

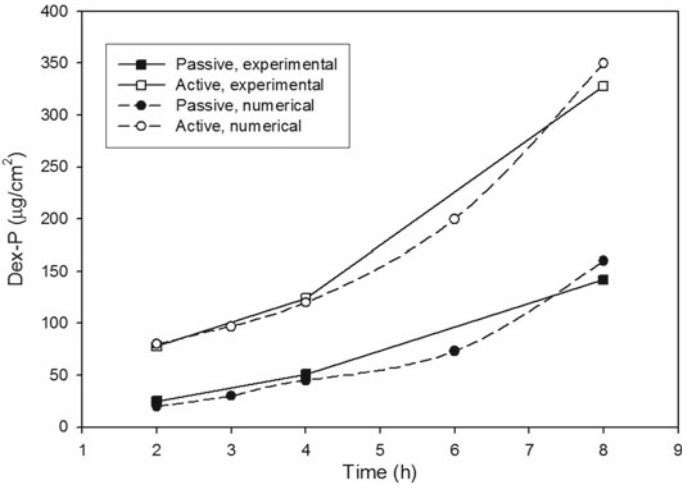

Fig. 17.7 Amount of Dex-P in skin after passive and active delivery (2 h, 0.3 mA/cm^2) for different initial drug loadings

8 mg initial loads). This result probably originates from changes in transport number of the drug, which is dependent on the molar fractions of DEX-P and other competing charge carrier ions, primarily Cl^- (REF 2).

17.4 Summary and Outlooks

Iontophoresis is to apply a current to deliver charged drugs through the skin. Different from electroporation which mainly acts on the skin structure, iontophoresis primarily works on drugs themselves and drives their transport via an electric field. Iontophoresis is a method based on the application of small currents to administer charged drugs through the skin, conducting them through an electric field. Unlike electroporation, which affects the physical integrity of the barrier (skin) in iontophoresis the drug penetrates existing skin openings such as existing skin structure abnormalities and hair follicles causing the drug to diffuse into the bloodstream. An important future application of iontophoresis may be seen as the application of biomacromolecules for therapeutic purposes. Biomacromolecules such as DNA, RNA, (poly)saccharides, peptides, and proteins can be administered orally with a big question of their fate in the digestive tract and intravenously with slightly better administration, but with the issue of real active concentration at the remote sites that are actually treated. Iontophoresis will, in the future prospective, enable their application in situ. Gene editing, silencing, and enhancement via DNA/RNA local administration may represent a major advantage of iontophoresis over other therapeutic methods. Also, for the purpose of influencing gene expression, that is, mRNAs DNA/RNA mimic interacting molecules can also be used. Such molecules are, for example, peptide nucleic acids (PNA) with extremely high sequence specificity. PNAs, aptamers, and similar biomacromolecules are chemically resistant molecules that can be administered locally by iontophoresis. In addition to this so-called direct gene manipulation, it is possible to use iontophoresis indirectly using viral vectors by influencing, for example, telomerase genes, genes, and generally the processes involved in neurodegenerative changes (Alzheimer's disease). It is similar to other peptides and smaller proteins. The assumption is that the risk of systemic inflammation using this method is significantly lower than in intravenous administration. There are already numerous studies explaining these potentials, but this field is still under-explored. In addition to the administration of biomacromolecules, it is also possible to envisage the use of other specific small molecules that would significantly contribute to better imaging of specific local areas of interest. Diagnostic imaging is an area that needs to be further developed in the future. Many imaging methods involve imaging the broad areas of the human body or even the entire human body to obtain local information. The question arises as to why contrast molecules need to be added throughout the bloodstream if only a narrow area of the organism is recorded.

Numerical simulations in this direction are useful to do **in silico** many experiments, which cannot be done in real physical world and to better explain the physics behind the process, so it is a high challenge for the future to develop mathematical models that accurately describe effects of technology on the biological features. One of the greatest challenges in the materials science is reduction of time and material spent on finding optimal parameters in different settings. Regarding the numerical simulations, they have been made to show the effects of transdermal iontophoresis

on the electrotransport across the skin. Concentration profiles were taken at a cross-section of the epidermis, in order to observe relative change in drug distribution within the epidermis. The influence of the current on the drug release concentration for 1.5 and 3 mA for Dex, Dex-P and Dex + Dex-P drug was investigated both experimentally and numerically. By fitting some numerical parameters, we can get the numerical results which corresponds well with experimental results. Numerical simulations will open the door for faster delivery of results in biomaterials field, reducing the time gap between the idea and clinical application of pharmacokinetics and pharmacodynamics in drug delivery.

Important Notes

- Iontophoresis is a technique used to increase the penetration of drugs through the skin.
- Skin is a resistant barrier that protects organism from many external influences. Also, given its size, the skin can simply be used for applying different types of therapies.
- By using a low potential (0.1–2 V), the outermost layer of the skin, stratum corneum resistance is reducing and the penetration of the drugs is significantly enhanced.
- It is possible to create mathematical model of transdermal iontophoresis by using Nernst–Planck definition of species flux through homogeneous media resulting from the electrochemical potential gradient.
- The use of finite-element study may result in good convergence of the model.

Questions for Future Research

- **How the efficiency of iontophoresis can be further enhanced?** A particular aspect of iontophoresis improvements should be addressed in the so-called individualization of iontophoresis therapy, that is, iontophoresis on demand. If we talk about the application of iontophoresis on the skin, we will find that the skin differs significantly in different patients. Even the skin of the same patient on the same part of the topic area may contain significant variations. In this sense, it is necessary to create devices that will be able to recognize these differences and at the same time adequately change the usage parameters. The use of iontophoresis in the future must be perfectly adaptable.
- **How can we improve streamline experimental studies by information technologies?** In the era of significant development of information technologies, the application of such are not yet sufficiently developed in the direction of industry and biomedical support. One of the major challenges is the machine learning approach, mathematical modeling, the use of neural

networks, and artificial intelligence in everyday industrial and scientific use. We already need machines to predict the behavior of complex systems in real-world conditions. In other words, if large series of scientific and/or industrial tests are to be done today to optimize a parameter, the assumption is that AI technologies will be able to predict in silico the flow of experimentation/testing in the future. The future of science and industry can be seen as a virtual simulation that will offer us at least one optimal solution. Based on the recommendation of the machine, we approach a much smaller number of tests that in reality need to confirm the in silico recommendation and result in obtaining a real end product with less use of time and especially resources. However, in order to reach this goal, it is necessary to transfer the human experience to the machines, which will have more autonomy and creativity in the future.

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Glossary

Dermis The layer of skin that lies beneath the epidermis and above the subcutaneous layer.

Epidermis The outermost of the three layers that make up the skin, the inner layers being the dermis and hypodermis.

In silico Performed by computer simulation.

Iontophoresis A process of transdermal drug delivery by use of a voltage gradient on the skin.

Nernst–Planck flux The flux of ions under the influence of both an ionic concentration gradient and an electric field.

N,N′-Methylenebisacrylamide A cross-linking agent used during the formation of polymers such as polyacrylamide.

Stratum corneum The outermost layer of the skin, consisting of keratinized cells.

Transdermal A route of administration wherein active ingredients are delivered across the skin for systemic distribution.

Vasoactive drugs Drugs that have the effect of either increasing or decreasing blood pressure and/or heart rate through their vasoactivity, that is, vascular activity.

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Chapter 18
Parameters and Strategies to Overcome Barriers to Systemic Delivery

Radhika Narayanaswamy, Sara Aly Attia, and Vladimir P. Torchilin

Abstract In the preceding chapter, the possibilities to couple experiments with computation techniques to characterize and optimize the performance of a drug delivery system have been described. In fact, while it is important to address the problems with the physical properties of a drug delivery system for optimal delivery performance, the impact of the physiological, bio-chemical and chemical barriers within the biological system on the efficiency of systemic drug delivery should not be overlooked, either. Keeping this in mind, in this chapter we will give an overview of the various physiological, chemical and bio-chemical barriers in systemic drug delivery, using oral drug delivery route as an example. We will also discuss the challenges in drug delivery to specific targets such as solid tumors and brain since these targets pose unique barriers that need specific knowledge on to tweak them suitably for enhanced drug delivery and achieve therapeutic benefit for several life-threatening illnesses and incurable disorders. It is hoped that, with more awareness of the various barriers in systemic drug delivery and any issue with the drug property, potential losses of therapeutics in a biological body can be minimized and the efficiency of therapies can be increased at the clinical level.

Keywords Barriers · Nanotechnology · Tumor targeting · Brain · Systemic delivery

18.1 Introduction

The process of drug discovery and development is quite expensive and divided into several stages. These include identification of a target, finding hits by screening millions of compounds (candidates with desirable interaction with the target) and identifying the leads with optimized activity suitable for testing in pharmacological models. Further optimization of bio-pharmaceutical properties can generate the

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drug-like compounds (drug candidates) selected for further development. The whole process starts with screening of ~10,000 compounds that tapers down to very few compounds suitable for further development. Only a very small percentage of the drug candidates find success in clinical testing owing to poor pharmacokinetic properties, lack of clinical efficacy, toxicity, adverse drug effects and a small percent because of commercial limitations. This failure is not preferred by pharmaceutical companies and the health care industry since it is clear that this is not due to the lack of biological activity or target interaction in vitro. It is important to evaluate several other barriers and drug properties crucial for **systemic delivery** at early stages of the drug development process. Pharmacokinetics, toxicology, **metabolism**, substrate properties and physicochemical properties are some of the important areas to be addressed early in the drug development process to produce the best drug for therapeutic use. An overview of the various barriers in systemic **drug delivery** could help address them well before the final drug product is developed. With this goal in mind, this paper was designed to provide insight on the various physiological, chemical and biochemical barriers that hinder the systemic drug delivery process. A discussion on the properties of drugs that need to be checked prior to their use in the clinic is included. The paper deals with tumor-specific barriers that hinder molecules and nanoparticles from getting to their target site with some examples of currently used nanopreparations. Apart from tumor drug delivery, brain drug delivery is especially challenging as well since there are specific barriers hindering the delivery process in brain such as those in the tumor. Considering the uniqueness of the barriers and the need to specifically address them through nanotechnology approaches, the paper discusses the various barriers in tumor and brain and provides some examples from the current nanotechnology approaches for overcoming them (Das 2006).

18.2 Factors that Affect the Developability of Drug Candidates

Systemic site-specific delivery is one of the ultimate goals of the drug delivery process that has gained tremendous momentum and attention. However, there are still various barriers and limitations that can impact the optimal drug delivery, including physiochemical properties and pharmacokinetic profiles (Mathias and Hussain 2010). After the drug molecule enters the human body, it must cross multiple restrictive barriers to reach its target site. Decreasing the possibility of drug absorption in the loading step, preventing the initial burst release of the drug and achieving an accurate kinetic constant of drug release are the main challenges in obtaining an optimal drug delivery (Etezadi et al. 2019). We consider the case of an oral drug delivery as an example to illustrate the barriers to drug delivery and discuss the implications for the properties of the drug molecule to be considered for effective systemic delivery via the oral route.

18.2.1 Solubility

Solubility is one of the fundamental parameters needed to achieve the concentration of the drug candidate in the systemic circulation for a desired pharmacological response. Low aqueous solubility remains an ever-present obstacle that hampers the rapid development of new chemical entities (NCEs). Dissolution of the drug is the rate determining step for oral absorption and consequently it's in vivo **bioavailability**. The characteristics of physiological environment, like buffer species, pH, bile salts, intestinal motility and gastric emptying rate, profoundly affect the drug dissolution rate and absorption extent (Mudie et al. 2010). In this case, poorly aqueous soluble drugs demand higher and more frequent drug doses to reach a therapeutic plasma concentration and lead to inadequate and variable drug bioavailability, limited therapeutic efficacy, gastrointestinal mucosal toxicity and higher incidences of side-effects. Hence, enhancing the dissolution rate of poorly soluble drugs is a fundamental key factor for optimizing a drug's bioavailability and effectiveness (Patel and Jain 2014; Savjani et al. 2012).

18.2.2 Permeability

The **permeability** as well as the solubility are fundamental factors that determine the appropriate delivery systems for getting the highest effectiveness. Although many new chemical entities are designed with a high potential therapeutic effect, many of these compounds exhibit unfavorable poor membrane permeation characteristics. Drug molecules must cross a lipid-like barrier imposed by epithelial mucosal layers to show a therapeutic effect and this is the main reason behind it. Moreover, drug transporters are also considered as one of the determinants of the cellular drug permeability (Peterson 2019; Mandal et al. 2017). For instance, the xenobiotic transporter p-glycoprotein (PGP) is a membrane efflux transporter of the ABC superfamily and the protein product of MDR1 gene. It is typically lipophilic, located in the intestinal epithelium and plays a potential role in determining the oral availability of drug molecules. It is the main cause for the cancer resistance to several chemotherapeutic agents by pumping the drugs out of the cells, keeping the intracellular concentration at sub-lethal levels (Sharom 2014; Watkins 1997). Therefore, the drug should have sufficient hydrophilic properties to dissolve in the aqueous environment surrounding the biological membranes as well as a sufficient level of **lipophilicity** to aid partition into the membrane for achievement of passive absorption via a trans-cellular pathway (Peterson 2019).

18.2.3 Drug Stability

Stability is an inevitably encountered issue in industrial applications as it represents the limiting step in the development of such platforms (Wang et al. 2013). Drug stability is often challenged in gastrointestinal tract (GIT) owing to the wide pH range between about pH 1–2 in the stomach and above pH 7 in the lower small intestine. Moreover, there are differences in residence time due to the various factors controlling gastric emptying, in addition to the variability in GI transit time. In this regard, the extent of drug ionization is a crucial issue in controlling the dissolution rate and passive permeability across the GIT. Thus, drug molecules should be designed in a way matching the physiological pH, otherwise they will be liable to degradation by either acid- or base-catalyzed hydrolysis even before reaching the site of absorption (Helen Chan and Stewart 1996; El-Kattan and Varma 2012).

18.2.4 Metabolic Instability

Drug metabolism refers to a metabolic process in which the chemical structure of a drug is transformed into metabolites to promote the elimination from the body (Issa 2017). Metabolic instability is a primary factor in the lead optimization stage of drug discovery that often excludes promising drug candidates from any prospective experimental studies (Ulenberg et al. 2015; Baillie 2008). The plethora of metabolizing enzymes in gut mucosa and liver are considered as impeding elements that limit the systemic exposure of drug molecules that have been absorbed from the GIT (Gavhane and Yadav 2012). Drug metabolism markedly affects drug action since the drug concentration and elimination rates are mainly determined by metabolic activity. Moreover, the inter-individual variations in drug response contribute considerably to drug metabolism (Pelkonen et al. 2005).

Each of the drug property mentioned above should be considered properly and tested to ensure improved bioavailability for orally delivered drugs. The physiological, chemical and bio-chemical barriers that are obstructions to drug delivery are to be eliminated along with optimization of the various physio-chemical properties of the drug in order to achieve successful systemic delivery of therapeutics and improve the drug development process (Fig. 18.1) (Cao et al. 2019).

18.3 Barriers to Systemic Drug Delivery

The oral pathway is currently the most popular alternative approach for systemic drug delivery due to the extensive surface area of the gastrointestinal tract (GIT) which is lined with a viscous mucosal layer that paves the way for drug attachment and consequent absorption. In addition, the GIT has various types of cells that help

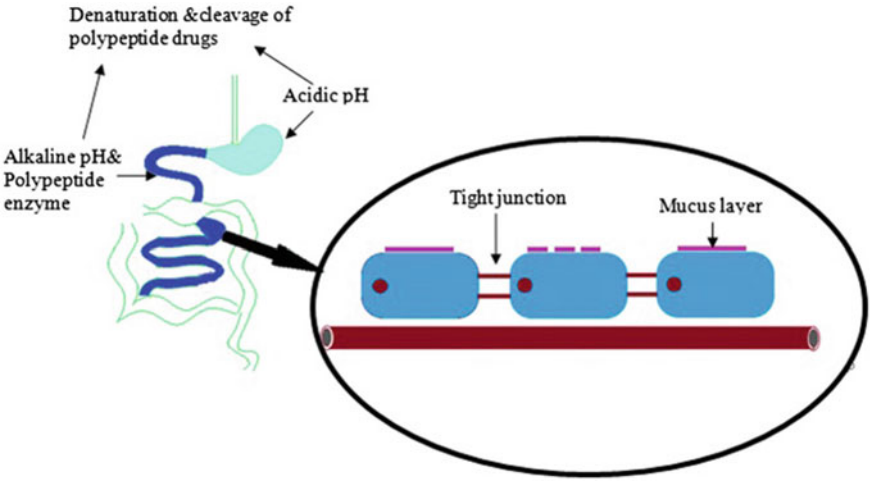

Fig. 18.1 Simplified representation of the major barriers of orally administrated therapeutic agents that hinder the optimal systemic drug delivery process at the physiological and biochemical level. Reproduced from Cao et al. (2019) with permission from Springer Nature

in drug absorption like endocrine cells and mucin-secreting goblet cells, as well as specialized M cells associated with Peyer's patches. Also, there are various options by which the drug can be absorbed, including the paracellular, trans-cellular, carrier-mediated and the facilitated transport pathway. Despite these potential advantages, many drugs are not suitable for oral administration since the majority of the new chemical entities display poor systemic availability and diminished efficacy. The obstacles to effective oral administration can be broadly classified into physiological, bio-chemical and chemical barriers (Date et al. 2016; Homayun et al. 2019).

