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Delivery Systems for Biopharmaceuticals. Part I: Nanoparticles and Microparticles

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Abstract: Pharmaceutical biotechnology has been showing therapeutic success never achieved with conventional drug molecules. Therefore, biopharmaceutical products are currently well-established in clinic and the development of new ones is expected. These products comprise mainly therapeutic proteins, although nucleic acids and cells are also included. However, according to their sensitive molecular structures, the efficient delivery of biopharmaceuticals is challenging. Several delivery systems (e.g. microparticles and nanoparticles) composed of different materials (e.g. polymers and lipids) have been explored and demonstrated excellent outcomes, such as: high cellular transfection efficiency for nucleic acids, cell targeting, increased proteins and peptides bioavailability, improved immune response in vaccination, and viability maintenance of microencapsulated cells. Nonetheless, important issues need to be addressed before they reach clinics. For example, more *in vivo* studies in animals, accessing the toxicity potential and predicting *in vivo* failure of these delivery systems are required. This is the Part I of two review articles, which presents the state of the art of delivery systems for biopharmaceuticals. Part I deals with microparticles and polymeric and lipid nanoparticles.

Keywords: Biopharmaceuticals, proteins, peptides, nucleic acids, cells, polymeric nanoparticles, lipid nanoparticles, microparticles.

1. INTRODUCTION: BIOPHARMACEUTICALS

1.1. Concepts

Biopharmaceuticals were first introduced in the eighties of the twentieth century and since then several definitions for these products appeared, which sometimes make difficult an accurate description. The general definition of biopharmaceuticals includes therapeutic proteins and peptides produced by genetic engineering (i.e. recombinant DNA techniques), monoclonal antibody hybridoma, cell-based products and nucleic acids (deoxyribonucleic acid, DNA, and ribonucleic acid, RNA) therapeutics. Furthermore, products obtained by fermentation, non-recombinant cell culture proteins and other products from live organisms are also classified as biopharmaceuticals. Accordingly, the main classes of biopharmaceuticals are cytokines, hematopoietic growth factors, hormones, enzymes, blood/plasma products, monoclonal antibodies, vaccines, cultured cells and tissues, products for gene therapy, antisense oligonucleotides and aptamers [1-4].

So far the number of biopharmaceutical products has been increased, which is related to their high therapeutic potential, compared to conventional drug molecules. Some patents have expired since 2007, and several biosimilar (i.e. biopharmaceuticals whose patent has expired) have been introduced [5, 6].

1.2. Bioavailability Limitations

According to their molecular sensitivity and the physiological harsh conditions (e.g. acid stomach pH and enzymatic degradation), biopharmaceuticals are usually administered by parenteral routes (e.g. intravenous, subcutaneous or intramuscular), which is unpleasant for patients. The development of alternative efficient delivery systems for these molecules is required, aiming to improve their activity and patient compliance [4, 7].

In this context, pharmaceutical technologists have a crucial role, since they deal with drug formulation. For the clinical success of therapy, the formulator should have a good knowledge about drug molecular characteristics, overcoming problems related to its stability and bioavailability. The most important features that should be addressed are the

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molecular degradation mechanisms of biopharmaceuticals, which may occur during production, isolation, purification, storage and delivery (i.e. during upstream and/or downstream processing). The plasma half-life, molecular size and immunogenic potential have also been recognized as important parameters that interfere with biopharmaceutical *in vivo* performance [5].

Protein degradation processes may occur by both chemical and physical mechanisms. The former include cleavage or linkage formation, whereas the latter comprise tridimensional conformation changes, adsorption and aggregation or precipitation phenomena. Several efforts have been done to improve biopharmaceuticals bioavailability, such as the linkage of molecules to proteins surface (e.g. polyethylene glycol, PEG, and polysaccharides) or the development of appropriate delivery systems (e.g. microparticles and colloidal carriers), improving their half-life. In contrast, nucleic acids require chemical modifications to loss functionality, despite physical degradation could also be responsible for this. Moreover, nucleic acids must enter the cell and reach nucleus for gene expression. This means that high transfection efficiency is fundamental to obtain therapeutic success. On the other hand, a low circulation time, enzymatic susceptibility and immunological responses have been pointed out as limitations for nucleic acids administration. Therefore, the administration of these molecules is usually performed by means of viral (e.g. recombinant virus that are replication-incompetent) or non-viral vectors (e.g. colloidal carriers, such as lipoplexes and polyplexes) [4, 5, 8].

