

# Delivery Systems for Biopharmaceuticals. Part II: Liposomes, Micelles, Microemulsions and Dendrimers

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**Abstract:** Biopharmaceuticals are a generation of drugs that include peptides, proteins, nucleic acids and cell products. According to their particular molecular characteristics (e.g. high molecular size, susceptibility to enzymatic activity), these products present

some limitations for administration and usually parenteral routes are the only option. To avoid these limitations, different colloidal carriers (e.g. liposomes, micelles, microemulsions and dendrimers) have been proposed to improve biopharmaceuticals delivery. Liposomes are promising drug delivery systems, despite some limitations have been reported (e.g. *in vivo* failure, poor long-term stability and low transfection efficiency), and only a limited number of formulations have reached the market. Micelles and microemulsions require more studies to exclude some of the observed drawbacks and guarantee their potential for use in clinic. According to their peculiar structures, dendrimers have been showing good results for nucleic acids delivery and a great development of these systems during next years is expected. This is the Part II of two review articles, which provides the state of the art of biopharmaceuticals delivery systems. Part II deals with liposomes, micelles, microemulsions and dendrimers.

**Keywords:** Biopharmaceuticals, proteins, peptides, nucleic acids, liposomes, micelles, microemulsions, dendrimers.

## 1. INTRODUCTION

Peptides and proteins are a new generation of drugs that are included in the so-called biopharmaceutical products, which have shown powerful and selective therapeutic activity against acute and chronic diseases, including cancer, viral or autoimmune pathologies. However, peptides and proteins transport to target organs or tissues presents several limitations. For example, their high molecular weight hinders the passage through cell membranes; the molecular sensitivity to the environment originates enzymatic and pH-dependent inactivation; a short lifetime, which is related to immunological responses that leads to a fast body clearance. To overcome these drawbacks a suitable carrier system should be selected, which should guarantee drug protection while fulfill therapeutic needs [1]. Peptides and proteins can have hydrophobic or hydrophilic nature, depending on their amino acid composition, and the choice of the more appropriate

delivery system depends on both, water solubility and administration route [2].

Regarding biopharmaceutical products for clinical uses, despite proteins are the most explored, nucleic acids (deoxyribonucleic acid, DNA, and ribonucleic acid, RNA) and cells have also been employed. Nucleic acids therapies are used in the treatment of severe diseases (e.g. cancer, AIDS and genetic disorders), comprising the administration of genes, aptamers and antisense oligonucleotides. To carry out the therapeutic effect, these molecules must enter the cells, direct to the nucleus and express the encoded protein, in a process usually called nucleic acids transfection. During this course some physiological limitations need to be overcome, such as passage throughout the cell and nucleus membranes, intra and extracellular enzymatic degradations and molecular systemic circulation time. Besides, DNA and RNA are large hydrophilic and negatively charged molecules, which mean that their administration alone is not much effective. Accordingly, the association of nucleic acids with delivery systems has been employed. In this regard, two main approaches have been presented, comprising the use of viral and non-viral vectors. Alternatively, improvements on the administration of DNA or RNA alone have been described, using physical methods that facilitate the entrance of these molecules into the cells. Regarding delivery systems, viral vectors (i.e. recombinant viruses in which the genes responsible for

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replication were removed) have shown promising results, but some concerns are pointed out, related to patient safety and incorrect gene expression. Therefore, non-viral vectors have been described as advantageous compared to viral ones, despite some limitations to clinical application (e.g. *in vivo* failure of the systems) have been raised [3-6].

In addition, apart from using viral or non-viral vectors, nucleic acids therapies can be performed by *in vivo* or *ex vivo* methods. The former involves DNA or RNA association to vectors or use of physical techniques and subsequent administration to the patient, by local implantation or systemic intravenous injection. In contrast, *ex vivo* techniques comprise the initial removal of diseased cells from the patient body, followed by their *in vitro* genetic manipulation and amplification, and re-introduction in the patient. Therefore, it is easy to conclude that *ex vivo* techniques are more efficient for therapy, since cells can be augmented and selected *in vitro* before patient implantation, although sometimes it is not possible to access diseased cells and *in vivo* therapy remains as the unique option [7, 8].

The present article corresponds to Part II of two review manuscripts, which provides the readers with the state of the art of biopharmaceuticals delivery systems. Part II deals with liposomes, micelles, microemulsions and dendrimers. According to their size, cells are associated to microparticles, which have been addressed in Part I.

## 2. DELIVERY SYSTEMS FOR BIOPHARMACEUTICALS

The development of suitable and efficient delivery systems for biopharmaceutical products remains a challenge for technologists, due to their particular molecular properties. In this area, different delivery systems, such as liposomes, polymeric and lipid nanoparticles, micelles and dendrimers, have been explored. Ideally, delivery systems should be non-toxic, nonimmunogenic and compatible with physiological conditions. Moreover, they should have production methods easy and reproducible, to gain the attention of biopharmaceutical industries. The *in vivo* success of delivery systems depends on their long-term physicochemical stability; having enough blood circulation time, avoiding recognition and uptake by the cells of the reticuloendothelial system; efficient targeting effect in the tissues or organs of therapeutic action. In contrast, a suitable delivery system should protect biopharmaceuticals from enzymatic and chemical degradations, enhancing its bioavailability and therapeutic activity. Currently, there are several delivery systems under clinical trials. Therefore, according to the growing interest and studies performed in this field, it is expected that in a near future, some delivery systems for biopharmaceuticals enter in the clinical practice [6, 9-13].