18.3.1 Physiological Barriers

The mucus layers lining the epithelial surfaces of the GIT are a chief physiological barrier determining the fate of drugs delivered. Defined in a general manner, the intestinal mucosa consists of a thin layer of muscle cells called the muscularis mucosae, connective tissue "lamina propria" and the epithelium. The epithelium is composed of a monolayer of polarized cells protected by mucus. For systemic circulation delivery, any drug molecule crosses the epithelium first to reach the capillary network of the connective tissue. Mucus is a complex polymer-based hydrogel composed of mucin as the main protein that can be either secreted or remain cell-bound, and is composed of globular proteins, carbohydrates, lipids, salts, DNA and cellular debris. Regardless of the importance of the mucus as a first line of defense against pathogens and toxins and having lubricating properties that enhance the

passage of food, chyme and feces via the GIT, it has multiple barrier properties that impact the eventual drug delivery. For instance, mucus acts as dynamic barrier (Date et al. 2016; Leal et al. 2017; Cone 2009; Lieleg et al. 2010). Due to the continuous secretion and shedding of the mucus, the drug has to diffuse in an upstream way to reach the epithelium. In addition to counteracting the peristalsis movement in a horizontal flow along the mucosal surface, the drug exerts local effects at the mucosal surface and may fail to reach the systemic circulation. Moreover, the mucus forms a steric barrier as a result of its viscosity and the high molecular weight of the mucin network. Besides the dynamic and steric barrier properties of the mucus, it also acts as an interactive barrier because of its ability to form hydrophobic and ionic interactions that limit the free diffusion of compounds within and through the mucus (Boegh and Nielsen 2015).

Furthermore, the characteristics of the drug molecules, especially the chemistry and size properties must be precisely taken into consideration as they play a potential role in determining the absorption sites and pathways across the intestinal epithelia in the GI tract. The drug passing through the absorptive epithelia travels via the paracellular route (diffusion through the spaces between epithelial cells) and trans-cellular route (diffusion across the cells). The trans-cellular pathway is divided into passive trans-cellular transport, carrier-mediated transport and **endocytosis** or trans-cytosis. In most cases, the trans-cellular route is limited to the passage of hydrophobic large drug molecules, while the para-cellular pathway is mostly the preferable route for the hydrophilic small drug molecules. In addition, the structure of the outer regions of the membrane bilayers and the high molecular density of the hydrophilic polar heads represent a formidable barrier to the passage of a broad spectrum of drug molecules through the cell membranes (Homayun et al. 2019; Yeh et al. 1998; Reinholz et al. 2018).

Apart from all the principal roles that efflux transporters play in drug absorption and disposition, they also constitute a restrictive barrier by limiting drug uptake from the intestinal lumen into the systemic circulation (Hamman et al. 2007). As mentioned earlier, P-glycoprotein (P-gp) is one of the challenging barriers that impede the delivery of various therapeutic agents, leading to alteration in the drug pharmacokinetics properties and consequently the treatment outcome (Amin 2013).

It was reported that there was a tenfold increase in the oral bioavailability of paclitaxel in mice treated with P-gp blocker (van Asperen et al. 1997). In another study, it was shown that the administration of verapamil, a P-gp inhibitor, along with lumefantrine, resulted in a significant enhancement of lumefantrine bioavailability with a concomitant decrease in clearance (Wahajuddin et al. 2014).

18.3.2 Biochemical Barriers

The oral bioavailability of drug candidates is not only limited by poor solubility or poor membrane permeability. Pre-systemic metabolism or degradation has an

unavoidable impact on the drug bioavailability (Pereira de Sousa and Bernkop-Schnürch 2014). The wide disparity of the proteolytic enzymes present in the GI tract represents an immense challenge for delivery of many therapeutic agents. The GI tract contains a plethora of endo- and exopeptidase enzymes with broad specificity and significant degradation effects on **peptides**. In brief, endopeptidases are responsible for hydrolyzing the bond internal to the terminal bonds of the peptide chain, while exopeptidases hydrolyze the bond linking the NH2-terminal or the COOH-terminal amino acid to the peptide chain.

The harsh acidic condition of the stomach can drastically denature many of the orally administrated drugs and alter their efficacy. Also, many biopharmaceuticals are limited due to their liability to degradation by gastric enzymes such as pepsin and gelatinase. Notably, in addition to the stomach and gastric acid, there are also pancreatic enzymes that can readily decompose nucleic acids as well as reduce the gastric residential stability of biomolecules. Moreover, the brush border, the microvilli-covered surface of the epithelial cells found in the small intestine, houses more than a dozen of peptidases that have extensive degradation effects on proteins and peptides (Homayun et al. 2019; Woodley 1994; Yun et al. 2013).

Among the many drug metabolizing enzymes located in the small intestine, the CYP450 enzyme system is of particular importance, being the key pathway for drug metabolism. CYP enzymes are distributed in an uneven pattern along the GI tract, being generally higher at the proximal end of the small intestine. CYP450, a superfamily of hemeproteins, are classified into families and sub-families based on the similarity of amino acid sequence. As the major congener among the CYP3A subfamily, the CYP3A4 isoenzyme accounts for approximately 60% of CYP450 in the liver and about 70% in intestine. Diverse numbers of clinically important drugs have been identified as substrates, inducers or/and inhibitors of CYP3A4. As a result of the key role of CYP3A4 in drug metabolism, significant inhibition of this enzyme could result in unfavorable prolonged drug–drug interaction and fatal toxicity while the induction of the enzyme can negate drug efficacy. Additionally, the extensive metabolism by CYP3A4 contributes to significant inactivation of some orally administrated drugs as well as poor and inconsistent bioavailability of various drug candidates. Therefore, understanding of the site-dependence of metabolic enzyme expression as well as inter-individual variability in enzyme expression either genetically or environmentally should be a major goal and the drug dose is accordingly adjusted (Gavhane and Yadav 2012; Thelen and Dressman 2009; Zhou et al. 2007).

In addition, the gut microbiome is of considerable interest, since it actively participates in determining a drug's pharmacological activity, owing to its ability to express enzymes that either metabolically activate or inactivate drugs by various metabolic processes such as reduction, hydrolysis, dihydroxylation, acetylation, deacetylation, proteolysis, deconjugation and deglycosylation (Swanson 2015). Given this, a thorough understanding of proteolytic enzyme activity, stability and degradation of drug molecules during the intestinal transport process can facilitate oral delivery (Renukuntla et al. 2013).

18.3.3 Chemical Barrier

Hydrogen bonding potential is considered a principal factor affecting peptide permeation (Rafi et al. 2012; Wang et al. 2014). The octanol–water partition coefficient predicts cell membrane permeation with a sigmoidal curve for small organic molecules. However, in case of the larger peptides there is an energy input needed to solvate the polar amide bonds in the peptide in order to permit its entry through the cell membrane. This is the concept behind hydrogen-bonding potential. The lower the potential, the better the permeation of the drug into the cell (Burton et al. 1996; Hattotuwagama and Flower 2006).

18.4 pH Responsive Carriers as a Solution to Oral Drug Delivery Challenges

As mentioned previously, oral drug delivery is limited by the persistent challenges associated with strong gastric acid and pre-systemic enzymatic degradation, leading to poor systemic exposure. Therefore, numerous pH responsive carriers have been investigated for a range of applications in order to enhance the stability of therapeutic agents to achieve adequate bioavailability levels (Liu et al. 2017). For example, pH-responsive polymeric microspheres of poly (methacrylic-g-ethylene glycol) were loaded with insulin and administered orally in healthy and diabetic Wistar rats. It was observed that the insulin remained in the complexation gel and was protected against any proteolytic enzymes. While the gels were not swollen in the acidic environment of the stomach, due to the formation of intermolecular polymer complexes, in the basic and neutral environments of the intestine, the complexes dissociated, resulting in rapid gel swelling and insulin release. Also, it was reported that, within 2 h of administration of insulin containing polymers, there were noticeable dose-dependent hypoglycemic effects in both healthy and diabetic rats and these effects lasted for up to 8 h following the dose administration (Lowman et al. 1999). Tozaki et al. developed chitosan capsules for colon-specific delivery in order to enhance insulin absorption from the rat colon. In order to evaluate the efficacy of chitosan capsules, carboxy fluorescein (CF) (a model water soluble compound) release from chitosan capsule was observed in an artificial gastric juice (pH 1), an artificial intestinal juice (pH 6.8) and a suspension of rat cecal contents. The results showed that there was a remarkable increase in the CF release in the presence of rat cecal contents. A notable insulin absorption and a corresponding strong hypoglycemic effect was observed relative to insulin solution when insulin-loaded chitosan capsule was orally administered in rats (Tozaki et al. 1997).

The combined approach of chemical modification with pH responsive hydrogels for insulin oral delivery was investigated by another study. Conjugation of polyethylene glycol to the amino terminus of the B-chain of insulin produced a PEG–insulin conjugate. The authors explored the in vitro release profile by loading PEG–insulin

conjugate and human insulin into pH responsive poly (methacrylic acid-g-ethylene glycol) (P(MAA-g-EG)) hydrogels. It was noted that the release of PEGylated insulin was lower than that of human insulin at all pH levels considered. It was suggested as due to the stronger affinity between the PEGylated insulin and the hydrogel. Since PEGylated insulin would maintain higher concentrations within the hydrogel as it moves through the GI tract, it leads to the maintenance of prolonged insulin bioactivity, thereby providing an advantageous release pattern for oral insulin delivery (Tuesca et al. 2009).

18.5 Barriers to Tumor Drug Delivery

The barriers to the delivery of anti-cancer therapeutics are typically relevant to solid tumors that are characterized by some unique properties. Some of these properties are barriers to effective delivery of chemotherapeutics while the others favor drug delivery using nanotechnology approaches. Herein, the goal is to discuss the tumor-specific features that are relevant when considering systemic site-specific drug delivery (Sriraman et al. 2014).

18.5.1 Heterogenous Tumor Vasculature

As a tumor grows, there is a rising metabolic demand, but it is insufficient to rely on the **vasculature** from surrounding host tissue for its blood supply. At this point, the tumor switches to an angiogenic state, actively forming its own blood vessels for sustenance. The imbalance between pro-angiogenic and anti-angiogenic signaling in the tumor microenvironment is the main cause of pathological angiogenesis. VEGF-A, basic fibroblast growth factor (bFGF) and interleukin-8 are a few important pro-angiogenic factors that overwhelm the angio-static signals such as those associated with angiostatin and endostatin that induce the pro-angiogenic switch (Schaaf et al. 2018). The chaotic arrangement of tumor vasculature is attributed to an imbalance in the angiogenic regulators, VEGF and angiopoietins.

The high oxygen/nutrient demand ratio and the tumor outgrowth are supported by inciting the formation of new blood and lymphatic vessels from pre-existing vessels. A structurally chaotic, tortuous vascular compartment and the associated heterogenous blood flow hinder the penetration and a deeper more even delivery of drugs to solid tumors (Kratz and Warnecke 2012). In fact, the pro-tumorigenic environment characterized by high interstitial pressure, **hypoxia** and acidosis is mainly contributed by the structurally and functionally aberrant tumor vasculature. It furthermore acts as a barrier to the infiltration of T-cells (Fig. 18.2).

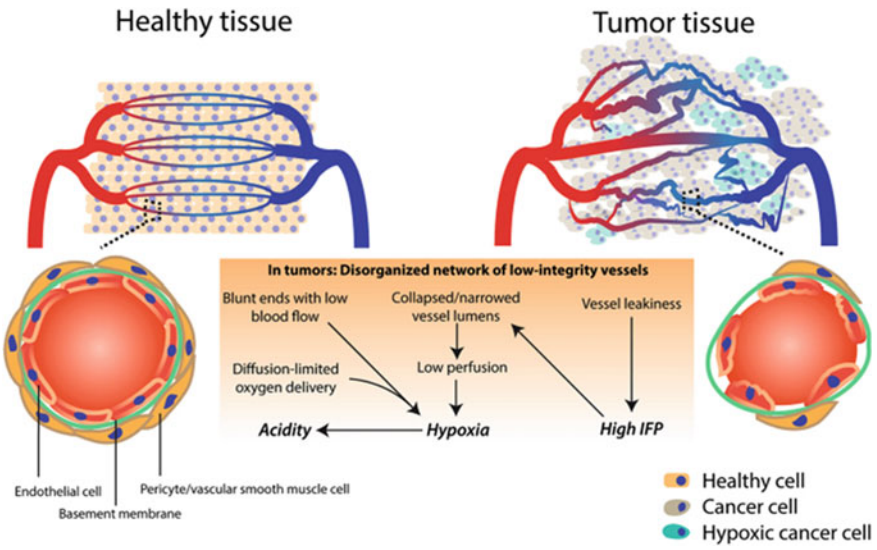

Fig. 18.2 Schematic comparative illustration of the vasculature network of healthy tissue versus tumor tissue. Reproduced from Schaaf et al. (2018) with permission from Springer Nature

18.5.2 Hypoxia and Acidic Regions in Tumor

An irregular organization of the tumor vasculature is also the main cause for hypoxia in certain regions of the solid tumor. Some cancer cells within the solid tumor are positioned far away from the blood vessels (>100 μm, i.e., over the oxygen diffusion limit) which results in strongly hypoxic regions within the tumor. Apart from regions of hypoxia, the tumor has acidic regions owing to the irregular structural features of the tumor vasculature. The acidic condition in certain tumor regions is due to the anaerobic mode of glycolysis that succeeds a hypoxic or less oxygenated condition in the tumor. Hypoxia and acidification are often the main reasons for a lowered, unfavorable therapeutic responses from solid tumors. These conditions often tweak the cancer microenvironment to favor choice of malignant cells that are more metastatic and malignant. Also, these issues favor angiogenic stimulators over the inhibitors, making the conditions more suitable for cancer metastasis and aggressiveness. Cells resistant to hypoxia-induced apoptosis are clonally selected in the presence of these adverse conditions within the tumor. Even the actin positive smooth muscles lining the tumor vessels do not behave normally as contractile muscle cells, and thereby impose serious challenges to any pharmacological modulation of tumor blood flow (Carmeliet and Jain 2000).

Reactive oxygen species (ROS) induced apoptosis is a therapeutic approach to treatment of malignant neoplasms. A recent study developed a biomimetic nanoflower composed of self-assembling nanozymes. These bio-mimetic flowers produced reactive oxygen species in both hypoxic and normoxic conditions and

in the absence of any external stimuli. Highly ordered MnO_2@PtCo nanoparticles that relieved hypoxic conditions and induced cellular apoptosis by a ROS-mediated mechanism thus produced remarkable tumor-specific inhibition. These nanoparticles induced intracellular oxidative damage at variable oxygen tension conditions and showed tumor inhibition in xenograft-bearing mouse models. Since the nanoparticles acted in a pH-dependent way, the therapeutic outcome was specifically directed against tumors and not normal tissues. The hypoxic environment in the multicellular tumor spheroids could be alleviated by these nanoparticles by initiating intracellular bio-chemical reactions that generate ROS and ultimately induce their death (Wang et al. 2018).

MnO_2 has been used as a tumor microenvironment responsive agent for use in **theranostics**. MnO_2 decomposes in the presence of H+ or glutathione (GSH) in the tumor microenvironment to favor T1 magnetic resonance imaging. Apart from that, MnO_2 splits H_2O_2 into water and oxygen to relieve tumor hypoxia. With suitably functionalized shells and coatings for controlled release, hollow MnO_2 nanostructures could accommodate huge quantities of therapeutic agents. Thus, a stable intelligent theranostic nanoplatform with hollow MnO_2 nanostructures was generated with polyethylene glycol coating, loaded with chlorine e6 (Ce6), a photosensitizer and the anti-cancer drug doxorubicin. Synergistic therapeutic effect was obtained after combining **chemotherapy** and photodynamic therapy in a single low-dose therapy. The therapy reverses the immune-suppressive tumor microenvironment. A combination of the therapy with immune check point inhibitor, PDL-1 checkpoint blockade, use CTL migration to inhibit both the primary tumors and the distant ones, even without light exposure. Thus, a synergistically effective combination of chemotherapy, photodynamic therapy and cancer immunotherapy, along with the tumor microenvironment modulation was developed to battle cancer (Yang et al. 2017).

PEGylation of mesoporous silica nanorods for drug delivery under hypoxic conditions has been suggested. The nanoparticles functioned in a pH dependent way, improved mitoxantrone (anthraquinone agent) release in cancer cells and had promising in vitro effects supporting efficient cell killing. PEGylation improved colloidal stability of the nanoparticles and reduced hemolysis as well (Wani 2017).

Clustered nanoparticles (iCluster) of ~100 nm initial size have been developed to overcome multiple barriers in the tumor microenvironment. The intrinsic tumor extracellular acidity triggered discharge of a platinum prodrug-conjugated poly(amidoamine) dendrimers of ~5 nm after iCluster accumulation at tumor sites. The dendrimer prodrugs internalized are further intracellularly reduced to release cisplatin to kill cancer cells. Poorly permeable pancreatic cancer, drug-resistant cancer and metastatic cancer models in vivo demonstrated superior activity and broad applicability of the iCluster nanoparticles. Acidic extracellular pH was used as a stimulus to trigger release of small particles at the tumor site. Only sub ~30 nm micelles effectively penetrated the poorly permeable pancreatic tumor, and the size shrinkage property of the nanoparticle post release worked in favor of the therapeutic goal. To penetrate intractable tumors and reach hypoxic cancer cells away from blood

vessels, small PAMAM dendrimer prodrugs of ~5 nm size were demonstrated to be very helpful (Li et al. 2016).

18.5.3 Interstitial Fluid Pressure

High interstitial fluid pressure observed within several tumors is the main barrier to transcapillary transport. The trans-capillary flow of water and molecules out of capillaries into the lymphatic or venous side of the vascular system is highly important for the tissue homeostasis. The flow is determined by the hydrostatic and colloidal osmotic pressure in the capillaries and in the interstitium. Osmotic and hydrostatic interstitial fluid pressures are high in solid tumors. The tumor interstitial fluid pressures are very uniform in the core of the tumor but drop steeply as they head toward the periphery. The inherent anomalies in tumors such as the blood vessel leakiness, interstitial fibrosis, abnormalities in lymph vessels and contraction of interstitial space all contribute to increased interstitial tumor fluid pressure. The therapeutic efficacy of the anti-cancer agent is considerably reduced in the presence of reduced transcapillary transport in tumors. Tumor interstitial fluid pressure is lowered by certain compounds that work through a variety of mechanisms of action. Hyaluronidase, PDGF antagonist and nicotinamide are a few of them known to work by degrading hyaluronan, decreasing stromal fibroblast contraction and decreasing microvascular pressure, respectively (Heldin et al. 2004).