Cell-based products must preserve their viability after administration, and should not trigger immune responses. To assure these commitments, cells should also be administered using suitable delivery systems (e.g. microparticles) [9].

Despite the strategies that have been performed to achieve an efficient delivery of biopharmaceuticals, this trend remains challenging for pharmaceutical technologists. This article is the Part I of two review manuscripts, which provides the state of the art of delivery systems for biopharmaceuticals. Part I deals with microparticles and polymeric and lipid nanoparticles.

2. DELIVERY SYSTEMS FOR BIOPHARMACEUTICALS

Table **1** summarizes the information presented in this article related to the *in vivo* studies, using polymeric and lipid microparticles and nanoparticles, as delivery systems for biopharmaceuticals. The interested reader can find more detailed information about these studies in the next sections.

2.1. Microparticles

Microencapsulation is a technology that has been obtaining promising results in biotherapeutics field, for both cell and tissue engineering and development of other biopharmaceutical formulations. Microparticles are particulate dispersions or solid particles having a diameter less than one millimeter, irrespective of the specific interior or exterior structure [49]. These systems have been demonstrating benefits in overcoming some difficulties, related with traditional methods of administration, namely greater effectiveness, lower toxicity and superior long-term stability [50]. Additionally, they present other advantages, including: modulate the delivery of the encapsulated contents; improve bioavailability; target the encapsulated substances to specific sites (e.g. by conjugation of specific recognized molecules onto their surfaces); protect the enclosed substances against degradations (e.g. acid and enzymatic) and clearance after administration; relative easiness of preparation and formulation; high loading efficiency [49, 51].

The term "microparticles" comprises two subcategories, namely microcapsules and microspheres. Despite many authors use these two terms interchangeably, the structure of each ones is different and specific. In general, microspheres are spherical microparticles consisting of a homogeneous structure with one continuous phase where the encapsulated substance and matrix agent (usually a polymer) are a uniform mixture. In contrast, microcapsules refer to a spherical reservoir-like structure with a well-defined core, surrounded by an envelope, composed of a material distinctly different from that of the core. This type of microparticles contains at least one discrete domain of active substance and, occasionally, has more [52].

Different substances (e.g. polymers, proteins, lipids, polysaccharides, organic and inorganic materials) and various techniques (e.g. o/w single emulsion solvent evaporation, double emulsion, spray drying, ionotropic gelation, coacervation, polymerization, supercritical CO_2) are available to produce a variety of microparticle types and sizes, under controlled conditions. The growing interest towards the uses of microparticles for biopharmaceuticals (e.g. proteins, nucleic acids, and stem cells) delivery has been highlighted in several research papers. In the following subsections we will refer to the most recent and promising trends.

2.1.1. Polymeric Microparticles

Polymeric microparticles have been used as a favorable tool in delivering biopharmaceuticals that can possess different release and degradation rates, depending on the type and ratio of polymer used in the formulations. The most frequent used polymers in microparticles have good biodegradability and biocompatibility properties. Some examples are chitosan [53-58], poly(D,L-lactic-co-glycolic acid) (PLGA) [59-63], poly(lactic acid) (PLA) [23, 64] and poly (ε-caprolactone) (PLC) [65].

Chitosan, a natural polymer, and its derivatives are promising materials to develop pH-sensitivity controlled release systems, due to their cationic character. Zou *et al.* [53] produced bovine serum albumin (BSA)-loaded pH-responsive chitosan microspheres. BSA is a protein that has good antigenic properties and string cellular immunity. The results demonstrated that the sodium tripolyphosphate cross-linked chitosan microspheres showed higher swelling ratio and more pH-responsive properties than microspheres cross-linked by glutaraldehyde. The delivery system exhibited a pH-responsive release behavior, probably due to a different hydrolysis degree of chitosan, under varying acidic environment [66]. Kavianinia *et al.* [54] prepared chitosan microspheres crosslinked with pyromellitic dianhydride for colon-specific delivery of BSA, which was used as a model protein. In order to avoid rapid release of encapsulated protein,

Table 1. Examples of *in vivo* studies using delivery systems for biopharmaceuticals: microparticles, polymeric and lipid nanoparticles.