Table 1 [14-47] summarizes the information described in this article, related to the drug delivery systems for biopharmaceuticals. The interested reader can find more detailed information about each study in the next sections.

### 2.1. Liposomes

Liposomes were invented in the 1960s of the twentieth century and consist of sphere-shaped vesicle carriers, formed

by one or more lipid bilayers, encompassing an aqueous core. The lipids commonly used to produce liposomes are phospholipids, particularly cationic phospholipids. Nonetheless, anionic and neutral phospholipids, or other amphiphilic lipids (i.e. lipids with a positively charged hydrophilic head and a hydrophobic tail) can be employed. According to their composition and production procedures, liposomes can have, respectively, different surface charges (anionic, neutral or cationic), and nanometric or micrometric sizes. Besides, since phospholipids are present in the membranes of natural living cells, a low *in vivo* toxicity for liposomes is expected [48].

Since their invention, liposomes have been extensively studied as drug delivery carriers, and it is surprising that few formulations reached the pharmaceutical market. The main reasons for this are the *in vivo* failures of such systems, occurrence of unexpected instability phenomena and the complex regulatory issues that must be accomplished before passage to clinics [9, 49].

Regarding biopharmaceuticals, the use of liposomes for improve insulin delivery is the most explored and was first suggested in 1976 by Patel and Ryman [32]. The authors observed a significant reduction on the blood glucose level after oral administration of insulin-loaded liposomes to diabetic rats, compared to the results obtained after the administration of the same amount of free insulin. Since then, according to the high clinical interest in administering insulin by non-parenteral routes, several research groups worldwide have been exploring this application. However, until now none reach clinics, despite having several under clinical trials [50]. Therefore, we will only refer to the most recent and promising results reported regarding the application of liposomes to improve insulin delivery.

Niu *et al.* [33] studied the effects of bile salts, size and dose on the oral bioavailability of insulin-loaded liposomes, administered to diabetic and non-diabetic rats. The authors observed highest bioavailability values of 8.5 and 11% in non-diabetic and diabetic rats, respectively, for sodium glycocholate liposomes. Both prolonged hypoglycemic effect (over 20h, with peak at 8-12h) and increased blood insulin levels were observed after administration of all the insulin-loaded liposome formulations. The mechanistic study performed showed that liposomes were absorbed intact through the M-cell or the epithelia pathway, which means that particle size influence the results. Furthermore, authors observed that the pharmacological action of insulin-loaded liposomes was dose-dependent. In other study, the same research group focused on the mechanisms responsible for the enhancement on oral bioavailability of insulin-loaded liposomes containing bile salts. The cellular uptake and transport of bile salts insulin-loaded liposomes and conventional insulin-loaded liposomes were evaluated *in vitro* using Caco-2 cell lines. The former showed prolonged residence time, which was size and concentration-dependent, suggesting more stability in the gastrointestinal tract. Furthermore, the potential cytotoxic effects of bile salts were found to be insignificant. The gastrointestinal residence profiles of insulin-loaded liposomes were assessed by *in vivo* imaging, after oral administration to rats. Authors observed that higher residence times resulted in higher serum levels of insulin, i.e. the liposomes containing

Table 1. Examples of delivery systems for biopharmaceuticals: liposomes, micelles, microemulsions and dendrimers.

Biopharmaceutical product	Delivery system	Relevant effect	References
Apolipoprotein B (ApoB)-specific siRNA	Poly (L-Lysine) dendrimers	Knockdown of ApoB in healthy mice without hepatotoxicity.	[14]
Calcitonin	Mucoadhesive liposomes	Regulated blood calcium level, after oral administration.	[15-17]
Chlamydial gene	PAMAM dendrimers	Development of an efficient method for modification of chlamydial gene function.	[18]
	Polyamidoamine dendrimers	High transformation efficiency for <i>Chlamydia trachomatis</i> .	[19]
Cyclosporine A	O/W microemulsions	Improved bioavailability.	[20]
DNA	PPI dendrimers	Effectiveness for systemic <i>in vivo</i> administration.	[21]
Epimedium polysaccharide-propolis flavone immunopotentiator (EPI)	Liposomes	Efficient adjuvants, originating higher antibody titers, T lymphocyte proliferation and concentrations of interferon- $\gamma$ and interleukin-6, and lowest mortality and morbidity, compared to EPI suspension and solution.	[22]
Fibroblast cell lines	PAMAM dendrimers	Introduction of RRRK peptides improved efficiency for cell transfection.	[23]
Gonadotrophin-releasing hormone	Liposomes	Sustained release.	[24]
Growth hormone releasing peptide	W/O microemulsions	Effective delivery into the dermal layer.	[25]
HIV transactivator protein TAT	W/O microemulsions	High plasma half-life; Enhanced oral bioavailability.	[2]
Human retinal pigment epithelial cells	PAMAM dendrimers	Efficient transgene delivery in cells.	[26]
Human serum albumin	Polymeric micelles	Triggered targeting system.	[27]
Insulin	W/O microemulsions	Improved oral delivery.	[28]
	W/O microemulsions	Improved oral bioavailability of insulin with slight effects on blood glucose levels in diabetic rats.	[29]
	Non-alcoholic microemulsions	<i>In vivo</i> increased insulin absorption after nasal administration.	[30]
	W/O microemulsions	Effective delivery into the dermal layer.	[25]
	W/O microemulsions	Enhanced <i>in vitro</i> transdermal permeation through both excised mouse skin and goat skin.	[31]
	Liposomes	Reduced blood glucose level after oral administration to diabetic rats, compared to the administration of free insulin.	[32]
	Liposomes	Improved bioavailability in non-diabetic and diabetic rats; Prolonged hypoglycemic effect and increased blood insulin levels	[33]
	Liposomes containing bile salts	Enhanced oral bioavailability.	[34]
	Liposomes containing glycocholate	Retained the encapsulated insulin in simulated gastrointestinal media and in <i>ex vivo</i> gastrointestinal media.	[35]
	Biotin-modified liposomes	Enhanced oral absorption Enhanced hypoglycemic effect in diabetic rats after oral administration, compared to conventional liposomes.	[36, 37]
Folic acid and polyelectrolyte-surface modified liposomes	<i>In vivo</i> improved oral bioavailability and hypoglycemic effect compared to subcutaneous administered insulin solution.	[38]	