Pancreatic ductal adenocarcinoma considered the fourth leading cause of cancer deaths in the United States show high resistance to chemotherapy and is extremely aggressive in nature. Extremely high interstitial fluid pressures (IFP) and a dense extracellular matrix have been identified as the main causative factors that generate difficulty in distribution and delivery of therapeutic agents to the tumor. Interstitial pressures exceeding the combined intravascular forces and the elastic forces on the vascular wall produce a collapsed vasculature. As opposed to really low IFP that range from −2, 0.1 to 8 mm Hg recorded in various parts of the pancreas in normal tissues, the pancreatic ductal adenocarcinomas had dramatically elevated IFP as high as 130 mm Hg. It was noted that a significant intra-tumoral depletion of hyaluronan (HA) reduced IFP within 2 h and peaked within 24 h. Increased numbers of patent tumor vessels and an improved penetration of chemotherapeutics across tumor bed accompanied the lowering of IFP in tumor. Targeting other ECM components such as collagen, versican, decorin or inhibiting contractile forces generated from fibroblasts were suggested alternatives to lower interstitial tumor pressure (Provenzano and Hingorani 2013). Salnikov et al. in an earlier study demonstrated that inhibiting TGF-beta lowered IFP in a carcinoma model and also promoted vessel maturation (Salnikov et al. 2000).

18.6 Challenges with Nanotechnology Approach in Tumor Delivery

When the nanoparticles are administered in vivo, they face numerous challenges to delivery. One of the first they face is the mononuclear phagocytic system (MPS). The nanoparticles ideally remain in the circulation for prolonged periods of time to reach the intended tissue site and exert their action. However, as soon as nanoparticles enter the system, proteins are adsorbed onto their surface forming a corona that affects the fate of the delivery system. Opsonization is the process that causes protein adsorption on surface of the nanoparticles and it allows macrophages or other mononuclear phagocytic system cells to facilitate clearance of the nanoparticles before they reach the site of action.

Various physicochemical properties of the nanoparticles such as their composition, size, charge and presence of specific targeting moieties determines which protein interacts with the nanoparticle. Ultra-small superparamagnetic ionic nanoparticles surrounded by PEG-(polypropylene sulfide) prepared at sizes of 30, 40 and 100 nm diameter showed increased macrophage uptake with increasing size of the nanoparticles. This defeats the purpose of PEG coating for evading MPS. Larger particles accumulate in the liver and spleen more rapidly than the smaller ones. Also, in a study using Al_2O_3 nanoparticles of different sizes (10, 40, 150 and 10,000 nm), it was observed that nanoparticles of 10 nm had the lowest accumulation in the liver. Spleen uptake showed no correlation with the size of the nanoparticles (Hoshyar et al. 2016). Also, the type and chemistry of the nanoparticles have an impact on the way in which the cells process them. It has been observed that large inorganic particles are retained in the Kupfer cells and motile macrophages of liver sinusoids, and the smaller organic ones are rapidly degraded and eliminated by them. The strongest MPS uptake happens with cationic nanoparticles, followed by those with an anionic surface charge and those with net zero charge. Coating the nanoparticles with polyethylene glycol was a suggested method to extend their circulation times in the blood and evade attack by the reticuloendothelial system. However, other studies have shown a possibility for induction of anti-PEG antibodies and reduced cellular internalization with the use of PEG in drug delivery. Since even with PEGylation, the MPS attack on nanoparticles is not resolved to the fullest, the challenge remains for systemic delivery.

18.6.1 Renal Clearance

The three layers of the kidney glomeruli include a glomerular basement membrane, a fenestrated endothelium and a lining epithelial layer. The basement membrane is composed of a heterogenous network of several extracellular matrix components including collagen, fibronectin and heparin sulfate and carries a net negative charge. Slits of size range 4–6 nm in width that allows nanoparticles of sizes lower than

the cut-off size to be present in the epithelial lining. Those nanoparticles filtered out from the blood in glomeruli are moved to proximal convoluted tubules and finally eliminated in urine. Nanoparticles filtered from glomeruli are endocytosed by the cells in the proximal convoluted tubules before finally making their way to bladder (Wilhelm et al. 2016).

18.6.2 The Concept of "The Enhanced Permeation and Retention Effect" and Its Role in Nanoparticle-Based Drug Delivery

The concept of "enhanced permeation" of nanoparticles during drug delivery can be attributed to the tumor blood vessel hierarchy and organization. The density of the tumor vasculature is the highest at the interface with the tumor and it gradually reduces from periphery to the center (Overchuk and Zheng 2018; Hobson and Denekamp 1984). The turnover rate of the endothelial lining in the tumor vasculature is about once in 1000 days versus 10 days in solid tumors (Hobson and Denekamp 1984). Feeding arteries, mother vessels, glomeruloid microvascular proliferations (GMP), vascular malformations, capillaries and draining veins are all part of the process of angiogenesis (Nagy et al. 2008). The increased spacing (100–500 nm) between the endothelial cells in mother vessels, that also lack a well-defined morphology, allows relatively large-sized molecules to accumulate within the interstitial space. The nanoparticle extravasation through mother vessels happens via intercellular pathways, and the alternative pathway via vesiculo-vacuolar organelles (VVOs) works for transcellular extravasation of nanoparticles (Dvorak and Feng 2001). Thus, the enhanced permeation part of nanoparticle-based drug delivery is based on the extravasation and accumulation of nanoparticles in a tumor microenvironment that develops primarily from the chaotic tumor vasculature, and the enhanced retention effect occurs due to lack of effective lymphatic drainage (Maeda et al. 2000, Maeda 2001). Since the healthy blood vessels are lined with endothelial cells with relatively tight junctions, nanoparticles greater than 10 nm size cannot effectively extravasate from normal vessels.

Abraxane—an albumin-stabilized nanoparticle used for paclitaxel delivery—and doxorubicin—a pegylated liposome-based doxorubicin delivery system—show enhanced tumor localization through the enhanced permeation retention effect (Miao et al. 2015). Though the main driving force for passive targeting of nanomedicine is the EPR effect, the usefulness of process faces challenges such as the non-uniform pore size of various tumor endothelia. Large volumes of liquid accumulation in the tumor area happen when the tumors have large openings in the endothelia. The resulting increase in interstitial fluid pressure matches the microvascular pressure and prevents extravasation of nanomedicines. The drug concentration maybe increased in tumor vicinity and not inside the tumor cells even when the EPR effect is in action. Torchilin et al. studied the effects of a polyethylene glycol micelle formulation vs.

polyethylene glycol liposome formulation on tumor accumulation in a murine Lewis lung carcinoma model. Since the carcinoma showed heterogeneity in the size of tumor vasculature fenestrations, the micelles proved to be a better choice for tumor accumulation over liposomes, despite enhanced blood circulation times the latter showed. The heterogeneity of tumor vasculature and the size-dependent enhanced permeation retention effect for retaining nanoparticles was clearly demonstrated (Weissig et al. 1998).

18.6.3 Extra-Cellular Matrix (ECM) of Tumor

The ECM structure is maintained by cancer-associated fibroblasts (CAFs) that produce collagen I in the structure of ECM. Apart from this, the ECM also has a versatile functionality responsible for cell migration, anchorage and signaling events that will influence tumor growth, survival and progression. The composition and structure of the ECM depends on the tissue site and the various steric, hydrodynamic and electrostatic interactions of the nanoparticles with the ECM's components. Under pathological conditions, the ECM fibers undergo stiffening that promotes angiogenesis and tumor growth. The recruitment of endothelial cells for vascularization, invasion into the surrounding stroma and finally metastasis happens following the alterations in tumor ECM. The composition of ECM is very diverse and functionally different. Thus its nature is very critical in designing an optimal nanocarrier for improved efficacy of tumor delivery (Mitchell et al. 2018).

18.6.4 Matrix Metalloproteinases

As mentioned above, there are several proteins and factors in the ECM that support angiogenesis and tumor progression (Venning et al. 2015). The most important of those proteins are the zinc-dependent endopeptidases that degrade ECM components and promote tumor invasion and metastasis (Itoh and Nagase 2002; Merdad et al. 2014). Though several MMP inhibitors have been developed, most failed in clinical trials owing to low solubility, toxicity issues and poor bioavailability. However, the MMPs are ideal for developing smart nanosystems since, when in solid tumors, these enzymes could be used as trigger for payload release from stimuli-sensitive nanoparticles (Salzano et al. 2016; Torchilin 2014).

Combining the benefits of PEGylation for long-circulating nanocarriers and TAT peptide for targeting, Zhu et al. developed a promising efficient drug delivery vehicle. The polyethylene glycol chains were linked to the nanocarrier via the pH-sensitive MMP linkers. The PEG chains were cleaved in the presence of MMPs at the tumor site and the TAT peptides exposed to effect specific tumor targeting. The result was specific targeting of tumor by TAT peptides and increased internalization

inside tumors via cleavage of PEG chains that would otherwise hinder nanoparticle internalization (Zhu et al. 2013).

Among the several MMP inhibitors developed including Prinomastat, Batimastat and Tanomastat, Marimastat (MATT) was one with very few side effects. However, its use was not approved since it prolonged the survival in gastric cancer patients only as much as the placebo. MATT is a broad spectrum MMPI peptidomimetic that acts by mimicking MMP substrates and that works in a reversible, competitive manner. It was expected that the MMP inhibition by MATT could maintain the physical barrier of tumor microenvironment, suppressing cancer metastasis and angiogenesis. Lysolipid, containing thermosensitive liposomes loaded with MATT, was used for delivery to inhibit breast cancer metastasis. Lung metastasis and angiogenesis in 4T1-tumor-bearing mice was significantly inhibited in the presence of MATT-loaded nanoparticles. Since MATT-loaded nanoparticles gave better inhibition of metastasis over primary tumor growth, it was developed as an adjuvant therapy for metastatic breast cancer (Lyu et al. 2019).

Li et al. developed an **amphiphilic** dendrimer for paclitaxel and siRNA codelivery used for cancer therapy in a synergistic manner. siRNA was incorporated in the hydrophilic cavity. Huge quantities of paclitaxel stored in hydrophobic interlayer with a PEG coated outer layer offered prolonged circulation times. A tumor microenvironment-triggered release via MMP2/9-triggered mechanism was made possible with tumor microenvironment-specific polypeptides that enhanced cellular penetration, uptake and tumor accumulation of nanoparticles with therapeutic agents. The location of siRNA was optimal for protection from degradation as opposed to simple adsorption onto the nanoparticle surface (Li et al. 2018).

Phage-displayed peptides have been ideal targeting ligands for various tumor-specific receptors in cancer therapy. Phage peptides displayed in fusion with phage protein sequences have shown great specificity for various cancer cell types as well as minimal toxicity in vivo. Bio-panning method enrich the target-specific peptides and also produce the peptides in large quantities via infection in *Escherichia coli* (Molek et al. 2011). Recently, a phage peptide sequence MT1-AF7p was identified with specific targeting ability for MT1-MMP. MT1-MMP has been involved in several aggressive disease processes and tumor invasion apart from embryonic development and reproduction. The study developed a novel peptide with increased affinity and prolonged half-life by modifying MT1-AF7p. Two peptidomimetics with mutations at histidine 4 were obtained that showed higher affinity and specificity to the MT1-MMP receptor in vivo. The study also confirmed the benefit of the use of molecular simulation to optimize peptides for cancer detection (Ren et al. 2018).

18.7 Challenges Specific to Brain Drug Delivery by Nanotechnology

Brain tumors are highly aggressive and invasive in nature and most of them do not have an effective therapy. When considering nanotechnology approach for brain delivery, the nanoparticles face a range of biological, physical and chemical hurdles before reaching the site of action. The important aspect to keep in mind while delivering drug to brain is the fact that the microvasculature in brain is highly restrictive to prevent uncontrolled flooding of brain with nanoparticles or molecules may lead to changes in neuronal pathologies affecting personality, memory, movements and the senses (Krol 2012).

As soon as a nanoparticle is delivered to brain, its surface is modified by body fluid or blood-derived proteins (i.e. opsonization happens) and this process initiates recognition by the RES leading to a rapid blood clearance. This surface modification to the nanoparticle can happen any time during its passage through organ transit of the lung, stomach, blood, skin, etc. Also, the process of nanoparticle binding to proteins influences the overall surface charge, increases size and covers functional groups. The first task hence would be to identify or predict the modifications that happen to nanoparticles post-administration to avoid their rapid elimination (Walczyk et al. 2010; Dobrovolskaia et al. 2009; Aggarwal et al. 2009; Vroman et al. 1980; Tenzer et al. 2011; Barran-Berdon et al. 2013; Dell'Orco et al. 2010; Monopoli et al. 2011; Lacerda et al. 2010).

Achieving a local high nanoparticle (Np)/drug concentration and Np residence time in contact with the target cells allows sufficient drug release at the target. As soon as the nanoparticle is administered, it is diluted in ~5 L of blood. Considering the blood velocity encountered and homogenous distribution in blood vessels, most nanoparticles or hydrophilic macromolecules show tissue extravasation at a size range between 5 and 12 nm. Larger pores may sometimes allow nanoparticles of 24–60 nm diameter (Sarin 2010). But brain allows only macromolecules of 1 nm or smaller to extravasate freely (Bradbury 1993). Other molecules are strictly regulated by receptor-mediated endocytosis. Moreover, specific delivery of drugs to brain is of utmost importance since the blood vessel density is very high and the distance between capillaries is very short. This results in a shorter travel distance for the administered nanoparticles that distribute everywhere in the brain (Vinchon-Petit et al. 2010). Also, after crossing the extremely tight 20–30 nm thick endothelial layer that constitutes the blood–brain barrier (BBB) of the brain, the nanoparticles get further diluted in the cerebrospinal fluid (CSF) (Grant et al. 1998). The BBB is a diffusion barrier in brain that allows only small molecules to passthrough. However, in certain pathological conditions such as cancer the BBB is affected. The sheer number of cells poses a serious challenge to targeting specific disease sites in the brain using nanoparticles.

After the long journey of the nanoparticles through its blood vessels, only a small proportion of the nanoparticles are likely to reach the microvessels close to the target tissue. Most of the nanoparticles are diluted, modified or retained in transit tissues.

And of course, the main challenge is to design specifically targeted nanoparticles that effectively cross BBB and aid uptake by neuronal cells or glial cells.

The chief cellular barriers affecting transport into brain cells include the following:

(1) **Blood–brain barrier** (BBB); (2) Glial cells, or neuroglia and (3) Two types of neurons (with white matter and without) (Krol 2012).

The general interaction of nanoparticles with cells facilitating their entry acts either via the active mechanisms such as receptor-mediated endocytosis or passive mechanisms to directly enter the plasma membrane by penetration. Brain cells have a protective layer of glycocalyx that acts as a hurdle, and the lipid composition of cell membrane is highly variable. Depending on the type of nanoparticles used, additional problems maybe encountered in the endocytic lysosomal pathway. The pathway degrades protective coatings on nanoparticles and triggers release of potentially toxic ions such as cadmium from quantum dots or high concentrations of amino acids from poly-amino acid nanoparticles. An escape into the cytosol can interfere with several other effects such as DNA condensation, cytoskeletal or mitochondrial functions and transport mechanisms (Li et al. 2011; Brightman 2002; Sohaebuddin et al. 2010).

The BBB poses drug delivery issues due to the following:

(1) The presence of tight junctions closing the gaps between cells
(2) Reduction in rate of pinocytosis to regulate uncontrolled entry into the cell at the luminal side
(3) Lack of a pore morphology blocking passage between cells in the endothelium
(4) Presence of an enzymatic barrier against foreign molecules and nanoparticles
(5) A P-glycoprotein efflux transporter system that eliminates most small molecules before their passage to the basal side of the endothelial cells.

Only very small molecules (<400–500 Dalton) or extremely hydrophobic substances can penetrate into the BBB. A receptor-mediated shuttle facilitates entry of higher molecular weight entities via BBB and there is evidence to suggest such. Good substrates for P-glycoprotein efflux include small hydrophobic drug molecules, and this important BBB defense mechanism significantly impacts nanoparticle concentration in brain parenchyma. It was observed that myocardial toxicity associated with doxorubicin was reduced using pluronic nanoparticles for cancer therapy and importantly the formulation selectively inhibited expression of P-glycoprotein in tumor cells reversing multi-drug resistance (Banks 2009; Gabathuler 2010).

Transferrin, insulin and low-density lipoproteins maintain receptors on the BBB to facilitate Np delivery (Gabathuler 2010). Earlier studies showed enhanced accumulation of citrate-stabilized gold nanoparticles at very high concentrations in brain. It was also confirmed later that the accumulation happened after the BBB and in specific brain stem regions in neuronal or glial cells. Once the BBB pathway is overcome by the nanoparticles, they were taken up by the glial cells that have a macrophage-like function in the brain or they cross the basal membrane separating glial and endothelial cells or they directly enter the CSF (Jong et al. 2010; Sonavane et al. 2008; Sousa et al. 2010). Pericytes, astrocytes, oligodendrocytes are glial

cells, each with a unique role in the perfect sealing of the BBB in ways to make the Np delivery to brain that is extremely complex (Krol 2012; Guerra et al. 2017) (Fig. 18.3).