Biopharmaceutical product	Delivery system	Relevant effect	References
Anti-tumor necrosis factor monoclonal antibody	PLGA nanoparticles	Induced cytotoxic T lymphocyte proliferation, tumor antigen-specific cytotoxicity and cytokine production, in mouse tumor models.	[10]
Bacillus anthracis antigen	Chitosan nanoparticles	*In vivo* higher levels of serum specific antibodies and cytokines, after nasal immunization.	[11]
Bevacizumab	Albumin-PLGA nanoparticles	Reduced frequency of intravitreal injections in the treatment of eye posterior segment neovascularization.	[12]
Calcitonin	Glycol chitosan and glycol chitosan coupled with thioglycolic acid chitosan nanoparticles	Increased lung tissue mucoadhesion, biocompatibility, after intra-tracheal administration to rats; Prolongued hypocalcemic, with high bioavailability.	[13]
	PLGA nanoparticles	*In vivo* sustained increase of drug plasma level; Increased bioavailability, after subcutaneous administration.	[14]
Chlamydia trachomatis membrane peptide	PLA–PEG nanoparticles	*In vivo* effective subcutaneous mice immunization, with higher production of specific T-cell cytokines and antibodies.	[15]
Cysteine proteinase type I	SLN	Induced specific Th1 immune responses to control *Leishmania major* infection; Effective delivery to peritoneal antigen presenting cells.	[16]
DNA plasmid encoding the production of three types of cysteine proteinase	SLN	*Leishmania major* infections inhibition after SLN-DNA vaccination to mice.	[17-20]
Hepatitis B surface antigen (HBsAg)	Chitosan and trimethyl chitosan nanoparticles	High serum and nasal antibody titers, after nasal and intramuscular administration; High titers of specific immunoglobulins, after intramuscular administration.	[21]
	SLN	High cellular uptake, lower cytotoxicity and induction of greater TH1 type of immune response.	[22]
	PLA microsphere coated with cationic polymers	Induced strong cell and humoral immune response.	[23]
Influenza antigen (hemagglutinin)	PLGA nanoparticles	*In vivo* safety and enhanced immune responses using particle molding technology (Particle Replication in Nonwetting Templates – PRINT®).	[24]
Insulin	Alginic acid nanoparticles	Pharmacological availability of 100% and bioavailability of 80%, after sublingual administration to diabetic rats.	[25]
	Chitosan nanoparticles	Glycemic level prolonged reduction, after oral administration.	[26]
	Chitosan/alginate nanoparticles	*In vivo* improved oral relative bioavailability; Prolonged nanoparticles intestinal residence time; No systemic toxicity.	[27]
	Chitosan and decanoic acid nanoparticles	Reduced *in vivo* serum glucose level.	[28]
	Eudragit L100-cysteine/reduced glutathione nanoparticles	Prolonged reduction in blood glucose levels, after administration into the ileum loop of healthy rats.	[29, 30]

(Table 1) Contd….