(Table 1) Contd....

Biopharmaceutical product	Delivery system	Relevant effect	References
	Liposomes	Reduced plasma glucose after pulmonary administration to rats.	[39, 40]
Insulin growth factor 1	W/O microemulsions	Effective delivered into the dermal layer.	[25]
Interferon- $\alpha$	Liposomes	Higher bioavailability and liver targeting.	[41]
Interleukin-2	Liposomes	Well tolerated after pulmonary aerosol administration.	[42, 43]
Leuprolide	Liposomes	<i>In vivo</i> increased drug tumor uptake and prolonged its biological half-life.	[44]
TAT peptide (siRNA)	Polymer micelles	Enabled non-invasive supply of genes to the brain after intranasal administration.	[45]
Recombinant gp85 protein of subgroup J avian leukosis virus	Liposomes	Increased serum antibodies after intramuscular administration to chickens, and higher protection against leucosis virus, compared to Freund's adjuvant emulsion.	[46]
Salmon calcitonin	W/O microemulsions	Improved absorption after intraduodenal administration in rats.	[47]
siRNA	Arginine-decorated amphiphilic dendrimers	Enhanced cellular uptake.	[28]
Viral protein antigens	Liposomes	Enhanced immune responses.	[22], [46]

**Abbreviations:** ApoB - Apolipoprotein B; EPI - Epimedium polysaccharide-propolis flavone immunopotentiator; O/W - oil-in-water; PAMAM - poly(amido amine); W/O - water-in-oil; PPI - poly(propylene imine); siRNA - small interfering RNA.

bile salts showed higher residence time and higher serum levels of insulin, compared to conventional liposomes and free insulin solution. Based on these results, authors proposed the occurrence of a mechanism of enterocyte internalization to explain the enhanced absorption of insulin-loaded liposomes [34]. In other study, Hu *et al.* [35] evaluated the ability of liposomes containing glycocholate to protect encapsulated drugs from physiological degradation during their transit throughout the gastrointestinal tract, while enhance their oral absorption, compared to conventional liposomes. The authors used insulin as a model for hydrophilic macromolecules and evaluated the stability of insulin-loaded liposomes in simulated gastrointestinal fluids containing simulated media of various pH, bile salts and enzyme (pepsin and pancreatin) levels. Moreover, the liposome stability in

*ex vivo* fluids from rats was also tested. From the results, authors concluded that liposomes containing glycocholate retained significantly the encapsulated insulin in simulated gastrointestinal media and in *ex vivo* gastrointestinal media, compared to conventional liposomes, suggesting that the presence of this bile salt enhance the oral bioavailability of insulin.

Zhang *et al.* [36, 37] studied the effect of biotin-modified liposomes in the improvement of oral insulin delivery. From the experiments, authors concluded that the biotinylation significantly enhanced the oral absorption of insulin-loaded liposomes, by a mechanism of receptor-mediated endocytosis, with an increased cellular uptake and fast transport throughout the gastrointestinal tract. The observed hypoglycemic effect in diabetic rats (i.e. pharmacological activity and blood insulin levels) was significantly enhanced after

oral administration of biotin-modified liposomes, compared to conventional liposomes. The functionalization of liposomes for oral insulin delivery was also studied by Agrawal *et al.* [38]. These authors reported the enhancement of insulin bioavailability after the oral administration of folic acid and polyelectrolyte-surface modified liposomes to rats. The *in vivo* studies showed an approximate relative bioavailability of 20% and a two-fold hypoglycemic effect, compared to subcutaneous administered insulin solution. Moreover, both improved stability in simulated gastrointestinal fluids and cellular uptake were observed, in the *in vitro* and *ex vivo* studies.

The pulmonary insulin delivery by means of liposomes has been suggested by some authors. After the administration of aerosolized insulin-loaded liposomes into rat lungs, the plasma glucose levels were effectively reduced. Furthermore, it was observed that liposomes were homogeneously distributed in the lung alveolar. From these results, authors suggested the pulmonary route as a successful alternative for insulin and other proteins or peptides administration [39, 40].

The feasibility of using liposomes to obtain a sustained release of gonadotrophin-releasing hormone (GnRH), with consequent stimulation of luteinizing hormone secretion was studied *in vivo* by Schafer *et al.* [24]. The authors observed a significant decrease in the release rate of GnRH after liposomal encapsulation, with plasma levels up to 8-20h after administration, compared to the free hormone. The results of this study provided the required information to prepare GnRH-loaded liposomal formulations with modified release of the hormone. Accordingly, it was expected that more stud-

ies were carried out to confirm the results. However, we did not find any additional published data.