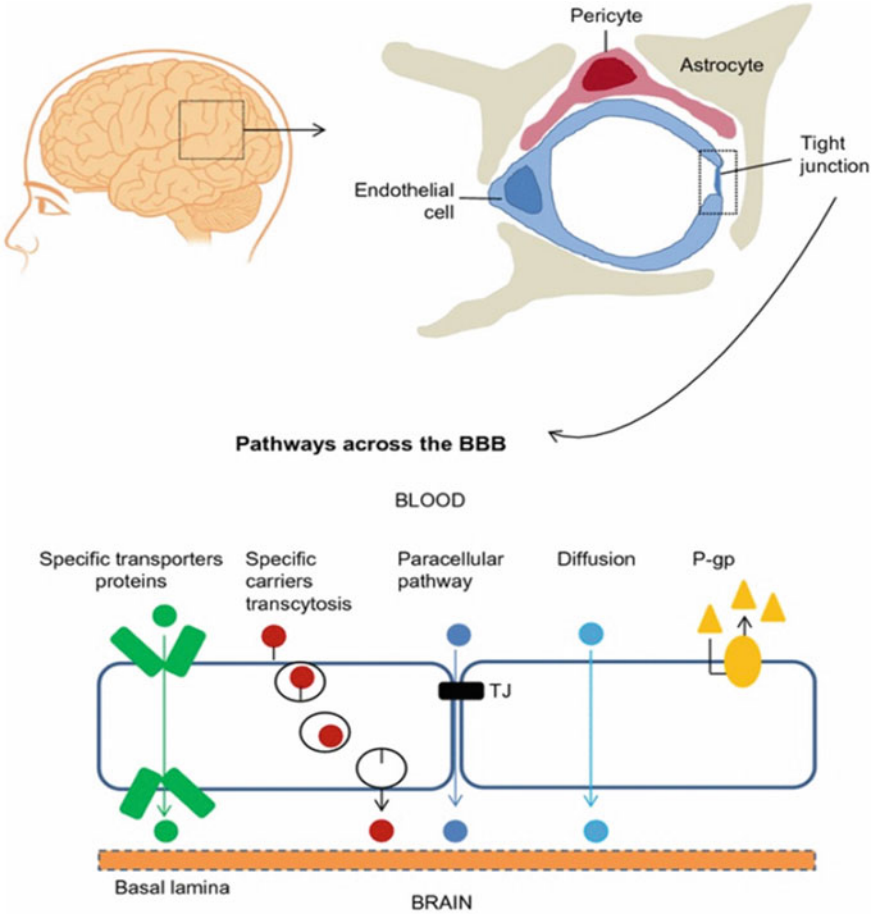

Fig. 18.3 Schematic representation of the blood–brain barrier (BBB). It is composed of endothelial cells, pericytes, astrocytes, tight junctions and basal membrane. Also, the diagram depicts the paracellular and trans-cellular pathways by which the drug molecules can be delivered to brain cells. Reproduced from Guerra et al. (2017) with permission from Springer Nature

18.8 Nanotechnology as a Solution to Brain Delivery via BBB

Nanotechnology approaches are ideal solutions to the various challenges a drug encounters in crossing the BBB. There are several nanocarriers currently available that are effective solutions to the difficulties encountered in the delivery process to brain or a tumor site (Fig. 18.4). Brain-specific phage-derived peptides in hierarchical forms (also known as nanoligand carriers) target cerebral endothelial cells specifically through transferrin receptor and advanced glycation end products receptor. These cross BBB to reach neuronal and microglial cells. NLC-Beta secretase 1 (BACE1) siRNA complexes delivered intravenously show BACE1 down-regulation in the brain very effectively without inflammation and toxicity. Thus, these nanoparticles can facilitate targeted delivery of nanoparticles with phage display peptides or cell-penetrating peptides and also provide a broader repertoire of receptors for targeting. BACE-1 is highly over-expressed in neurons in brain and triggers Ab (amyloid-b) production in Alzheimer's disease. A single IV administration of a siRNA coupled nanoparticle gave substantial reduction in Ab (amyloid-b) production, supporting nucleic acid medicine functionality in the brain via the delivery of NLC and served as a proof of validity of the engineered NLC approach to function

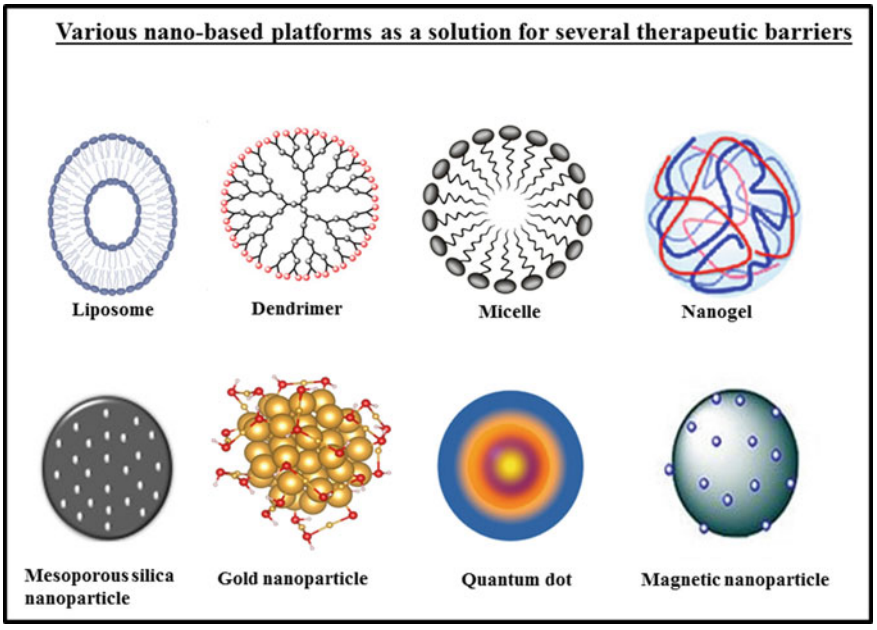

Fig. 18.4 Different nanocarriers employed to address the common challenges encountered in drug delivery process. The nanocarriers range from liposomes (that are lipid bilayers) to magnetic nanoparticles that are manipulated suitably to address specific needs in drug delivery

as a neurological nanomedicine. NLC has proven safety and minimal toxicity in vivo as was revealed by an absence of injury, necrosis or inflammation. Unlike cell penetrating peptides, these NLCs showed tremendous specificity to target and works by self-assembling of peptide ligands in targetable nanocarriers (Wu et al. 2019).

As discussed above, a major barrier to systemic drug delivery is the nanoparticle interaction with the protein corona that attracts RES attack on the nanoparticles for elimination. A new approach has been to use corona-mediated targeting for site-specific drug delivery. Special bio-inspired liposomes (SP-sLip) have been developed that are surface modified with short non-toxic peptide $AB_{1\text{-}42}$. These peptides specifically interact with the lipid binding domains of exchangeable **apolipoproteins** in blood. In the process of adsorbing to the lipid binding domains, the receptor binding sites of these apolipoproteins are exposed to achieve brain-targeted drug delivery. Doxorubicin loaded bio-inspired liposomes had an improved anti-cancer effect over non-targeted plain liposomes. Penetration of the BBB and significant distribution in the cerebral cortex and hippocampus was observed with the targeted liposomes with and without doxorubicin in a mouse brain tumor model. The specific liposomes were also proven to be highly immune-compatible. The apolipoproteins ApoE, ApoJ and ApoA1 associated with the bio-inspired liposomes via interaction with the lipid binding domains on the apolipoprotein surface. The receptor-binding domains subsequently exposed on the liposome allowed multiple receptor recognition specific for the apolipoproteins facilitating BBB passage via transcytosis (Zhang et al. 2019).

Effective migration of drugs through BBB to deliver drugs to a HIV reservoir organ such as central nervous system has been a major challenge. Nanodiamonds have been proposed for use recently, since they are naturally biocompatible and nontoxic in nature. The nanodiamond loaded with HIV-1 drug, Efavirenz, showed minimal toxicity and high drug loading capacity. Efavirenz is a cART drug that is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and suffers poor bioavailability owing to blood plasma protein binding. The study proved that conjugating Efavirenz with nanodiamonds that are suitably functionalized increased their therapeutic efficacy considerably. Chemical and biological characterization of nanodiamonds along with studying their toxicity profile gave results supporting their use for targeted delivery to the CNS during HIV infection. The fate of the nanodiamonds after drug release, however needs supporting in vivo studies for further understanding of the process (Roy et al. 2018).

Gold nanoparticles have been extensively used in theranostic applications. Surface-modified gold nanoparticles with brain-targeted exosomes demonstrated increased passage across the BBB and high binding to brain cells. After IV injection into mice, the targeted exosome-coated gold nanoparticles were seen accumulating in mouse brain. The nanoparticles specifically recognized and targeted neuronal cells with improved transport across BBB. Exosomes are nanosized vesicles produced by cells. Due to similar composition to the body's cells, exosomes are highly non-toxic and non-immunogenic. Specific rabies viral glycoprotein (RVG) peptide targeting the acetylcholine receptor (nAchr) allowed targeted delivery at the BBB. Tropism to brain cells was provided by the exosomes by fusing Lamp2b protein to an RVG

peptide specific for neuronal cells. The approach could broadly aid therapy of several brain diseases and disorders (Khongkow et al. 2019).

Latent human immunodeficiency virus (HIV) was non-invasively targeted with delivery of nanoformulation composed of Cas9/gRNA complexed with magneto-electric nanoparticles (MENPs). These particles crossed BBB and inhibited latent HIV-1 infection of microglial (HC69) cells. The MENPs showed an in-demand drug release with an external AC magnetic field and broke bonding between particle and drug. Reduced viral long terminal repeat (LTR) expression levels in the Cas9/gRNA treated latent HIV infected cells confirmed the efficacy of the method for crossing the BBB to kill the latent virus in the brain (Kaushik et al. 2019). Apart from the several innovative nanotechnology approaches developed to target BBB, computational analysis of Parkinson's disease inhibition by nanoparticles loaded with specific inhibitor has also been studied. With advancements in technology, the possibilities for suitably manipulation of nanotechnology for effective drug delivery purposes are manifold.

18.9 Summary and Outlooks

This chapter discusses hindrances to systemic drug delivery at the cellular, biochemical and chemical levels. It specifically emphasizes difficulties in drug delivery to solid tumors and brain and how innovative approaches in nanotechnology may help to resolve those issues. This chapter also deals with the numerous physicochemical properties of drugs and how optimal drug development is possible by fine tuning those properties. Encountering the various barriers to systemic drug delivery is inevitable since anything foreign is resisted and rejected. However, with the recent developments in nanotechnology and nanocarrier-based drug delivery, most of the barriers may be effectively overcome and desired results can be achieved.

Important Notes

- Systemic drug delivery faces several challenges and most of them are important to be considered and addressed at an earlier point in drug development process to avoid any serious losses in clinical trials.
- The challenges need to be addressed starting at the level of the drug considering its properties such as solubility, permeability and metabolic instability to name a few. However, the key barriers such as the various physiological, chemical and bio-chemical ones have to be kept in mind while developing a drug for delivering to a specific site.
- Ever since the development of nanotechnology approaches for drug delivery, most of the problems a drug molecule would encounter during its journey to

the target are highly minimized. Since barriers exist even with the nanoparticle delivery, suitable modifications such as PEGylation could enhance the circulation time and protect the nanoparticles from RES attack.

- Site-specific systemic drug delivery to brain or tumor is very challenging and limited by very unique barriers. With the development of nanotechnology approaches the drug delivery process has been revolutionized.
- Targeting the protein corona functions, the blood–brain barrier of the brain and the matrix metalloproteinases, the hypoxia, interstitial fluid pressure in tumors have been very easily achieved in recent years using nanoparticles as drug delivery vehicles. Nanodiamonds, gold nanoparticles, stimuli-responsive peptides, micelles, liposomes are a few to name that have helped overcome obstacles in drug delivery successfully.
- Multi-functional, smart nanocarriers tailored to escape the various challenges are ideal drug delivery vehicles to provide optimal benefits in therapy.

Questions for Future Research

- **How can the barriers in systemic delivery be completely eliminated?** Any foreign material that the body encounters faces resistance during the delivery process, even with the use of current nanotechnologies. Continuous efforts devoted to the development of strategies to over various barriers at the cellular and tissue levels are required to achieve success in systemic delivery.
- **How can the toxicity assessment studies at the earlier stages be sufficient to prove reduction in toxicity with not just the drug molecule but with the carrier as well?** Different types of materials ranging from biologically compatible ones such as lipids to more "chemical" ones such as metal ions-based quantum dots go into the making of nanoparticles. Effective strategies to evaluate the toxicity of both the drug molecules as well as the carrier are needed for the development of technologies for effective systemic delivery.
- **What is the validity of assessing the performance of a systemic delivery technology in a small-scale preclinical setting?** Currently, assessment made on nanoparticle toxicity and physical properties (such as polydispersity, stability, shelf-life and so on) are held in a laboratory scale before the delivery method proceeds to clinical trials. Methods to enhance the validity of the preclinical testing can help eliminate bigger expenses at a large scale in the clinical phase.

Glossary

Amphiphilic Possessing both water-loving and lipid-loving properties.

Apolipoproteins Proteins that bind to fat and cholesterol to form lipoproteins are apolipoproteins and they help with the transportation of lipids in blood, CSF and lymph.

Bioavailability The rate and degree at which the administered drug is absorbed by the systemic circulation (body's circulatory system) is bioavailability.

Blood–brain barrier A semipermeable border that is highly selective and separates the circulating blood from the extracellular fluid and the brain in the central nervous system (CNS). The barrier has specialized cells and tight junctions blocking entry of most pharmaceutical agents into the CNS and is a challenge to brain delivery of most drugs.

Chemotherapy Use of powerful chemicals to kill rapidly dividing and growing cells in the body is chemotherapy.

Drug delivery The process or method of administering a pharmaceutical compound to achieve a therapeutic effect in animals or humans is drug delivery.

Endocytosis Invagination of cell membrane to enclose macromolecules and particles from surrounding medium to allow substance entry into the cell is endocytosis.

Hypoxia Deprivation of adequate oxygen supply at the tissue level in the body or a region of it is hypoxia.

Lipophilicity The ability of a chemical compound to dissolve in fats, oils, lipids and in non-polar solvents such as hexane or toluene is lipophilicity. Lipid-liking behavior of a drug is drug lipophilicity.

Metabolism Chemical alteration of drug inside the body to result in metabolites that are either inactive or similar to or different from the original drug in therapeutic activity or toxicity is drug metabolism.

Peptides Short chain of amino acids linked by peptide bonds are peptides.

Permeability A drug's ability to cross a biological membrane that is critical to affect its absorption and distribution is permeability. Several drug properties such as lipophilicity, size and transport mechanisms such as active transport, passive transport, diffusion etc. determine the level of drug permeability across a biological barrier.

Reactive oxygen species Oxygen-containing chemically reactive chemical species is reactive oxygen species. Peroxides, superoxides, singlet-oxygen are examples.

Solubility Dissolution of solute in a solvent to give a homogenous system is solubility. It is an important parameter to achieve the desired drug concentration in systemic circulation to attain a desired pharmacological response.

Stability The ability of a pharmaceutical dosage form to maintain its physical, therapeutic, chemical and microbial properties during storage and use by patient is drug stability.

Systemic delivery Intravenous, subcutaneous, oral or pulmonary administration of a drug product systemically (to the whole circulation) into the body of a patient is systemic drug delivery.

Theranostics Integration of imaging and therapy in a single system that allows both treatment and monitoring at the same time is theranostics.

Vasculature The arrangement of blood vessels in the body or in a part of the body is vasculature.

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Chapter 19
Blood Interactions with Nanoparticles During Systemic Delivery

Wing-Fu Lai, Eric M. Huang, and Wing-Tak Wong

Abstract In the preceding chapter, different physiological, chemical, and biochemical barriers in systemic drug delivery have been introduced. In this chapter, we will talk about the interactions between nanoparticles and blood components, and will delineate strategies to enhance the compatibility of the nanoparticles in blood. In fact, in order to achieve systemic delivery, blood circulation plays an important role because it is the blood that helps deliver the therapeutics to tissues bodywide. A good understanding of the interactions between nanoparticles and various components in blood is, therefore, pivotal to proper design of nanoparticulate systems as effective systemic carriers. The objective of this chapter is to introduce the methods of manipulating the pharmacokinetics and biodistribution of nanoparticulate systems by manipulating the interactions between nanoparticles and blood components during systemic delivery. Strategies to improve the hematocompatibility of the nanoparticulate systems will also be discussed for enhancing the use of the carriers in practical interventions at the preclinical and clinical levels.

Keywords Hematocompatibility · Hemolysis · Systemic administration · Complement activation · Thrombogenicity

19.1 Introduction

Over the years, several routes of drug delivery using nanoparticulate systems have been examined, ranging from intratracheal instillation and intratissue injection to intravenous administration. Among them, systemic delivery is the most challenging

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one. This is because when a carrier is administered intravenously, the clearance by the reticuloendothelial systems (RES) and the interactions with diverse blood components will diminish the chance of the carrier to reach tissues for action. Despite this, systemic delivery has its unique merits, including higher **bioavailability**, a higher rate of drug absorption, and a lower chance of removal by the first-pass metabolism in the liver. In addition, to tackle or ameliorate bodywide symptoms (e.g., chromosomal abnormalities and cellular aging), systemic delivery is the only choice.

In fact, systemic drug delivery has been adopted in different studies on disease treatment, and various levels of success have been achieved. For example, dystrophin expression has been restored bodywide in skeletal muscles of the dystrophic *mdx* mouse, with significant improvement in muscle function observed, after intravenous administration of morpholino phosphorodiamidate antisense oligonucleotides (AONs) on a weekly basis (Alter et al. 2006). Upon systemic administration of nanoparticles synthesized by using the three-way junction (3WJ) motif derived from bacteriophage phi29 packaging RNA (pRNA) as a scaffold harboring various functional motifs (including therapeutic anti-miRNA, Alexa647 as a fluorescent imaging module, and an epidermal growth factor receptor-targeting RNA aptamer as a targeting ligand), accumulation in tumor tissues and the onset of miRNA knockdown have been observed in orthotopic triple-negative breast cancer (TNBC) tumor-bearing mice (Shu et al. 2015). More recently, by using lipid nanoparticles for systemic delivery of factor IX mRNA, protein replacement therapy has been executed in a Factor IX (FIX)-deficient mouse model of hemophilia B (Ramaswamy et al. 2017). All these have demonstrated the versatility and technical feasibility of systemic delivery of therapeutics using various nanoparticulate systems as carriers.

19.2 Roles of Nanoparticulate Systems in Systemic Delivery

Our current understanding of the fate of an exogenous agent upon administration into a body is based on the absorption, distribution, metabolism, and excretion (ADME) concept (Fig. 19.1) (Markovsky et al. 2012). In general, upon intravenous injection, the first tissue that will be encountered by the nanoparticle is blood, which contains both acellular and cellular portions. The former comprises the plasma (in which over 90% is water) and various biomolecules; whereas the cellular portion consists of erythrocytes, leukocytes, lymphocytes, and platelets. Both the acellular and cellular portions may interact with the surface of the nanoparticle. This constitutes the so-called "synthetic identity" and "biological identity" of the nanoparticle, which denote the surface characteristics of the nanoparticle before and after exposure to serum (Albanese et al. 2014). Through the blood flow, the carrier will be transported to the heart through the right ventricle (Fig. 19.2) (Bertrand and Leroux 2012). After that, it will enter the pulmonary circulation. Due to their small diameter (around 2–13 μm), lung capillaries form the first sieving constraint for drug carriers (Bertrand and Leroux 2012). In general, rigid nanoparticles with a diameter of 10 μm will be

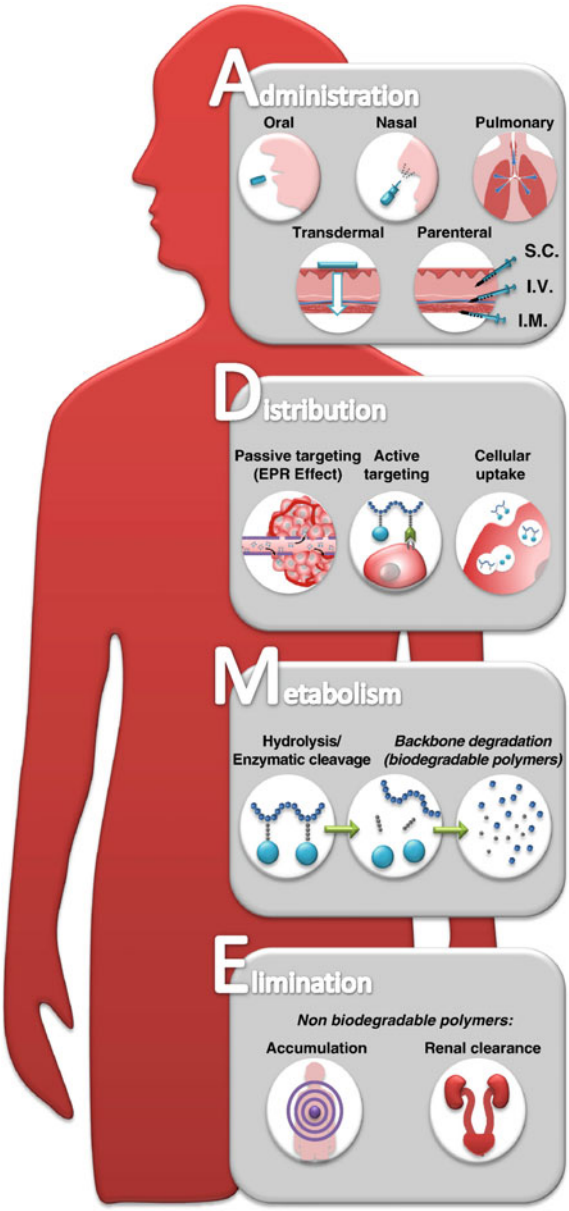

Fig. 19.1 The ADME concept. Reproduced from Markovsky et al. (2012) with permission from Elsevier B.V.

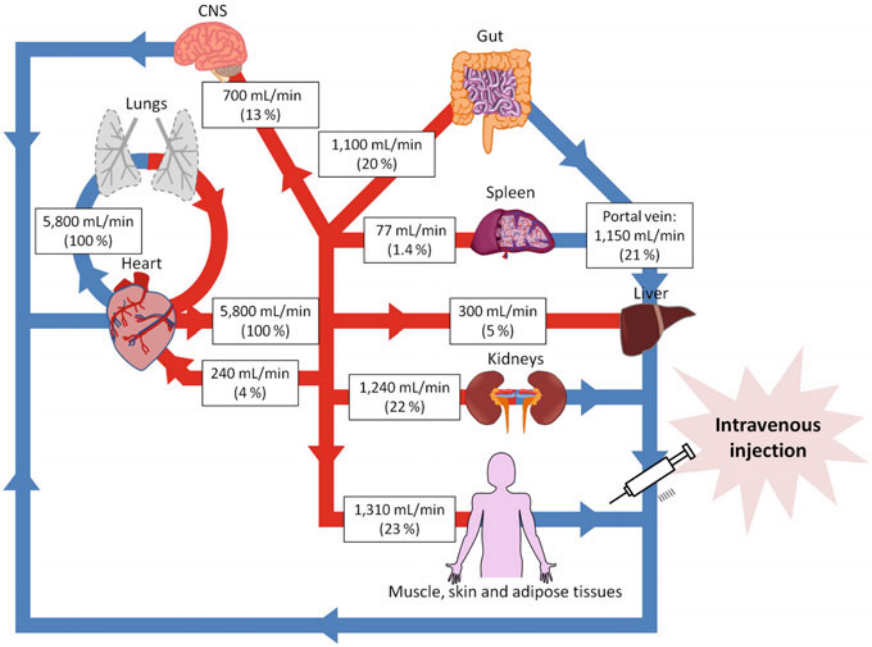

Fig. 19.2 Distribution of the blood flow in the systemic and pulmonary circulation. The percentage of the total blood flow is shown in parentheses in each organ. Abbreviation: CNS, central nervous system. Reproduced from Bertrand and Leroux (2012) with permission from Elsevier B.V.

permanently trapped inside lung capillaries (Kutscher et al. 2010); whereas those with a diameter less than 3 μm can escape from pulmonary retention (Kutscher et al. 2010). For those with a diameter of 3–6 μm, they may be initially trapped but can escape at the end (Kutscher et al. 2010). Due to the small diameter of lung capillaries, pulmonary retention has been exploited for systemic delivery of mRNA, which is complexed with polymer-lipid nanoparticles generated from poly(β-amino esters) and lipid-PEG, to the lung (Kaczmarek et al. 2016); however, in general, pulmonary retention is not desired. Proper control of the nanoparticle size, therefore, plays an important role. Those particles that successfully escape from pulmonary retention can return to the heart through the left ventricle, and enter the systemic circulation.

In addition, when a hydrophilic drug is administrated intravenously into a body, the drug can be eliminated easily via renal excretion. On the contrary, if the drug is more hydrophobic in nature, the extent of serum protein binding experienced by the drug will be increased. This may reduce the rate of renal clearance in the beginning; however, a hydrophobic drug can often be metabolized in the liver to enhance hydrophilicity, and at the end be excreted either into bile or into urine before it can effectively get into tissues bodywide. Because of this, systemic delivery of a free drug is generally difficult. This problem can be partially tackled by using nanoparticles, which may increase the molecular size to reduce the rate of renal clearance (whose

cutoff size has been estimated to be around 5.5 nm by using quantum dots (Choi et al.)) and may also protect the drug from attack by metabolizing enzymes in the liver. In addition, while bodywide distribution of a drug is desired when systemic aging symptoms (e.g., **telomere** shortening and cell senescence) are tackled, treatment of more localized age-associated diseases (e.g., Parkinson's diseases and primary tumors) may necessitate more localized accumulation of a drug to specific tissues so as to obtain enhanced therapeutic effects and to avoid unnecessary systemic toxicity. By changing the physical properties (including size and **zeta potential**) and surface chemistry of a drug, the use of nanoparticles can help manipulate the pharmacokinetic (PK) profiles of the parent drug to achieve the desired profile of tissue distribution.

While small-molecule compounds can easily diffuse through the capillary wall and get into tissues for action, nanoparticulate systems, owing to its bigger size, usually have to make use of the intercellular pores in the endothelium for getting into tissues. The pore size of the endothelial wall, therefore, becomes one of the barriers to the accumulation of the carrier in tissues. This explains the occurrence of passive targeting of a carrier to tumor tissues via the **enhanced permeability and retention (EPR) effect**. Apart from tumor tissues, a carrier may tend to accumulate in the liver, spleen, and bone marrow. This is because these tissues contain a large amount of macrophages, which can capture particulates and macromolecules in blood. Upon intravenous administration of a carrier, the carrier surface is subjected to **opsonization** and subsequently is recognized by the scavenger receptor on the macrophage for internalization. Minimizing opsonization is required if the development of a carrier for effective bodywide distribution is needed.

19.3 Manipulation of Pharmacokinetics and Biodistribution

The success of manipulation of the pharmacokinetics and biodistribution of a carrier can be examined by determining the concentration of a drug in all major tissues from the time of drug administration until the elimination phase. Based on the pharmacokinetic (PK) profile, various parameters can be calculated to quantitatively describe the way the drug or the nanoparticle is processed in a body. Examples of these parameters include the half-life ($t_{1/2}$), maximum concentration ($C_{\max}$), clearance (Cl), mean resident time (MRT), and the area under the curve (AUC). Successful enhancement of a drug carrier for blood retention is usually manifested by an increase in AUC, MRT, and $t_{1/2}$, as well as a decrease in Cl. Apart from determining the efficiency of a strategy for enhancing a drug carrier for blood retention, PK parameters can help optimize the dose and dose regimen during the execution of an anti-aging intervention so that the therapeutic agent can stay in the blood circulation long enough for therapeutic effects to manifest while having minimal side effects.

To alter the PK and biodistribution profiles of a carrier, several properties of the carrier can be modulated. One important property is the particle size. For instance, by using a mesoporous silica (MS) templating method, the size of poly(ethylene glycol) (PEG) hydrogel particles has been successfully controlled. During particle fabrication, mesoporous silica particles with different average diameters (viz., 1000, 500, 280, and 110 nm) are first constructed as templates (Cui et al. 2015). 8-arm-PEG-NH_2 with a hexaglycerol core structure is then infiltrated into the mesoporous silica particles, followed by crosslinking between 8-arm-PEG-NH_2 and succinimidyl carboxyl methyl ester-functionalized 8-arm-PEG (Cui et al. 2015). Upon the dissolution of the templates and the incorporation of Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488) into the PEG particles for fluorescence visualization, PEG particles, denoted as PEG40-1000, PEG40-500, PEG40-280, and PEG40-110, are successfully generated from mesoporous silica particles with an average diameter of 1000, 500, 280, and 110 nm, respectively (Fig. 19.3) (Cui et al. 2015). An ex vivo assay using human whole blood reveals that an increase in the PEG molecular weight, or a decrease in the PEG particle size, can reduce the association of the PEG particles with phagocytic blood cells (Cui et al. 2015). This is consistent with the observation in vivo, in which PEG particles with a smaller size (150 nm) have been found to be more effective in blood retention than the larger counterparts (>400 nm) (Cui et al. 2015).

Another important factor to be tuned is the surface properties. One example is the surface charge, whose effect on biodistribution has been demonstrated by a previous study (Arvizo et al. 2011), which examined the PK and biodistribution profiles of four types of gold nanoparticles that display different surface charges (neutral, positive, negative, and zwitterionic). Results showed that, upon intravenous and intraperitoneal administration, neutral and zwitterionic nanoparticles exhibit longer blood circulation time and more effective tumor uptake, whereas those having a negative or positive surface charge show shorter half-lives. The surface chemistry of a carrier can affect the profile of biodistribution, too. This factor, however, can be easily manipulated by both chemical and engineering methods. One example of the latter is the layer-by-layer (LbL) assembly approach, which has previously been adopted to generate electrostatically assembled nanoparticles for systemic delivery. By using carboxyl functionalized gold nanoparticles or carboxyl functionalized quantum dots as cores (with polylysine, dextran sulfate, and hyaluronic acid for layer deposition), an increase in the number of deposited layers has been found to lead to an increase in in vivo stability (Poon et al. 2011). In addition, the outermost deposited layer has been demonstrated to form a critical surface cascade, affecting not only the degree of non-specific particle uptake but also the ultimate tissue distribution profile (Poon et al. 2011).

Apart from the aforementioned factors, the shape of a carrier should be carefully designed in order to obtain the desired bodywide distribution. This is revealed by the difference in biodistribution experienced by fluorescent mesoporous silica nanoparticles (MSNs) (Huang et al. 2011a). While intravenously administrated short-rod MSNs predominately accumulate in the liver in mice, long-rod MSNs localize largely in the spleen (Huang et al. 2011a). In addition, short-rod MSNs are found to be more susceptible to be removed by renal and fecal excretion when compared with the

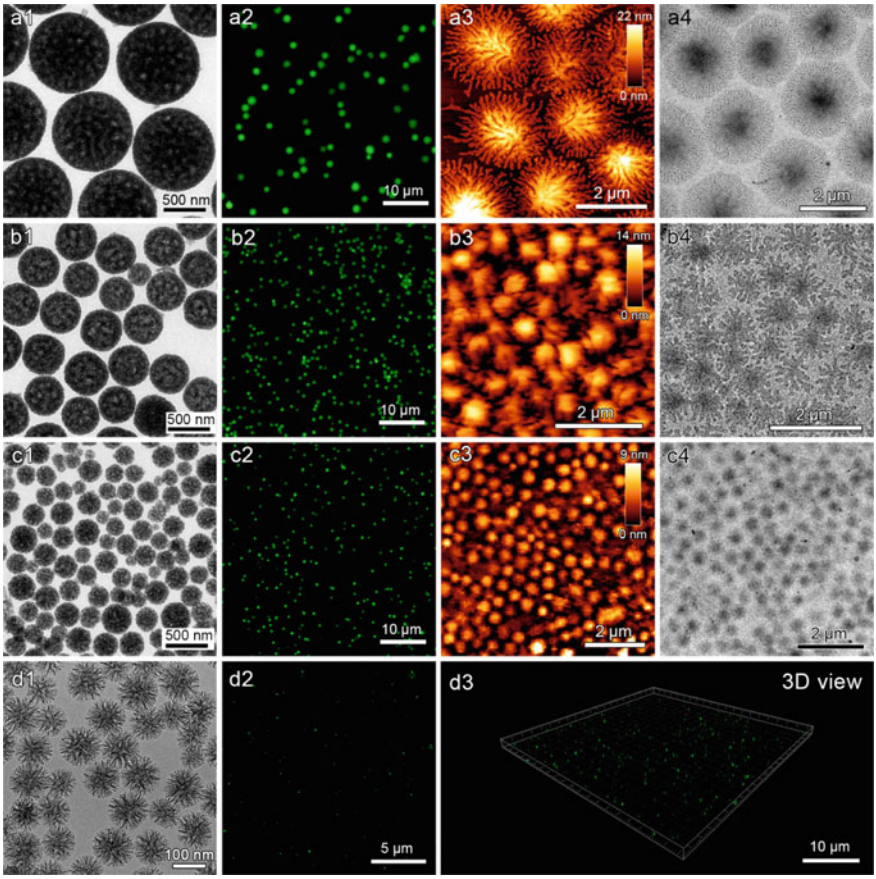

Fig. 19.3 Transmission electron microscopy (TEM) images of the mesoporous silica particles with different average diameters: **a1** 1000 nm, **b1** 500 nm, **c1** 280 nm, and **d1** 110 nm. Fluorescence microscopy images of different AF488-labeled PEG particles: **a2** PEG40-1000, **b2** PEG40-500, **c2** PEG40-280, and **d2**, **d3** PEG40-110. **a3**–**c3** Atomic force microscopy images and **a4**–**c4** TEM images of different PEG particles: **a3**, **a4** PEG40-1000, **b3**, **b4** PEG40-500, and **c3**, **c4** PEG40-280. Reproduced from Cui et al. (2015) with permission from American Chemical Society

long-rod counterparts (Huang et al. 2011a). Recently, the shape effect on biodistribution has been further corroborated by using magnetic mesoporous silica (MS) nanoparticles, which have been generated with different shapes and have been labeled with fluorescein isothiocyanate (FITC) for fluorescence monitoring (Fig. 19.4) (Shao et al. 2017). The spherical nanoparticles with a diameter of 200 nm are denoted as M-MSNP0, whereas those rod-shaped cylinders with dimensions of 200–250/120–150 nm and 400–450/120–150 nm are denoted as M-MSNP1 and M-MSNP2, respectively. Upon intravenous administration to tumor bearing mice, M-MSNP2 has been found to be most effectively deposited in the spleen and tumor (Shao et al. 2017),