Regarding gonadotrophins, the benefits of liposomal leuprolide encapsulation were studied *in vivo*, in tumor-bearing mice. It was observed an increased accumulation of leuprolide loaded liposomes in tumor, compared to free drug. From these experiments, authors concluded that the encapsulation of leuprolide in liposomes increase the drug tumor uptake and prolong its biological half-life [44]. In addition, the pulmonary administration of leuprolide was suggested as a promising alternative to the subcutaneous route, in order to increase drug bioavailability and patient compliance [51].

The use of drug-loaded liposomes to regulate blood calcium has been suggested since the eighties. Fukunaga and co-workers observed the decrease and increase of calcium levels, exerted by calcitonin and parathyroid hormone-loaded liposomes, respectively, after oral administration to rats [52]. Similar studies have been performed by several research groups. For example, Takeuchi *et al.* [15-17] reported an enhanced and prolonged decrease on calcium plasma, after oral administration to rats of mucoadhesive calcitonin-loaded liposome formulations. Moreover, these authors suggested that the mucoadhesion improves enteral peptide absorption, by promoting the adhesion of liposomes to the intestinal mucosa.

The intranasal route was suggested as an alternative for the administration of calcitonin, improving its absorption [53, 54]. However, more *in vivo* studies are necessary to confirm the advantages of using intranasal over oral route for calcitonin delivery.

The use of monoclonal antibodies to improve drug targeting by means of colloidal carriers has been extensively explored. In this context, several studies reported improvements on the therapeutic efficiency of drug-loaded liposomes that have been surface grafted with monoclonal antibodies [55]. For example, the tumor cells uptake of doxorubicin was improved after drug encapsulation in surface binding monoclonal antibody liposomes [56, 57]. Furthermore, it was suggested that these liposome systems may be used for a combined tumor-specific therapy and diagnosis [58], and for tumor targeted and synergistic therapy of bone metastases [59].

The liposomal encapsulation of cytokines, namely interferon- $\gamma$ , improves paracrine cytokine delivery in tumor vaccine. Accordingly, the interferon- $\gamma$  has been described as a promising adjuvant for vaccines against cancer and infectious diseases [60, 61]. In contrast, the encapsulation of interferon- $\alpha$  in liposomes showed good results for the local treatment of genital herpes. After intramuscular administration, it was observed a prolonged residence time of this cytokine, suggesting the potential of liposomes for the systemic therapy of genital herpes, achieving higher bioavailability and liver targeting [41].

Regarding cytokines, also liposomal encapsulation of interleukins, namely interleukin-2, has been explored. Anderson and co-workers carried out clinical trials to test the feasibility and toxicity of aerosol interleukin-2 liposome in individuals with pulmonary cancer metastases [42] and immune deficiency [43]. From the results, authors concluded

that interleukin-2 loaded liposomes are well tolerated, after pulmonary aerosol administration, although additional studies are required to determine the therapeutic efficacy of these systems [42, 43].

It has been described that liposomes alone have immunogenic properties, since they trigger immune responses within the body. Therefore, these systems have been used as adjuvants in vaccination, as antigen vaccine delivery systems or for immune-stimulation [62, 63]. For example, the enhancement of the immune responses after administration of veterinary liposome vaccines encapsulating viral protein antigens have been shown for chickens Newcastle disease [22]. The authors prepared epimedium polysaccharide-propolis flavone immunopotentiator (EPI) liposomes, EPI suspension and EPI solution, and compared the synergistic immune enhancing effect (i.e. the adjuvant effect) of these formulations in chickens vaccinated with Newcastle disease. The results showed that EPI liposomes were more efficient to use as adjuvants, since they originated higher antibody titers, T lymphocyte proliferation and concentrations of interferon- $\gamma$  and interleukin-6, compared to both EPI suspension and solution. Moreover, EPI liposomes presented the lowest mortality and morbidity. Zhang *et al.* [46] studied the potential of liposome vaccines in the prevention of avian leukosis virus. For this, authors prepared recombinant gp85 protein of subgroup J avian leukosis virus (ALV-J) loaded-liposomes. Before ALV-J liposome encapsulation, this recombinant protein was expressed in Rosetta (DE3) cells. The prepared liposome vaccine was tested in chickens and compared to Freund's adjuvant emulsion containing the same recombinant gp85 protein. The results showed increased serum antibodies in chickens, after intramuscular administration of both tested formulations. However, these values persisted longer in liposomes. Viremia tests indicated that liposomes provided higher protection against leucosis virus than Freund's emulsion, which is related to the higher serum antibodies levels. Furthermore, Quer *et al.* [64] reported the influence of cationic lipid composition in liposomes efficiency as vaccine adjuvants for influenza virus, and Zhuang *et al.* [65] suggested that PEGylation, i.e. the attachment of polyethylene glycol (PEG) molecules to liposomes surface, is a promising strategy to improve liposomes passive targeting, *in vivo* biodistribution and, consequently, the efficiency of encapsulated vaccines. Currently there are two vaccine liposomal formulations, usually called virosomes, approved for the intramuscular delivery of surface antigen influenza (Inflexal<sup>®</sup> V) and hepatitis A (Epaxal<sup>®</sup>) virus [9].