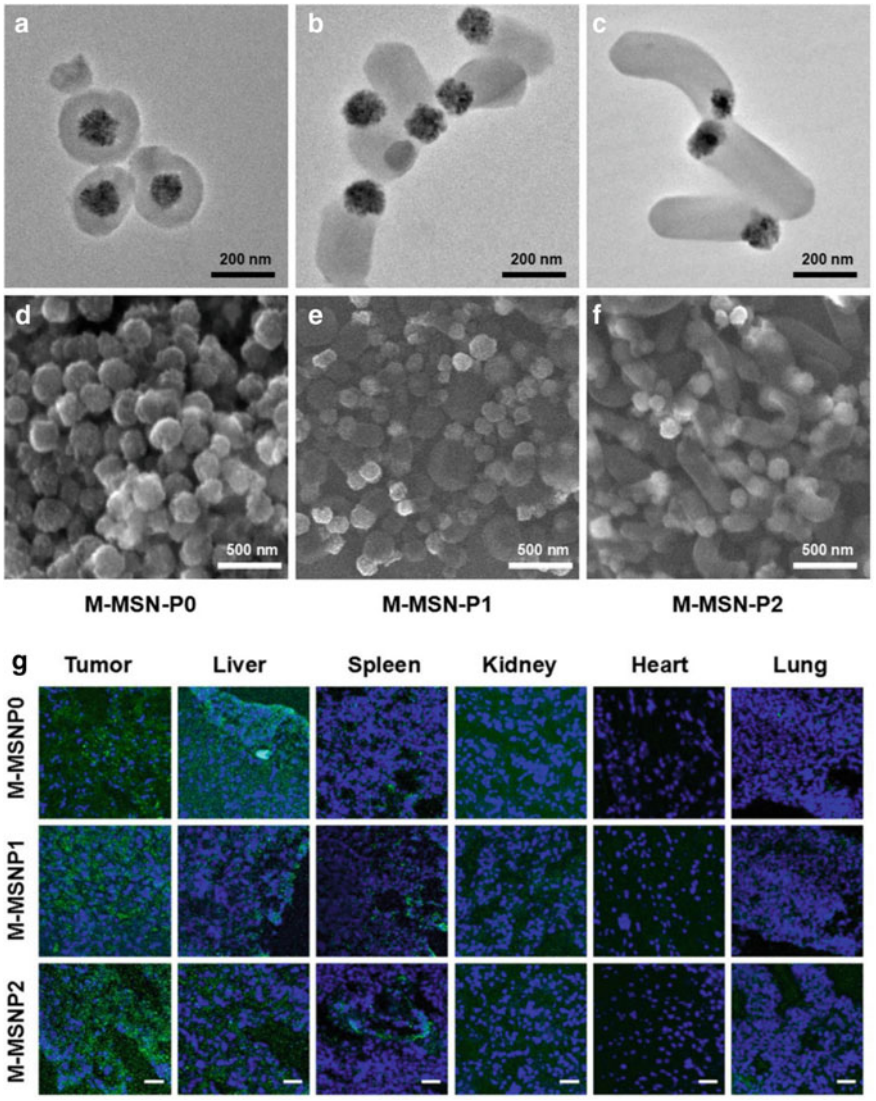

Fig. 19.4 **a**–**c** TEM and **d**–**f** SEM images of different magnetic MS nanoparticles: **a**, **d** M-MSNP0, **b**, **e** M-MSNP1, and **c**, **f** M-MSNP2. Scale bars in **a**–**c** represent 200 nm; whereas those in **d**–**f** represent 500 nm. **g** Confocal microscopy images of tissue sections taken from different tissues of HepG2 tumor-bearing mice 3 h after intravenous administration of FITC-labeled magnetic MS nanoparticles. Scale bars represent 100 μm. Reproduced from Shao et al. (2017) with permission from Elsevier B.V.

whereas M-MSNP0 has accumulated at a much higher level than those rod-like counterparts in the liver (Shao et al. 2017).

Finally, the number of times of drug administration may affect the PK profile. This is revealed by Dams and coworkers (2000), who have studied the pharmacokinetics and biodistribution of repeated injections of radiolabeled PEGylated liposomes. They have observed that, 4 h after the second dose in rats, a significant reduction in the blood content (from 52.6 ± 3.7 to $0.6 \pm 0.1\%$ injected dose, $P < 0.01$) occurs (Dams et al. 2000). Such reduction is accompanied by a dramatic increase in the uptake of the liposomes in the liver (from 8.1 ± 0.8 to $46.2 \pm 9.8\%$ injected dose, $P < 0.01$) and in the spleen (from 2.2 ± 0.2 to $5.3 \pm 0.7\%$ injected dose, $P < 0.01$). Similar observations have been found in rhesus monkeys (Dams et al. 2000). This suggests that previous administration of a carrier may lead to changes in the PK behavior of subsequently injected doses in time- and frequency-dependent manners. Because aging is a progressive process and tackling it requires repeated administration of an intervention, the impact of repeated administration on the PK profile, and hence on the efficiency of a biogerontological intervention, is a problem that shall be solved for future anti-aging medicine.

19.4 Enhancement of Hematocompatibility for Systemic Delivery

Because the extension of the blood retention time is one of the important tasks to be achieved during the development of a systemic drug carrier, the **hematocompatibility** of a carrier has to be seriously considered. This compatibility can be partially enhanced by modulating the effect of the carrier on **hemolysis** (Dobrovolskaia et al. 2008). Changes in hemolytic properties can be achieved by optimizing the surface properties (especially surface charge) of the carrier. This has been revealed in dendrimers generated from polypropylene imine (PPI) (Dutta et al. 2007; Agashe et al. 2006), polylysine (PLL) (Shah et al. 2000), carbosilane (Bermejo et al. 2007), and polyamidoamine (PAMAM) (Domanski et al. 2004). The presence of unprotected primary amines on the surface of these dendrimers has been found to lead to erythrocyte damage in a dose-dependent manner; whereas blocking of those primary amines has successfully reduced **hematotoxicity**. In addition to hemolysis, the extent of complement activation induced by a carrier largely affects hematocompatibility. Complement activation by a systemically administered carrier cannot only lead to rapid clearance of the carrier from blood circulation and hence failure of bodywide biodistribution, but may also result in life-threatening conditions such as hypersensitivity reactions and anaphylaxis. The latter is supported by a clinical study (Chanan-Khan et al. 2003), which has confirmed that the observed hypersensitivity led by PEGylated liposomal doxorubicin (Doxil) is mediated largely by complement activation. For this, unlike the case of vaccination in which local complement activation may be needed for enhanced antigen presentation to boost the vaccine

efficacy, complement activation by a carrier is not desired during systemic interventions. The surface charge of a carrier is one of the factors determining the extent of complement activation. As suggested by various systems (including polystyrene nanospheres (Nagayama et al. 2007), lipid nanocapsules (Vonarbourg et al. 2006), polypropylene sulfide nanoparticles (Reddy et al. 2007), and cyclodextrin-containing polycation-based nanoparticles (Bartlett and Davis 2007)), charged nanoparticles are generally more effective than the neutral counterparts in inducing complement activation. In addition, the choice and structure of the coating material can affect the capacity of the carrier to activate the complement system. For instance, while the presence of a PEG coating or a poloxamine 908 coating can reduce the activation of the complement system (Al-Hanbali et al. 2006; Vonarbourg et al. 2006), the incorporation of a dextran coating may lead to an increase in complement activation (Bertholon et al. 2006), whose extent increases with the coating thickness (Bertholon et al. 2006). Further studies are required to determine the relationship between the properties (including the thickness, density, and structure) of the polymer coating and the extent of complement activation.

Last but not least, **thrombogenicity** significantly governs the hematocompatibility of a carrier. This factor is especially important when a systemic carrier is developed. This is because when a carrier is used for systemic delivery, extended blood circulation time is required. In this case, the interaction of the carrier with components of the coagulation system will be long too. This may activate the coagulation cascade, leading to blood clotting and the occlusion of a blood vessel by thrombus. Thrombogenicity of a carrier can possibly be modulated by changing the surface properties of a carrier. This has been shown by the case of cetyl alcohol/polysorbate-based nanoparticles, in which the extent of platelet aggregation is reduced upon the incorporation of a PEG coating (Koziara et al. 2005). However, at the moment the association between the surface properties of a carrier to thrombogenicity is poorly understood. Apart from manipulating the carrier per se, co-administration of the carrier with anticoagulants may be a feasible method of reducing platelet aggregation. However, a recent study has observed that while micron-sized carbon particles require protein kinase C (PKC) for the upregulation of the glycoprotein integrin receptor GPIIb/IIIa to induce platelet aggregation (Radomski et al. 2005), carbon nanoparticles can induce integrin receptor activation in a PKC-independent manner (Radomski et al. 2005). This implies that platelet aggregation may be induced via multiple pathways, and the use of common anticoagulants may not necessarily be effective. To enhance the effectiveness of systemic delivery, elucidation of the exact mechanism underlying platelet aggregation induced by nanoparticulate systems is in dire need.

19.5 Other Factors to be Considered for Intervention Execution

Apart from the issues mentioned above, there are other technical factors to be considered during intervention execution. One is the route of administration. Oral/intranasal administration is usually not an option for many diseases such as cancer whose target site is not readily accessible by the oral and intranasal routes (Tatiparti et al. 2017). In addition, these routes may be associated with other issues such as enzymatic degradation in the intestine (Allemann et al. 1998; Bernkop-Schnurch and Krajicek 1998), mucociliary clearance in the nasal epithelium (Brime et al. 2000), and insufficient permeability across the intestinal epithelium into the systemic circulation (Haussecker 2014). Another option is subcutaneous injection which has the advantage of bypassing the first-pass effect of the liver and accessing to the circulation through capillaries or lymphatic drainage from interstitial space (Tatiparti et al. 2017). Nevertheless, the hydrophobicity and particle size of some drug carriers may trigger phagocytosis by the immune system (Tatiparti et al. 2017). Hitherto, intravenous injection is the most common method for systemic administration. In addition, as the administered agents are able to be passively delivered to tissues with an irregular fenestration (such as the bone marrow, liver, and spleen) (Lungwitz et al. 2005; Lai 2011), intravenous injection is suitable for treatments of localized diseases like primary cancer. Other administration means such as buccal/sublingual, pulmonary, and transdermal routes suffer from similar limitations mentioned above (e.g., compromised stability, immune response, etc.), leading to a dose loss issue. However, increasing the dose might not be an appropriate solution, as the additional dose required to compensate the dose loss might be too high to be practically applicable and safe, especially when a viral delivery system is used.

Crossing the vascular barrier is a crucial step for drug delivery to reach the target tissues, too. The most important feature determining the success of delivery within the vasculature is particle size (Tatiparti et al. 2017). Liver generally allows the entrance of particles with a size below 100 nm (Fang et al. 2011). In case of tumor tissues, carriers should be made in a smaller size as the capillaries have smaller pores (60–80 nm) and the endothelium there is covered with the continuous basal lamina which prevents the diffusion of larger nanoparticles (Wang et al. 2016; Kobayashi et al. 2013). Finally, one of the major mechanisms involved in the removal of drug carriers from the bloodstream is via urine by glomerular filtration in the kidneys with a pore size of approximately 8 nm (Jarad and Miner 2009; Wartiovaara et al. 2004; Huang et al. 2011b), which sets a lower limit for the size of carrier particles.

Immune response is also an obstacle for drug delivery (Ma et al. 2005; Draz et al. 2014). To overcome this limitation, non-immunogenic carriers are often used in order to avoid being destroyed before reaching the target (Tatiparti et al. 2017). Viral vectors might not be a suitable candidate in this case for their high susceptibility toward the immune response (Xue et al. 2015). Once a carrier reaches target tissues, effective cellular internalization is required. Plasma membranes are, however, made of negatively charged phospholipids. The surface charge often acts as a barrier for

the uptake of certain carriers or therapeutic agents such as siRNA (Tatiparti et al. 2017). To address this challenge, positively charged nanoparticles (e.g., cationic lipid nanoparticles) are often used as carriers (Lappalainen et al. 1994; Mishra et al. 2004). Alternatively, the effect of **endocytosis** has to be utilized (Meade and Dowdy 2007) by surface modification of the nanocarriers with agents such as folate (Rozema et al. 2007), transferrin (Konishi et al. 2008), and aptamers (Chu et al. 2006).

19.6 Summary and Outlooks

Similar to the case of hydrophobic drugs, intravenously administered carriers also undergo protein corona formation, leading to a further increase in the carrier size by 3–35 nm (Choi et al. 2007; Monopoli et al. 2011). This may change the ADME profile of the carrier as well. This has been demonstrated by the case of lipid nanoparticles composed of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE). Upon exposure to plasma, a protein corona consisting of various apolipoproteins (including Apo A-I, Apo C-II, Apo D, and Apo E) is formed, and causes a 13-fold increase in the uptake of the nanoparticles in PC3 prostate cancer cells (Barran-Berdon et al. 2013). The effect of the protein corona on physiological performance has been further confirmed by the case of dihydrolipoic acid- or cysteamine-functionalized quantum dots, whose size has been found to be increased upon exposure to protein serum, resulting in a reduction in renal clearance (Choi et al. 2007). Here it is worth noting that the composition of the protein corona could be different among different types of nanoparticles, or even among the same type of nanoparticles that show different dimensions. Taking the case of gold and silver nanoparticles as examples, the composition of the protein corona on these nanoparticles differs by over 60% even though the size and charge of the particles are the same (Walkey et al. 2014). In addition, the protein corona composition for amine- and carboxy-functionalized gold nanoparticles differs by almost 53%, whereas that for 15 and 30 nm gold nanoparticles differs by around 25% (Walkey et al. 2014). Apart from the physicochemical properties of the nanoparticles per se, the local environment and the duration of blood exposure may affect the protein corona composition (Lundqvist et al. 2011; Casals et al. 2010). Along with the fact that the composition of the blood changes constantly owing to convection and changes in cellular metabolism, precisely predicting the composition of the protein corona is challenging. Yet, taking this challenge is unavoidable because the protein corona composition largely determines the biological identity of a carrier. Taking the fact that the synthetic identity and the biological identity are largely interrelated to each other (Fig. 19.5), in order to develop an effective systemic carrier, proper manipulation of the synthetic identity to enable better control of the protein corona composition is required.

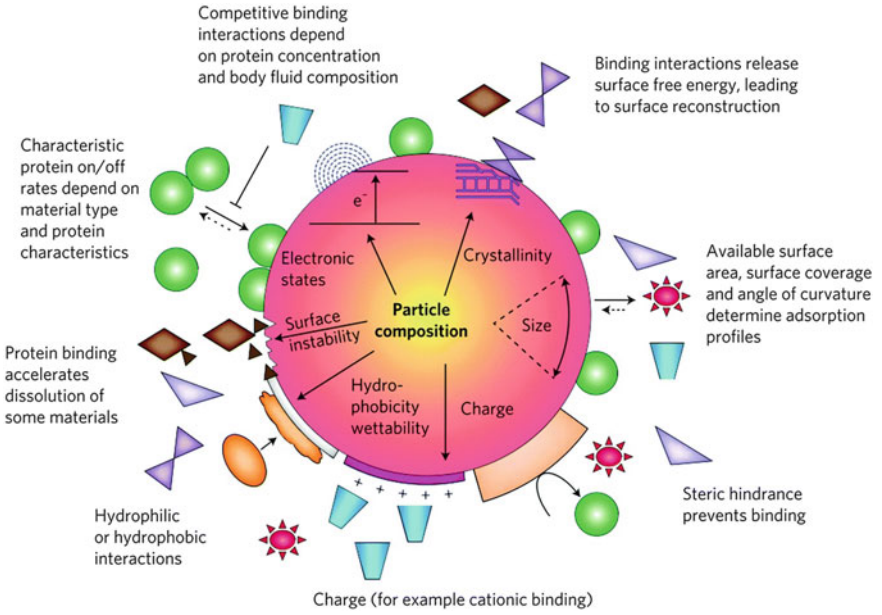

Fig. 19.5 An overview delineating the effects of the synthetic identity of a nanoparticle on protein adsorption. Reproduced from Nel et al. (2009) with permission from Springer Nature

Important Notes

- The ADME (absorption, distribution, metabolism, excretion) concept provides a framework for our understanding of the fate of an exogenous agent upon administration into a body.
- To alter the pharmacokinetics and biodistribution of a carrier, several properties (including the particle size, shape, and surface properties) of a carrier can be modulated.
- The hematocompatibility of a carrier can be partially enhanced by modulating the effect of the carrier on hemolysis.
- Thrombogenicity of a carrier is important when a systemic carrier is developed because extended blood circulation time of a carrier may activate the coagulation cascade.

Questions for Future Research

- **How can a carrier be able to reach tissues bodywide?** Aging occurs in all tissues and cells in a body. Carriers that can help deliver a bioactive agent to different parts of a body is vital for the development and execution of

a biogerontological intervention. Strategies to manipulate the pharmacokinetics and biodistribution of carriers are, therefore, required for practices in anti-aging medicine.

- **How can the physiochemical properties and the PK profile of a carrier be predicted before synthesis?** Right now carriers are usually developed in a trial-and-error manner. Their PK profiles and efficiency are known only after experimental evaluation. If the performance of a carrier can be estimated in silico based on the structure-activity relationship (SAR) and related models, candidates that are less likely to succeed can be excluded from further experimental studies. This may help facilitate the development of effective carriers.
- **How can a carrier be designed to tailor individual differences among subjects in a wider population?** The PK profile of a carrier is affected not only by the physical and chemical properties of the carrier per se but also by genetic variations and individual differences among subjects. Serious consideration shall be made on ways to accommodate such variations so that the delivery efficiency of a carrier among a wider population can be secured.

Glossary

Bioavailability The degree to which the active ingredient or moiety is absorbed from an administered dosage form and becomes available at the site of action.

Endocytosis A process that transports molecules from the extracellular milieu into cells via vesicle formation at the plasma membrane.

Enhanced permeability and retention (EPR) effect The effect of passive targeting caused by the extravasation of large molecules from the leaky tumor vasculature and hence the accumulation of those molecules in the tumor tissue.

Hematocompatibility Compatibility of a material with the components of the blood system.

Hemolysis Rupture of erythrocytes with release of hemoglobin into the plasma.

Hematotoxicity The toxicity of an agent to blood or hematopoietic tissues.

Opsonization A process in which opsonins interact with exogenous entities to make the entities more susceptible to phagocytosis.

Telomere A segment of DNA at the end of a chromosome. It gives protection to that chromosome.