Cationic lipids have been used to prepare liposome delivery systems for nucleic acids (i.e. lipoplexes). These systems are complexes established by electrostatic interactions, between the positively charged cationic lipid and the negatively charged nucleic acid molecule (i.e. DNA or RNA). The enclosed molecules are protected from enzymatic degradations during the way through reaching cell nucleus. Furthermore, cationic lipoplexes easily interact with the membrane of several types of mammalian cells, improving lipofection. Despite several research articles have been reporting the therapeutic effectiveness of cationic liposomes in cell cultures and animal models, an insufficient transfection efficiency and poor gene expression have been observed *in vivo*, in humans.

Nonetheless, among the non-viral nucleic acids delivery systems, liposomes are the most explored in clinical trials [6, 9].

## 2.2. Micelles

In the late 1960s of the twentieth century micelles have attracted significant interest as drug delivery systems due to their good pharmaceutical properties. Micelles are colloidal carriers with sizes within a range of 5-100 nm, which consist of amphiphiles or surfactants molecules, containing a hydrophilic head-group and a hydrophobic tail. These systems are formed when amphiphiles are placed in water. In an aqueous medium, low concentrations of these amphiphilic molecules exist separately. Increasing the concentration, aggregation takes place within a rather narrow concentration interval. The concentration of a monomeric amphiphile at which micelles appear is called the critical micelle concentration (CMC). Due to their small size, micelles present spontaneous accumulation via the enhanced permeability and retention effect in pathological areas with compromised vasculature. Attaching specific targeting ligand molecules (e.g. target-specific antibodies, transferrin or folate) to the micelle surface, it is possible to obtain micelle specific targeting to required areas [66].

Usually, hydrophilic agents can be dissolved in an internal aqueous phase to form water-in-oil (W/O) emulsions, microemulsions or conventional reverse micelles. The latter are ternary systems, which have an oil phase containing the surfactant aggregates with a low water content and a minimum water-to-phospholipid mole ratio of more than 15 [67].

The use of polymer-based micelles has gained much attention because of the high diversity of polymers, their biocompatibility, biodegradability, and the multiplicity of functional groups that they display for the conjugation with drug molecules [68].

One of the disadvantages of normal self-assembled polymeric micelles is that micelles are not stable and they may dissociate upon dilution [69]. On the other hand, lipid-core micelles formed by self-assembled amphiphilic polymers (e.g. PEG-PE (phosphatidylethanolamine)) demonstrate both *in vitro* and *in vivo* high stability, good biocompatibility, and prolonged blood circulation time [70].

In the study of Gao *et al.* [27] a degradable block copolymer methoxy poly(ethylene glycol)-poly(b-amino ester) (PEG-PAE) containing piperidine and imidazole was synthesized and used to encapsulate human serum albumin (HSA). This albumin encapsulated polymeric micelle maintained a stable micellar state at physiological pH 7.4 with a particle size of around 56.0 nm in water, and proved to be promising as a pH-triggered targeting agent and an effective drug delivery system in cerebral ischemia models.

In another study, Koyamatsu *et al.* [71] showed that a reverse polymer micelle composed of biodegradable PLGA and biocompatible PEG has potential application as an oral protein delivery carrier, using HSA as a model drug. To achieve a high colloidal stability of the reverse micelle in an aqueous medium, the micelle's surface was decorated with a phospholipid modified with PEG (PEG-phospholipid). The micelle system developed in this study showed the following advantages as HSA drug carrier [71]: high biocompatibility;

high biodegradability; low cytotoxicity; high protein loading efficiency ranging from 27% to 82%; a long term stability as a freeze-dried precursor (a solid nanoparticle) prior to the surface modification with the PLGA and the PEG-phospholipid; and is a functional drug carrier which releases proteins in response to the pH of targeted tissues and organs.

The development of effective strategies to enhance siRNA delivery to the brain is of great interest in clinical and pharmaceutical fields. The study of Kanazwa *et al.* [45], showed that the use of polymeric micelles with surface-loaded Tat peptide (a cell-penetrating peptide derived from HIV-Tat) for intra-nasal administration of nucleic acids enables the non-invasive and high rate transfer of genes to the brain. In another study, Huo *et al.* [72] developed novel polyion complex micelles composed of rabies virus glycoprotein (RVG) peptide tagged PEGylated polyasparthydrazide as carriers for the delivery of siRNA to the brain. The *in vivo* biodistribution studies conducted by these authors indicated that RVG modified micelles have the ability to pass through the blood brain barrier and enter the brain. Salzano *et al.* [73] developed PEG2000-PE polymeric micelles for co-delivery of paclitaxel and survivin siRNA. The use of this new system showed to be able to reverse drug resistance in the treatment of aggressive tumours. Besides these results support further studies able to verify the *in vivo* activity of the developed delivery system.

Studies on polyplex micelles have been performed in recent years with significant success. However, some problems remain, concerning *in vivo* applications and the development of systems available for clinical use has not yet succeeded. To overcome this, in the near future polymeric micellar gene carriers with further safety and functionality should be developed [74].

Polymeric micelles have also demonstrated good effectiveness as carriers of chemotherapy drugs, genes, and proteins in various preclinical glioblastoma multiform studies, improving the delivery of these therapeutic agents to brain tumors. However, despite these advantages, there are currently no micelle formulations targeting brain cancer in clinical trials [75].

As the presence of water can be detrimental to therapeutic agents, such as peptides and proteins, Wang *et al.* [76] described a simple procedure for the preparation of anhydrous reverse micelles (ARMs). In this method, a mixture of an aqueous phase containing insulin and an oil phase containing phosphatidylcholine was emulsified to prepare W/O emulsions, which were subsequently lyophilized. After addition of oil, the lyophilized W/O emulsions formed ARMs, which can be used as carriers for peptides and proteins, or other therapeutic agents that are unstable in aqueous solutions. These ARMs showed promise characteristics for the oral delivery or parenteral extravascular administration of peptides with sustained therapeutic efficacy.

## 2.3. Microemulsions

Microemulsions have emerged as promising delivery systems, which allow sustained release of drugs for different administration routes (e.g. oral, transdermal, topical, nasal, intravenous, ocular, and parenteral) [77].

Microemulsions can be defined as homogeneous, thermodynamically stable dispersions of water and oil, stabilized by surfactants, with a droplet size usually in the range of 20-200 nm [2, 78]. In other words, microemulsions are transparent (or translucent) systems of two immiscible fluids, stabilized by a surfactant (nonionic, zwitterionic, cationic, or anionic) or a mixture of surfactants, frequently in combination with a co-surfactant [77]. These systems can be classified in oil-in-water (O/W), water-in-oil (W/O), and bicontinuous microemulsions. The existence of polar, nonpolar, and interfacial domains in microemulsions allows the encapsulation of hydrophobic and hydrophilic therapeutic agents such as peptides and proteins. O/W microemulsions are promising in improving the bioavailability of hydrophobic peptides, such as cyclosporine A. W/O microemulsions have shown potential to improve the oral delivery of hydrophilic proteins or peptides, such as insulin [79].

The following are the most important properties of the microemulsions [77, 78]: enhancement of the solubilization and dissolution efficiency of poorly water soluble drugs; protection of labile drugs; improvement of drug targeting; production of controlled release and compatible delivery systems and ease of manufacture and scale-up. The type of microemulsion can influence significantly the extent and rate of drug delivery.

It has been shown that the oral administration is one of the routes that benefit more from the use of microemulsions. The mechanisms to enhance oral bioavailability using W/O microemulsions include the protection from enzymatic degradation and alterations in mucosal membrane fluidity. As an example, the *in vitro* peptide stability studies performed by Liu *et al.* [79] demonstrated the protective effect of a peptide incorporated in a W/O microemulsion against enzymatic degradation. The HIV transactivator protein TAT delivered orally using a W/O microemulsion as carrier system was shown to have a longer half-life comparing with the free drug.

Based on the results of acid-protection efficiency and stability tests, different microemulsions have shown promising results for oral delivery of insulin. As stated above, as insulin is a water-soluble peptide it requires a W/O microemulsion as a carrier. Studies demonstrated that, in this case, the addition of chitosan to the water phase improved the protection of insulin in the aqueous core [80].

Microemulsions encapsulating insulin were developed by Sarma *et al.* [29], using a low shear reverse micellar approach, in order to preserve the secondary structure of this drug. Microemulsions were prepared using triacetin as the oil phase, insulin solution as the aqueous phase, didocecyltrimethylammonium bromide as the surfactant, and propylene glycol as the co-surfactant. The results obtained in this study showed the ability of microemulsions to improve the oral bioavailability of insulin with slight effects on blood glucose levels in diabetic rats. Therefore, in order to better analyze the potential of reverse micellar systems for efficient management of hyperglycemia further studies need to be carried out.

A non-alcoholic microemulsion containing GRAS excipients and lispro insulin (a fast acting insulin analog) for

nasal delivery was reported by Sintov *et al.* [30]. The *in vitro* permeation and the pharmacokinetic/pharmacodynamic studies showed a remarkable increase in insulin absorption in rabbits promoted by the developed nasal microemulsion with no need of the addition of an absorption-enhancing adjuvant.

Fan *et al.* [47] prepared W/O microemulsions containing the following excipients: medium chain triglycerides; Tween® 80 and Span® 80 or soybean phosphatidylcholine; propylene glycol and phosphate saline; to encapsulate salmon calcitonin (sCT), a highly hydrophilic polypeptide. The developed W/O microemulsions showed potential to significantly improve the absorption of sCT by intraduodenal administration in rats. In this study the effects of the addition of polymers such as hydroxypropylmethylcellulose (HPMC K15M) and carbomer (Carbopol® 980), into the aqueous phase, on the properties of microemulsions were also evaluated. The addition of Carbopol® 980 caused disparate effects on entrapped sCT absorption and HPMC K15M had unfavorable effects, although further studies should be performed to confirm these results. Efficient transdermal delivery of peptides and proteins has attracted great interest in the pharmaceutical industry, in an attempt to develop a variety of delivery systems to force these molecules into the skin epithelium. However, there are only a few studies of transdermal delivery of peptides and proteins using microemulsions. Goebel *et al.* [81] summarized the research conducted about microemulsions as colloidal carriers for dermal delivery of peptides. The studies demonstrated the improved delivery of these drugs incorporated into microemulsions compared to conventional carriers.