Thrombogenicity The capacity of a material to induce or promote the formation of thromboemboli.

Zeta potential The electrical potential at the boundary of the hydrodynamic shear plane of a charged particle.

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Chapter 20
New Directions for Use of Systemic Drug Delivery in Anti-aging Medicine

Carmela Rita Balistreri

Abstract Anti-aging medicine has become a popular topic in recent years. It considers biological aging a revertible process that physiologically serves no positive function. Different technologies in systemic delivery and various strategies that characterize and optimize the performance of systemic delivery have been covered in this book. These technologies are essential for the establishment of a technical platform for interventive biogerontology. As the last chapter of this book, we would like to highlight some directions for future applications of systemic drug delivery in anti-aging medicine. We believe that biological aging and aging-related diseases are strongly associated with genetics/genomics and are pre-programmed. Several interventive approaches such as cellular/tissue reprogramming, microbiota supplementation, seno-therapeutics and pharmacological targeting will be discussed in this chapter.

Keywords Aging · Anti-aging medicine · Developmental programming · Innovative interventions and recommendations

20.1 Anti-aging Medicine: A Basic Overview

Population aging has been a social issue in Western countries in recent years. Life expectancy has been increasing steadily over the years, which may not necessarily be associated with an amelioration of health (Lunenfeld and Stratton (2013). Several factors have contributed to the aging process, of which geriatrics, education and medicine play the most important roles (such as the use of vaccination, antibiotics and disinfectants) (Harper 2014).

Aging is significantly associated with the onset of several defined age-related diseases (ARD) such as chronic inflammatory diseases, cardiovascular diseases (CVD), type 2 diabetes (T2D), osteoporosis, neurodegenerative diseases and cancers

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(Edwards 2012) (Fig. 20.1). By 2030, approximately 20% of the population will be aged 65 or older, and ARD will become a serious health problem. For instance, CVDs will be responsible for 40% of total deaths and ranked as the top cause (Jones et al. 2019; Kirkwood 2017). This estimation has led the governments and scientific community to invest in public health research, such as disease-prevention and health-promotion programs for investigating the major causes of morbidity in the elderly and reducing the treatment costs (Edwards 2012; Zolotor and Yorkery 2019). At the beginning of 1990, a branch of medical science, refereed as "anti-aging medicine", has become a popular topic in recent years (Lopreite and Mauro 2017; Kirkland 2013; Flatt et al. 2013). Anti-aging medicine aims to promote **healthspan** and lifespan by

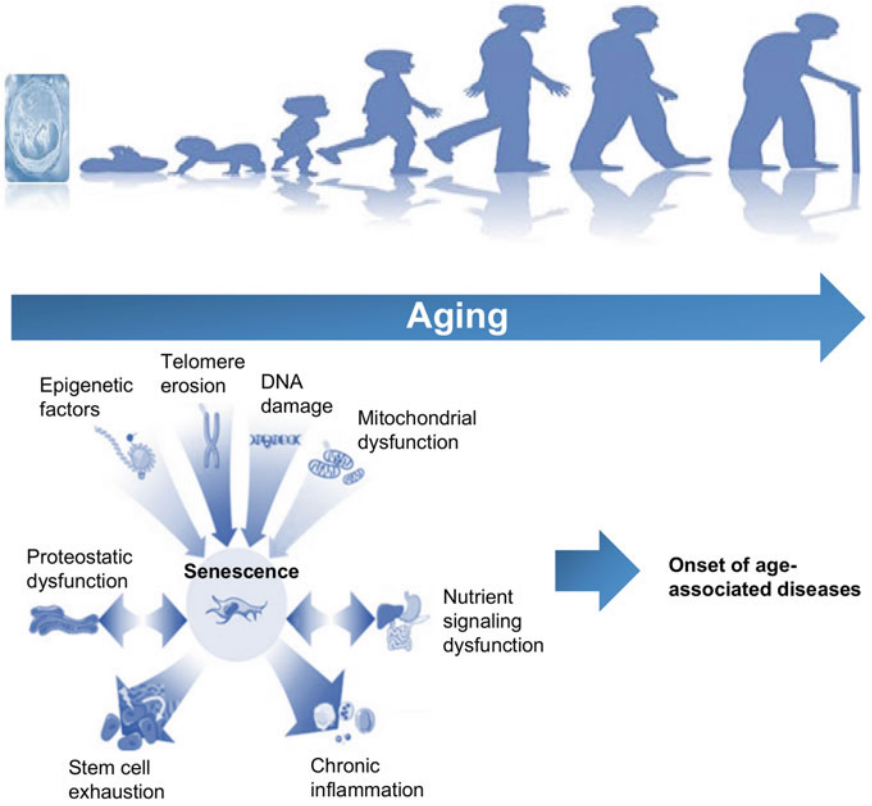

Fig. 20.1 The close relationship between aging, the onset of age-related diseases and the use of anti-aging medicine. The cells experience a decline in their activity and show diverse alterations over time (including genomic instability, epigenetic deregulation, loss of proteostasis, mitochondrial dysfunction, telomere shortening, autophagy, impaired stress resistance and deregulated nutrient signaling). As a result, senescence and apoptosis can occur, as well as a decline in their ability to be replaced because of the exhaustion of stem cells. This determines an alteration in both homeostasis and functions of tissues, organs and systems, as well as the causation of inflammation, fibrosis and consequently the onset of age-related diseases

using specific dietary and exercise regimes and performing biomedical interventions designed for delaying or reversing the aging process (Lara et al. 2016; Costa et al. 2016; Lemaître et al. 1806). Accordingly, conventional and alternative medical disciplines have been utilized for creating an integrated approach to achieve the best "anti-aging" results. Thereby, anti-aging medicine, as a holistic discipline, considers that ARDs affect the whole body instead of only a part of it. Various organizations (e.g. the American Academy of anti-aging medicine, https://www.a4m.com/) are offering related courses globally to physicians who are interested in studying age-related medicine and intervention approaches, particularly in the prevention of ARD. More importantly, advances in scientific knowledge has established a crucial platform of information for providing concepts regarding suitable life styles for extending lifespan and tackling aging.

Considering aging as a reversible process, anti-aging medicine has been playing a role in the aging process (Lemaître et al. 1806). Accordingly, some theories suggested that aging progresses as a result of other fundamental processes of life and that the aging process itself serves no specific function (Anton et al. 2005). Although this concept may be considered extreme, there are different approaches to manipulate aging. Age-associated senescence may be considered as a combination of many pathophysiological processes that could be prevented, delayed or even reversed (Gadecka and Bielak-Zmijewska 2019; Cevenini et al. 2010; Cabo et al. 2014). Currently, diverse biotechnological innovations such as genomics, transcriptomics, proteomics and metabolomics have been used in anti-aging medicine and shown great potentials to slow-down the aging process (Gadecka and Bielak-Zmijewska 2019; Cevenini et al. 2010; Cabo et al. 2014). In addition, these technologies have been used to study the molecular and cellular mechanisms related to the aging process, such as genomic instability, epigenetic deregulation, loss of **proteostasis**, mitochondrial dysfunction, cellular senescence, exhaustion of stem cells, inflammation, telomere shortening, autophagy, impaired stress resistance and deregulated nutrient signaling (Cabo et al. 2014; López-Otín et al. 2013; Riera et al. 2016; Balistreri et al. 2013). Based on the existing knowledge, innovative therapeutic strategies can be developed against age-related functional decline and the onset of pathological conditions of tissues, organs and systems of an organism. Here, we report and discuss new concepts related to the advantages in using anti-aging medicine and present novel interventive strategies, including cellular/tissue reprogramming, microbiota, supplementation and senotherapeutics (Fig. 20.2).

20.2 Endothelium (Re)programming as a New Concept for Anti-aging Medicine

It was suggested that aging and ARDs may be the results of an altered developmental programming during the early life, which has been demonstrated by our previous work and the research done by other groups. The results illustrated a close relationship between an altered fetal development and a series of altered physiological

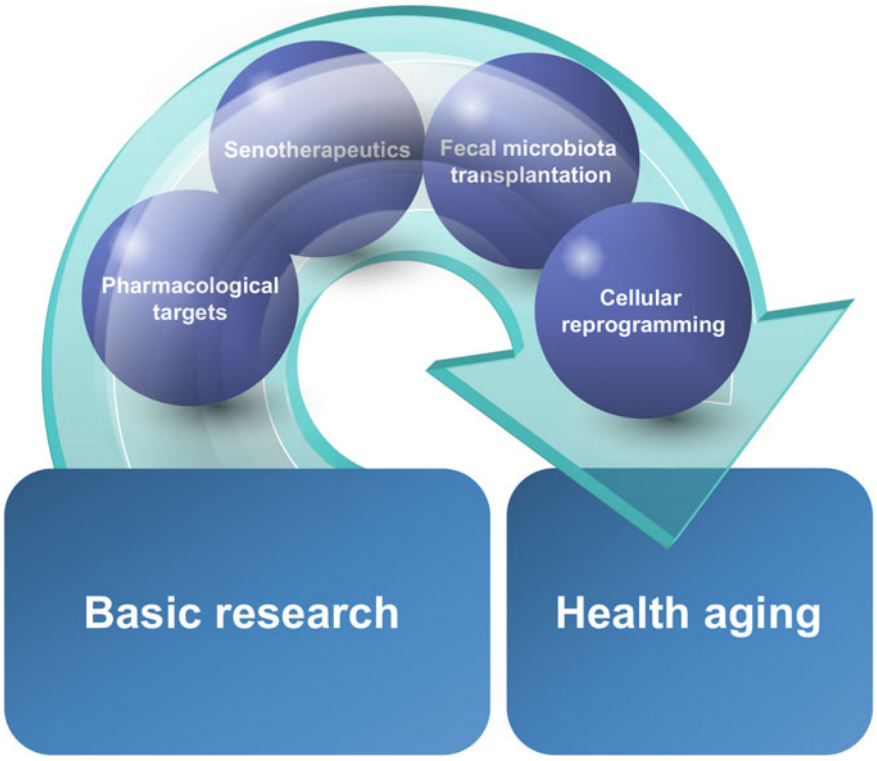

Fig. 20.2 Innovative approaches for decelerating aging of cells

functionalities such as early onset of diseases in the nervous, endocrine, immune, cardiovascular and hematopoietic systems (Faa et al. 2014; Alexander et al. 2015; Kwon and Kim 2017; Miranda et al. 2017; McGowan and Matthews 2018; Walker and Spencer 2018; Balistreri et al. 2019; Vaiserman et al. 2018). Accordingly, it has been demonstrated that the onset of specific ARDs and the consequent functional decline (senescence) can happen in an early stage of life (Vaiserman and Lushchak 2019; Wells 2017). The fetal period, specifically the embryonal phase, has been suggested to be a "critical window" highly vulnerable to various environmental stressors, which consequently influences the health conditions in adult periods (Kwon and Kim 2017; Miranda et al. 2017; McGowan and Matthews 2018; Walker and Spencer 2018; Balistreri et al. 2019; Vaiserman et al. 2018; Vaiserman and Lushchak 2019; Wells 2017; Phillippe and Phillippe 2017). Fetal period constitutes a critical stage of development due to a high rate of cellular proliferation and the plasticity of developing systems. These observations are in agreement with the new concepts regarding the high susceptibility of our systems to maternal, environmental and intrauterine stressors during embryonic development (Kwon and Kim 2017; Miranda et al. 2017; McGowan and Matthews 2018; Walker and Spencer 2018; Balistreri et al. 2019; Vaiserman et al. 2018; Vaiserman and Lushchak 2019;

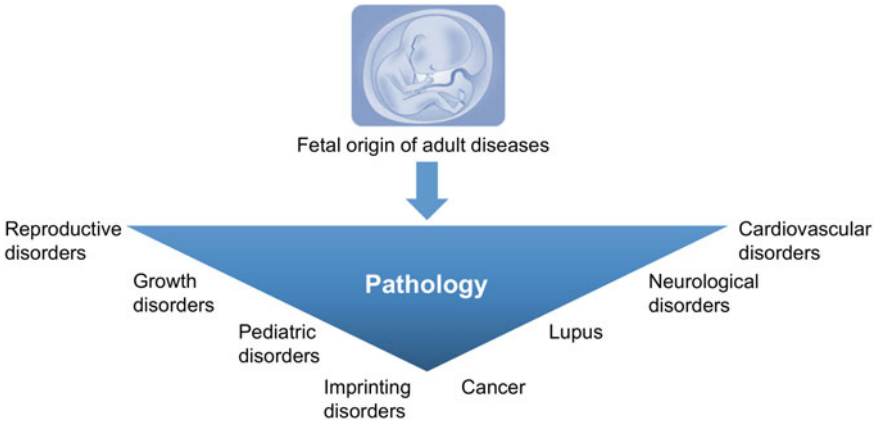

Fig. 20.3 The fetal origin of diseases

Wells 2017; Phillippe and Phillippe 2017). Unfavorable developmental conditions can influence the (epi)genetic and physiological processes of fetal development and thereby permanently modify the architecture and functionalities of the hypothalamic–pituitary–adrenal (HPA) axis and systems of the progeny (Kwon and Kim 2017; Miranda et al. 2017; McGowan and Matthews 2018; Walker and Spencer 2018; Balistreri et al. 2019; Vaiserman et al. 2018; Vaiserman and Lushchak 2019; Wells 2017; Phillippe and Phillippe 2017), resulting in a high vulnerability to disease development during adulthood. Accordingly, maternal pathological conditions, including **intrauterine growth restriction** (IUGR), **hypoxia**, malnutrition and placental insufficiency, are significantly associated with the risks of several pathologies in neonates (Fig. 20.3) (Kesavan and Devaskar 2019; Balistreri 2016, 2017; Barker 2007). For instance, IUGR neonates causes prematurity and related immediate medical problems, as well as a notable susceptibility to hypertension, CVDs, type 2 diabetes and neurodegenerative diseases in adults. Based on these concepts, several theories and models of the brain and cardiovascular systems have been developed (Alexander et al. 2015; Walker and Spencer 2018; Barker 2007).

The abovementioned observations underlined that the health conditions in adulthood are highly associated with the early developmental programming. Several developmental programming events and processes, including epigenetic mechanisms and the (re)programming of the endothelium, are essential for the embryonal origin, homeostasis and functions (Balistreri et al. 2019; Kesavan and Devaskar 2019; Balistreri 2016, 2017; Barker 2007) in all of the systems particularly cardiovascular, **haematopoiesis** and immune systems. Concerning the (re)programming of the endothelium, we have proposed a model (Balistreri et al. 2019). Specifically, we suggested that developmental adversities (of both maternal and paternal) permanently affect the **hypothalamic–pituitary–adrenal (HPA) axis** and **endothelium** with profound effects on their subsequent functions (Walker and Spencer 2018; Balistreri et al. 2019; Vaiserman et al. 2018). This is the consequence of epigenetic

(re)programming that induces these effects through a strong interaction of maternal endothelium with developing placenta and fetal hypothalamus. Developing placenta and hypothalamus influence the expression of fetal genome. In particular, the developing fetal hypothalamus regulates the release of hormones and their functions as well as the functions of maternal hypothalamus. This determines the secretion of various hormones that are crucial for the fetus and are responsible for various pregnancy conditions, such as IUGR and **preeclampsia** (Balistreri et al. 2019). The combination of all these programming conditions causes long-term structural and functional alterations in the systems and eventually results in increased risks of diseases and an accelerated aging process.

Apart from this, parental adversities are also associated with stressors, health conditions (such as hypertension and type 2 diabetes) and an unhealthy lifestyle such as smoking, abusing alcohol and drugs, diet, sedentariness and exercise. These factors have profound biological effects on both fetal development and the subsequent functionalities of HPA axis and specific systems (McGowan and Matthews 2018). Furthermore, these effects are species-, gender- and age-specific and dependent on the timing and duration of exposure, as emphasized by McGowan and Matthews (McGowan and Matthews 2018). This has led to the proposed two-level therapeutic approaches, where the first level is based on healthspan-promoting interventions which focus on unhealthy lifestyles of the parents before and during pregnancy. The interventions are based on recommendations on healthy diets (Hillier and Olander 2017; Biagi et al. 2019), physical activities (Thompson et al. 2017), lowing the levels of stress (Ng et al. 2019) and abstaining from smoking, alcohol and drugs (Hill et al. 2019). Hillier and Olander (2017) have underlined that dietary intake before and during pregnancy can significantly impact the health conditions of both the mother and the child, such as gestational weight gain. To ensure that appropriate interventions are designed to improve dietary intake during pregnancy, it is important to understand the dietary changes of pregnant women before the interventions. Thus, they have conducted 11 studies and found an increase in energy intake during pregnancy. Furthermore, most of the studies observed a significant increase in fruit and vegetable consumption, a decrease in egg consumption, a decrease in fried and fast food consumption and a decrease in coffee and tea consumption before and during pregnancy. It was also suggested that age, education and the intention to pregnancy were associated with healthy dietary changes. However, these factors were only assessed in a small number of studies. Consequently, interventions might be applied to dietary styles, such as the Mediterranean diet. Accordingly, Biagi and coworkers (2019) have examined the beneficial effects of adhering to the Mediterranean diet during pregnancy on the health of the child by considering the Mediterranean diet as a whole rather than focusing on the effects of its individual ingredients. Evaluating the data of 29 studies, they have underlined the protective role of the Mediterranean diet in fetal growth, prematurity, neural tube defects and other congenital pathologies, asthma and allergy, body weight and metabolic markers.