In the work of Russel Jones *et al.* [25] a high bioavailability of W/O microemulsions containing peptides and proteins, with molecular mass ranging from 900 Da to 150 000 Da, has been achieved after topical administration. Effective needle-free vaccine delivery has been achieved, and the stimulation of anabolic activity in muscles has been achieved using topically delivered insulin, Growth Hormone Releasing Peptide (GHRP-6) and Insulin Growth Factor I (IGF-I). The study of Malakar *et al.* [31] emphasized the efficacy of insulin-loaded microemulsions containing 10% oleic acid, 38% aqueous phase, and 50% surfactant phase with 2% dimethyl sulfoxide (DMSO) as permeation enhancer for enhanced *in vitro* transdermal permeation through both excised mouse and goat skin. The formulation developed by these authors showed maximum permeation flux and could be transdermally administered in the treatment of insulin-dependent diabetes mellitus with improved patient compliance.

## 2.4. Dendrimers

Dendritic polymers or dendrimers are a class of cationic polymers first reported in 1985, which have been extensively used in numerous biomedical applications, including drug and nucleic acid delivery, diagnostics and nanoengineering [82]. The name "dendrimer" originated from the Greek word "Dendron" meaning tree and "meros" meaning branch, which depicts a structure consisting of three main parts: an inner core, highly branched repeating units called layer of generations and peripheral multivalent functional groups, which play a key role in gene-complexing [82, 83].

Dendrimers are getting huge interest as advantageous drug carriers for delivering therapeutic agents and genes. Drugs or genes release from these systems may occur through two main mechanisms [84]: *in vivo* degradation of drug dendrimer covalent bonding, which depends on the presence of suitable enzymes or an environment capable of cleaving the bonds; or changes in physical environment, such as pH or temperature.

Dendrimers are highly symmetric, spherical, hyper-branched macromolecules and their three dimensional architecture provides a high degree of surface functionality and versatility meaning that these systems have several properties which differentiate them from other polymers [84]. These properties include [21, 82]: chemical homogeneity; possibility of increasing the generation by repeated attachment of chemical groups, and a high density of functional groups on the surface for numerous ligand attachments.

Due to the well-defined three dimensional dendrimer structure and many surface functional groups, drug molecules can be loaded both in the interior as well as attached to the surface groups. Thus, dendrimers can function as drug carriers either by encapsulating drugs within the dendritic structure or by interacting with drugs at their terminal functional groups via electrostatic or covalent bonds forming a prodrug [84].

Small organic molecules are often encapsulated into the dendrimers interior void space, while larger (bio)molecules preferably adsorb onto the dendrimer surface [85]. The forces responsible for these interactions are hydrogen bonding, van der Waals interactions, and electrostatic attraction between opposite charges of dendrimers and drugs. These macromolecular systems allows the improvement of solubility, stability, and deliver efficacy of several types of drugs [85].

Dendrimer-based delivery systems have demonstrated considerable effectiveness as non-viral vectors for gene therapy, promoting the cellular uptake of genes. They form complexes with several forms of nucleic acids, such as plasmid DNA or antisense oligonucleotides, protecting them from degradation [21].

The high transfection efficiency of dendrimers may not only be due to their well-defined shape, but may also be caused by the low pK of the amines (3.9 and 6.9) [84].

Even though there are many types of dendrimers used for gene delivery, there remains a continuing demand for those which are able to deliver effectively the molecules to cells. Cationic dendrimers are suitable as non-viral vectors for gene delivery because of their ability to form compact complexes with negatively charged DNA and RNA. They constitute a support for the attachment of drugs or genes and their release by various mechanisms [86].

The first synthesized dendrimers were polyamidoamines (PAMAM), which are the most investigated and, due to their facility of synthesis and modification, several types of these dendrimers with different core units and terminal groups have been developed. The PAMAM dendrimers have shown high levels of transfection in a wide variety of cultured cells [84].

Gérard *et al.* [18] developed PAMAM dendrimers for delivery of modified DNA, which allowed the study of

chlamydial gene function, enabling the development of novel dendrimer-based therapies. This method proved to be simple, extremely reliable, efficient, and easy to use for modification of chlamydial gene function. Kannan *et al.* [19] also described the development and use of a transformation system for *Chlamydia trachomatis* that utilizes delivery of DNA by complexation with dendrimers. This system proved to be reliable and showed high transformation efficiency.

Surface engineering has been found to reduce cytotoxicity and enhance dendrimers transport across epithelial cells [87]. For example, toxicity problems associated with cationic dendrimers can be overcome by PEGylation, which neutralizes their positive charge [84].

In order to overcome the drawbacks of dendrimers as gene vectors, Mastorakos *et al.* [26] used hydroxyl-terminated polyamidoamine (PAMAM) dendrimers functionalized with various amounts of amine groups. This safe dendrimer-based gene-delivery platform demonstrated efficient transgene delivery in hard-to-transfect human retinal pigment epithelial cells.

The study of Lee *et al.* [23] showed that the introduction of RRRK peptides from mouse fibroblast growth factor 3 (FGF3) on the surface of PAMAM generation 4 increased transfection efficiency and lower cytotoxicity in all cell lines, in comparison with that of the native PAMAM. These results showed that the introduction of RRRK peptides would make PAMAM-based vector an efficient cationic polymer for transfection of fibroblast cell lines.

In a recent study, the possibility of combining the advantages of the cationic structure of PAMAM dendrimer with the potential ability of carbon chains for interaction with biological membranes was investigated by Sabahi *et al.* [88]. The results obtained suggested that the optimal transfection efficiency was achieved by the modified dendrimers containing five to nine carbon chain lengths at degrees of conjugation around 10%, which showed substantial buffering capacity in the pH range of endosomes. The most probable explanations for the increased transfection efficiency are the improved hydrophilic-hydrophobic balance of the dendrimer; the synergistic effect of hydrophobic interaction of carbon chains with biological membranes and the proton sponge effect induced by the dendrimer amines.

In the last years, siRNA-mediated specific gene silencing has been used as a research tool and as a therapeutic agent for many diseases such as cancer, infections and metabolic disorders. Liu *et al.* [28] developed an arginine-decorated amphiphilic dendrimer as a non-viral vector for siRNA delivery, composed of a hydrophobic alkyl chain and a hydrophilic PAMAM polymer containing arginine terminals. This dendrimer was able to mediate improved siRNA delivery and potent gene silencing through enhanced cellular uptake of siRNA via arginine structural entities at the dendrimer surface. This study suggested that decoration of the dendrimer surface with arginine residues was an effective strategy to improve the delivery ability of dendrimers.

Poly(propylene imine) (PPI) dendrimers are another class of dendrimers that have been investigated for gene delivery. While most of the applications are focused on the use of dendrimer-based vectors for local or *ex vivo* administration,



Dufes *et al.* [21] demonstrated that specifically PPI dendrimers may have some properties, such as safety and efficient delivery of genes, which appear to make them particularly suitable to the systemic *in vivo* administration.

Activated dendrimers can carry a larger amount of genetic material than viral vectors. Amino-terminated PAMAM or PPI dendrimers have been reported as non-viral vectors, which enhance the transfection of DNA by endocytosis. There is commercially available a transfection reagent called SuperFect™ consisting of PAMAM activated dendrimers, which are one of the most widely applicable transfection agents and have become a standard tool used in cell and molecular biology. SuperFect-DNA complexes present high stability and provide more efficient transport of DNA into the nucleus than liposomes [21].

Carbosilane dendrimers have also been investigated as siRNA delivery vectors. Weber *et al.* [89] firstly characterized carbosilane dendrimers as effective carriers for siRNA to human immunodeficiency virus (HIV)-infected lymphocytes. Carbosilane dendrimers bound siRNA via electrostatic interactions and prevent siRNA degradation by RNase enzymes.

In recent years several studies have been reported about the potential use of phosphorus dendrimers in therapeutics. Because of their hydrophilic surface and hydrophobic backbone, phosphorus dendrimers are able to penetrate membranes. In their studies, Ferenc *et al.* [90] proved that the cationic phosphorus dendrimers have the potential to become efficient carriers of siRNA in anticancer therapies. However, more studies are needed regarding the properties of dendrimers, their toxicity and potential behavior in the human body.

Phosphorhydrazone dendrimers constitute a special family of dendrimers, possessing one phosphorus atom at each branching point. In fact, the properties of these compounds as transfection agents, as carriers of drugs, and as anti-HIV agents are mainly determined by the type of terminal groups that they bear [91].

Poly (L-Lysine) (PLL) dendrimers have also been used as non-viral vectors for siRNA-mediated specific gene silencing. Watanabe *et al.* [14] showed that intravenous delivery of apolipoprotein B (ApoB)-specific siRNA with a Poly (L-Lysine) (PLL) G6 dendrimer led to knockdown of ApoB in healthy mice without hepatotoxicity and with a significant reduction of low-density lipoprotein cholesterol in apolipoprotein E-deficient mice. A significant research has been carried out to develop dendrimer-based nanomedicine for the delivery of nucleic acids. However, the challenge of safe and efficient delivery of genetic material remains and current limitations of dendrimers, such as cytotoxicity and the release of the nucleic acid from the dendrimer complex needs to be carried out [83].

Can be considered essentially three means to optimize the dendrimer structure in order to lower the cytotoxicity and improve the delivery efficiency [92]: synthesis of new dendritic structure or use new core unit for dendrimer preparation; functionalization of the interior or exterior part of the dendrimer molecules; using other biocompatible/bioactive molecules to form effective complexes with dendrimers.

As stated above, the binding between dendrimers and nucleic acids is usually a complex process that involves various types of interactions. In a recent work, Pavan [93] discussed the computational efforts in the study of the interaction between dendrimers and nucleic acids. This author concluded that, actually, molecular simulation can provide complementary details that cannot be easily obtained through experiments.

### 3. CONCLUSIONS AND FUTURE PERSPECTIVES

Delivery of peptides, proteins and nucleic acids has been challenging, due to their poor bioavailability following administration. To overcome this limitation, several delivery systems have been proposed, namely, liposomes, micelles, microemulsions and dendrimers.

Liposomes improve drug delivery to specific sites, promoting targeting to disease cells and have shown promising results for the delivery of several biopharmaceuticals. However, *in vivo* failure, poor long-term stability and low transfection efficiency phenomena have been observed for these carriers, which are responsible for the small number of formulations currently available in clinic.

To date, several micelle delivery systems have been developed for therapeutic agents, such as peptides and proteins. However, some concerns have emerged, including toxicity, immunogenicity, low cellular uptake, short half-life, and tissue accumulation.

Microemulsions have also been shown to be suitable carriers to deliver therapeutic agents. However, more fundamental research is required for microemulsions loaded peptides and proteins, before being fully demonstrate their potential for use in clinic.

Dendrimer-based delivery systems have unique molecular architectures and have shown suitable properties to be used as tools for the development of genetic therapies. Recent successes in simplifying and optimizing the synthesis of dendrimers provided a large variety of structures with reduced production costs. Besides, new applications of dendrimers are rising and, in the future, an increasing number of commercialized dendrimer based drug delivery systems are expected.

### CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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