In this regard, strategies aiming to promote the adherence to this dietary pattern might be of considerable importance to public health. Accordingly, the World Health Organization (WHO) has proposed guidelines to provide information on an optimal

weight gain during pregnancy (Io 2009). These guidelines are based on the pre-pregnancy body mass index (BMI) independent of age, parity, smoking and race. For example, women with a pre-pregnancy BMI below 18.5 are supposed to gain 12.7–18 kg during gestation, while the weight gain recommended for pre-pregnant overweight/obese women are much lower at 6.8–11.3 kg for overweight women and 5.0–9.1 kg for obese women. Therefore, it is necessary to inform women before pregnancy about the importance of their nutritional intake to avoid starvation/caloric restriction (consuming all essential nutrients but having a 30–70% reduction in calories) of obese women, which have negative effects on the development of the unborn child. For example, it has been demonstrated that the levels of leptin decline in response to fasting, leading to a higher susceptibility to infection during pregnancy (e.g. influenza) due to the impaired immune response (impaired T cell activation, proliferation and differentiation) (Kahn et al. 2019). This is especially striking, as pregnancy itself has been reported to be a risk factor for severe influenza infection due to the contradictory demands for the maternal immune adaptation to pregnancy in addition to a required immune response to clear the infection. In contrast, women exceeding the recommended caloric intake could be exposed to an increased risk of developing gestational diabetes, pre-eclampsia and pre-term birth. Interestingly, normally progressing pregnancies are associated with a physiological insulin resistance known as the "diabetogenic state" which ensures stable glucose levels and facilitates nutrient availability to the fetus (Thiele et al. 2018).

In addition to dietary nutrition, supplemented nutrition and micronutrients also have the potential to modulate maternal conditions, including the responses of immune system. The importance of delivery technologies has been suggested because an enhanced delivery efficiency may increase the bioavailability of the micronutrients supplemented. This, on one hand, can reduce the dose required and hence avoid toxicity, and on the other hand can enhance the effects of the micronutrient administered. Due to the importance of the supplementation of micronutrients during pregnancy, pregnant women are receiving a wealth of recommendations. Recommended supplementations include mineral and vitamins such as folic acid (vitamin B9) and vitamin D.

A folic acid intake of 400 μg is usually recommended before pregnancy and during the first trimester in order to prevent profound developmental abnormalities in the fetus (e.g. neural tube defects) (Zhao et al. 2014). Folic acid is present in leafy green vegetables, granary bread, brown rice and liver. However, it is almost impossible to ensure adequate intake solely from food or folic acid fortification (e.g. salt). Therefore, deficiency remains an important challenge in Western countries as well as in developing countries. Dietary supplementation is deemed to be the only way to overcome this challenge. Folic acid is an essential vitamin and is known to be involved in one-carbon metabolism which is vital for amino acid metabolism and the methylation of DNA, RNA and proteins (Antony 2007). Therefore, folate acts as a methyl donor for epigenetic mechanisms and is well known for its potential to affect the methylation and consequently the gene expression in the placenta or the developing fetus (Håberg et al. 2009).

As the number of cases of vitamin D deficiency has generally increased over the past 50 years, vitamin D supplementation is recommended to pregnant women by the WHO at a dose of 5 μg per day especially during winter months. Vitamin D is present in very few sources such as salmon, mackerel and herring, cod liver oil and egg yolk. Vitamin D supplementation (e.g. from milk or margarine) has been generalized in some but not all countries (Gallo et al. 2019). Vitamin D is known to be involved in bone metabolism and to modulate immune responses and glucose metabolism (Gallo et al. 2019). The impacts of vitamin D deficiency are more severe in obese women as body fat is a key storage of the vitamin D produced in the skin, which reduces its availability to the body (Kaushal and Magon 2013). Given that vitamin D in fetus is solely supplied by the mother, the recommendation for supplementation is more important to pregnant women with a high BMI.

In terms of mineral supplementations in women during pregnancy, iodine, zinc and selenium have shown diverse beneficial effects on hormone synthesis. In addition to their roles in the protection against oxidative stress in the thyroid, these micronutrients interact with iodine in the form of thyroid hormones and facilitate the conversion of prohormone thyroxine (T4) to its biologically active form 3,5,3′-triiodothyronine (T3) (McAlpine et al. 2019). Supplementation with exogenous antioxidants such as vitamins E and C, beta-carotene, lipoic acid, coenzyme Q10, glutathione, polyphenols, phytoestrogens have also been recommended. Vitamin C (ascorbic acid) is an important hydrophilic antioxidant (Hovdenak and Haram 2012). It effectively reduces the levels of α-tocopheroxyl radicals and low-density lipoprotein (LDL) in cell membranes and thereby restores α-tocopherol and inhibits the generation of free radicals. Vitamin E (α-tocopherol) is the main hydrophobic antioxidant protecting cell membranes from oxidative damage by reacting with lipid radicals produced in the lipid peroxidation chain reaction. The dietary vitamin E supplementation has been shown to be associated with a reduced risk of atherosclerosis through reducing oxidative stress and inhibiting LDL oxidation (Hovdenak and Haram 2012). Thus, supplementation of nutrition and micronutrients might be a potential approach to modulate the complex and refined reprogramming during pregnancy. Therefore, the nutrition status before and during pregnancy is essential for reproduction.

20.3 Early Measures and Recommendations for Modulating Aging

In addition to the first level healthspan-promoting interventions, the second level therapeutic interventions during prenatal and neonatal periods or in adult life are also recommended. Extensive studies have been performed on pharmacological targets/targeting of pathways using mediators such as small molecular compounds and therapeutic nucleic acid materials (e.g. miRNA and siRNA). The execution

of such interventions necessitates the use of effective systemic delivery technologies to transport the therapeutic agents bodywide. Apart from manipulating age-associated symptoms and diseases in a fully developed adult, given the close relationship between endothelium and the human systems during fetal and adult life, we have proposed a therapeutic approach based on the use of agonists, antagonists and inhibitors of molecular pathways (i.e. Notch, Wnt, Hedgehog, Retinoic acid, Toll-like receptor, insulin/insulin-like growth factor-1 (IGF-1), mTOR) associated with the development, homeostasis and functions (Balistreri 2018; Balistreri et al. 2016, 2017). It might be an optimal strategy for improving the functions of human systems during altered development or the aging process (Balistreri 2018; Balistreri et al. 2016, 2017; Buffa et al. 2019).

Pharmacological targets/targeting are another type of anti-aging/disease treatment (i.e. anti-ARDs treatments) (Balistreri 2018). For example, modulation of IGF-1 availability could inhibit the activities of IGF-1. Accordingly, pharmaceuticals modulating the pregnancy-associated plasma protein-A (PAPP-A), a zinc metalloproteinase known to enhance the local bioavailability of the IGFs, have been developed. Such medications were found to be a promising approach as PAPP-A knockout mice have previously been found to have substantially extended healthspan, suggesting an important role of PAPP-A in aging and associated diseases (Conover 2010; Conover and Oxvig 2017). The mammalian target of rapamycin (mTOR) pathway is another nutrient-sensing pathway which plays a role in mediating the rate of aging (Ehninger et al. 2014). It is known to play a role in cell growth, metabolism and regulation of energy homeostasis (Ehninger et al. 2014). Therefore, mTOR has been deemed to be a major target for pharmacological interventions to modulate the nutrient response pathways and to decelerate the aging process. Inhibition of this pathway has repeatedly been demonstrated to confer the protection against aging-associated pathological conditions and to extend lifespan in different model organisms (Ehninger et al. 2014). Genetic inhibition of mTOR signaling caused life extension in worms, fruit flies and mice (Ehninger et al. 2014). Recent gene expression analysis showed that mTOR pathway is strongly associated with human health and longevity (Passtoors et al. 2013). Pharmacological interventions of telomerase activity are another promising anti-aging approach. Proper maintenance of telomeres (the nucleoprotein structures at the end of linear eukaryotic chromosomes) is crucial for genome stability (Chiodi and Mondello 2016). Age-related telomere shortening is known to play a major role in senescence and aging-associated conditions (Balistreri et al. 2014). Another treatment is the senotherapeutics that includes three therapeutic approaches: (i) using molecules that are able to selectively kill senescent cells (SCs) (senolytics); (ii) using compounds with the capacity to attenuate the proinflammatory effects of SCs or to modify the senescent **phenotype** (senomorphics); and (iii) preventing the accumulation of senescent cells (Balistreri 2018; Xu et al. 2018).

In recent years, pharmacological compounds targeting epigenetic regulators of gene expression are actively studied in the context of geroscience. Epigenetic mechanisms, including histone modifications, DNA methylation and microRNA (miRNA) expression, play an important role in regulating gene expression and genomic stability

throughout the lifespan. Epigenetic modifications are known to be finely balanced in normal tissues. However, they can be unbalanced in malignant and other transformed cells. Epigenetic dysregulation has been shown to contribute to the pathogenesis of age-associated pathologies such as cancers, atherosclerosis, type 2 diabetes, psychiatric and neurodegenerative diseases, as well as a decline in immune response. Therefore, the modulators of the activities of enzymes involved in epigenetic regulation might be clinically applicable. Epigenetic modifications are known to be potentially reversible. This feature makes them attractive targets for pharmacological interventions. Over the years, a series of medications have been developed targeting epigenetic regulators, including modulators of DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone acetyltransferases (HATs) and noncoding miRNAs, with potential effects against various types of tumors, myelodysplastic syndromes and neurodegenerative disorders (Arguelles et al. 2016). Apart from the methods mentioned above, other interventive approaches and recommendations might be used for modulating endothelium programming based on the mother's clinical conditions. The interventions on maternal (parental) microbiota might be an appropriate approach. Another approach is to modulate the stem cells of newborns by using regenerative medicine, such as cellular or tissue reprogramming. These will be described and discussed in the following paragraphs.

20.4 Fecal Microbiota Transplantation for Modulating Microbiota

At present, the research interest in gut microbiota has been growing due to its key role in modulating several mechanisms and processes associated with human health and diseases. Microbiota consists of many microorganisms (i.e. bacteria, viruses and mycetes) and plays a crucial role in the body (Milani et al. 2017). The microbiota resides in various anatomical structures of the body and is organized in niches. However, it is primarily located in the gut (Milani et al. 2017) and is defined as the gut microbiota (GM). The major bacterial species found in the gut include *Firmicutes* and *Bacteroidetes* (Milani et al. 2017).

As mentioned above, malnutrition or overnutrition during pregnancy can influence fetal programming. In addition, there is an increasing amount of evidence suggesting that environmental factors can influence intestinal microbiota in an early stage of life, which is also known as the "microbial programming phenomenon" (Balistreri et al. 2019; Balistreri 2018). In this context, obesity has been shown to be a key challenge for the health of both mothers and children. In children, obesity causes alterations in development of both microbiota and immune responses. Despite its significance, the weight during pregnancy, the composition and functional quality of maternal microbiota and its negative effects in progeny are unknown. Recently, Kozyrskyj and colleagues (Kozyrskyj et al. 2016) reviewed the literature of human studies and concluded that maternal obesity can modulate the composition and functions of gut

microbiota in newborns. Vertical transport of microbiota and their metabolic products have been hypothesized as possible mechanisms (Wang et al. 2019).

Based on the abovementioned observations, modulation of microbiota of both parents and newborns through the innovative method of fecal microbiota transplantation (FMT) (Wang et al. 2019) might be a potential approach. FMT directly modifies the recipient's gut microbiota in order to normalize the composition and to provide a therapeutic advantage. FMT has been approved by the FDA in 2013 for treating recurrent and refractory *Clostridium difficile* infection. Since 2013, its application has been restricted to gastrointestinal disorders, but it has also been administrated to patients affected by other diseases such as ARDs (Wang et al. 2019). Thus, FMT might be a beneficial therapeutic treatment for HS-ARDs. Although the long-term effects of FMT have not been completely elucidated, the current evidence showed few adverse effects (Wang et al. 2019). Consequently, it is important to determine the treatment duration for FMT as well as to follow-up with the potential side effects. In addition, further studies are needed for developing personalized FMT treatments.

20.5 Cellular Reprogramming for Tissue Regeneration

Another approach is the de novo generation of tissues such as endothelium cells. This approach involves the conversion of adult somatic cells to **induced pluripotent stem cells** (iPSCs) in vitro by delivering specific agents into the cells with a carrier. This technology has been defined as cellular reprogramming (Gurdon 1962). It has provided a new method for performing investigations on pathologies and for developing treatments and translational approaches. Currently, a new form of this methodology has been proposed by Takahashi and Yamanaka in 2006 (also known as the Yamanaka factors). It is essentially based on the use of specific transcription factors for a cellular lineage (Takahashi and Yamanaka 2006). Alternately, the use of microRNAs allows reorganization of the genetic expression of a somatic cell to a different cellular phenotype (Srivastava and DeWitt 2016). Thus, it has similar functions as direct reprogramming. With its principal advantages, it allows the control of the resident support cells of the niches within damaged organs or old tissues. As a result, it can facilitate the regeneration or repairment of old cells by transforming them directly to the cellular type which is required by specific tissues. Therefore, it can become a promising strategy for counteracting damage or aging of endothelium cells.

In fact, the endothelium is not only an essential element in the physiological development of fetal systems but also closely associated with physiological and disease conditions of all tissues and organs. An injury in endothelium exhibits a long-term effect known as endothelial dysfunction (Balistreri 2016, 2017). Hence, endothelium dysfunction precedes the onset of not only CVDs but also other pathologies related to age (Balistreri 2016, 2017). Thus, direct reprogramming might retard the onset of these diseases. Interesting results of the use of direct reprogramming in mice have

been obtained as demonstrated by the Abad group (Abad et al. 2013). Thus, reprogramming can be achieved in vivo. Further supportive evidence may be obtained from experiments on mouse BM transplantation followed by the identification of iPSCs in circulation, which may demonstrate the possibility of direct reprogramming HS in vivo (Abad et al. 2013). However, Abad and colleagues also demonstrated that Yamanaka factors induced a high grade of morality in mice, which was linked to the development of teratoma in multiple tissues (Abad et al. 2013). Certainly, this can limit its applications as a regenerative or anti-aging strategy (Abad et al. 2013). Alternatively, this methodology might be used for reprogramming a specific organ or tissue with considerations of the timing of its application and the conditions of the tissue or organ. Thus, the results obtained hitherto do not allow clinical applications. Several questions remain unanswered and require further investigations.

20.6 Summary and Outlooks

The current goal of researchers is to identify appropriate anti-aging interventions and their effects on the health of aged populations. Meanwhile, research in this area is becoming a business for several biotechnological companies who are developing a growing number of therapeutic agents, such as oxidant drugs, hormones, vitamins, diet supplements and various aesthetic drugs (Edwards 2012; Zolotor and Yorkery 2019; Lopreite and Mauro 2017; Kirkland 2013). In fact, these approaches are particularly costly but are extensively marketed by doctors, laboratories and companies to a gullible public perusing well-being (Edwards 2012; Zolotor and Yorkery 2019; Lopreite and Mauro 2017; Kirkland 2013). However, the majority of the existing treatments are rather palliative than curative and are certainly not feasible approaches to an extended lifespan (Edwards 2012; Zolotor and Yorkery 2019; Lopreite and Mauro 2017; Kirkland 2013). This indicates that effective anti-aging treatments for humans are still beyond reach. Despite this, with the increasing understanding of the aging mechanisms and the rapid development of systemic delivery technologies, one can be optimistic in identifying successful treatments in a foreseeable future. The development should start with various genetic, dietary and pharmacological interventions in short-lived model organisms (i.e. yeast, worm, flies, mice and rats) (Balistreri 2018). In addition, some of these treatments have also been demonstrated to retard the onset of ARDs and consequently to extend the healthspan (i.e. the length of time one lives in a good health). It is anticipated that, with the continuous development and optimization of anti-aging therapies, along with the emergence of novel strategies for anti-aging interventions and the sophistication of delivery technologies, the evidence in animal models will be converted into effective life-extending treatments in humans in a near future.

Important Notes

- Anti-aging medicine has become a popular topic in the recent decades. It considers biological aging a revertible process.
- Aging and the related diseases are results of an altered developmental programming.
- Innovative interventions and recommendations must be applied early. It is recommended that they should be applied not only to newborns but also to parents before pregnancy.

Questions for Future Research

- **How systemic delivery can be integrated into anti-aging medicine?** After decades of efforts, geriatrics and gerontology have become legitimate sciences. Over the years, anti-aging medicine has been investigating the complexity of the aging process, such as studying ARD. Here, we have focused on how the concepts and applications of anti-aging medicine can explain the origin of aging, such as fetal developmental programming.
- **How anti-aging medicine can be authentically achieved in real life?** Several biotechnological companies are promoting a growing number of therapeutic approaches, including oxidant drugs, hormones, vitamins, dietary supplements and various aesthetic drugs and techniques. In addition, the proposed approaches are particularly costly but are extensively marketed by doctors, laboratories and companies to a gullible public perusing well-being. However, in the majority of the cases, these treatments have revealed a palliative care rather than a curative approach or a longevity extension approach. This indicates that effective anti-aging treatments for humans are still beyond reach. Despite this, one can be optimistic in identifying successful treatments in a foreseeable future. Some of these have been described in this chapter together with associated limitations.

Glossary

Endothelium A continuous sheet of cells covering the innermost apical surface of blood and lymphatic vessels.

Haematopoiesis The formation of new blood cells.

Healthspan The length of time in an organism's life during which the physiological health is optimal.

Hypothalamic-pituitary-adrenal (HPA) axis The direct interactions between the adrenal gland, the hypothalamus and the pituitary gland.

Hypoxia A deficiency of oxygen in organs, tissues or cells to support their normal functions.

Induced pluripotent stem cells Pluripotent stem cells formed by reprogramming adult somatic cells that reach an embryonic stem cell-like state.

Intrauterine growth restriction The pathological inhibition of intrauterine fetal growth and the inability of the fetus to achieve its intrinsic growth potential.

Phenotype Genetically determined characteristics of an organism.

Preeclampsia A systemic syndrome that is typically characterized by the onset of hypertension and proteinuria after the 20th week of gestation and/or within 48 hours after delivery.

Proteostasis A state of equilibrium in which a functional proteome is maintained by a complex network of processes designed to control the synthesis, localization and degradation of proteins.

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