Biopharmaceutical product	Delivery system	Relevant effect	References
	Layer-by-layer of cationic and anionic polyelectrolytes microparticles	*In vivo* sustained serum insulin levels and decreased glucose serum levels.	[31]
	SLN	*In vivo* improved oral bioavailability.	[32, 33]
	SLN	*In vivo* increased relative pulmonary bioavailability.	[34]
Interleukin-2	SLN	Enhanced production of disease specific antibody; Improved immunological mechanisms.	[35]
Leuprolide	Polyacrylic acid nanoparticles	*In vivo* increased relative oral bioavailability of tablets containing nanoparticles.	[36]
	Thiolated chitosan-thioglycolic acid nanoparticles	*In vivo* increased area under the curve, elimination half-life, and maximum plasma concentration.	[37]
Measles antigen	Alginate coated chitosan nanoparticles	Local and systemic immune responses after oral administration.	[38]
Murine mesenchymal stem cells	Alginate-poly-L-lysine-alginate microcapsules	Mice secretion of erythropoietin.	[39]
Murine anti-bone morphogenetic protein-2 monoclonal antibody and human bone marrow mesenchymal stem cells (hBMMSC)	Alginate microspheres	Enhanced hBMMSC-mediated osteogenesis.	[40]
Nucleic acids (antisense oligonucleotide and siRNA)	Inorganic cations microparticles	Delivered to moist or aqueous target locations (e.g. lung tissues).	[41]
Ovalbumin	Poly(lactide-co-hydroxymethylglycolic acid) nanoparticles	Sustained release from the injection site to the lymph nodes; Induced antigen cross-presentation to specific $CD8^+$ T-cells.	[42]
	Polypropylene sulfide nanoparticles	*In vivo* nasal immunization with nanoparticles of 200 nm showed higher serum levels of specific immunoglobulins (IgG and IgA).	[43]
Pancreatic rat islets and curcumin	Alginate microparticles	Reduced fibrotic overgrowth and improved glycemic control in mouse model of type I diabetes	[44]
Peptide YY3-36	Mesoporous silicon microparticles	Improved *in vivo* bioavailability.	[45]
Porcine choroid plexus cells	Alginate-poly-L-ornithine-alginate microparticles	*In vivo* stimulated regeneration of the lesioned nigrostriatum.	[46]
Recombinant human epidermal growth factor	SLN and NLC	*In vivo* effectiveness of NLC and SLN in wound healing process.	[47, 48]

Abbreviations: BSA - bovine serum albumin; hBMMSC - human bone marrow mesenchymal stem cells; HBsAg - hepatitis B surface antigen; NLC – nanostructured lipid carriers; PEG - poly(ethylene glycol); PLA - poly(lactic acid); PLGA - poly(D,L-lactic-co-glycolic acid); siRNA – small interfering RNA; SLN – solid lipid nanoparticles.

due to the swelling and dissolution of chitosan in the acidic medium, the authors used crosslinked chitosan. The network of the crosslinked structures demonstrated the ability to control the *in vitro* BSA release from the microspheres. The presence of *β*-glucosidase, an enzyme used to simulate the colonic medium, increased by approximately 6% the BSA release, after 12 h in simulated colonic fluid (pH 7). Microspheres encapsulation efficiency and loading capacity were dependent on the initial concentration of BSA.

Homo and co-polymers of lactide and glycolide have been used as controlled delivery agents for microparticles, due to their advantages: Food and Drug Administration (FDA) approved for different clinical functions [67]; *in vivo* degradation into natural products (lactic and glycolic acid) that are metabolized by normal pathways [68], and adjustable physic-chemical properties [69]. Due to their hydrophobic nature, when the PLGA microspheres are in contact with aqueous medium and during the initial period of release, they prevent the penetration of water into the microspheres core, affecting the release rate and forming an acidic environment, because of the diffusion of acidic breakdown products, which can cause degradations of the encapsulated substance

[70]. To overcome this limitation, the inclusion of methoxy-poly(ethylene glycol) (mPEG) chains, i.e. a hydrophilic segment, acting as a surface modifier of hydrophobic PLGA network, has been widely proposed. Feng *et al*. [59] encapsulated BSA and lysozyme in PLGA-mPEG microspheres. The *in vitro* release data suggested that initially the release mechanism was mainly diffusion-controlled and, at latter stages of the release period, it was degradation/erosion-controlled. For lysozyme, a shorter lag period was obtained.

PEG was also used to increase the shelf-stability and biocompatibility of lysozyme-loaded poly(L-lactide) microparticles prepared using solution-enhanced dispersion, avoiding the changes in the secondary structure of the protein [64]. The addition of PEG molecules in suitable amount and molecular weight affected the particle size and polymeric network structure of the microparticles and, consequently, changed protein release rate.

Wan *et al.* [60] incorporated hyaluronic acid, a hydrophilic anionic biopolymer, into PLGA microparticles with the aim to suppress the initial burst release of BSA, by reducing the protein surface enrichment. The co-encapsulation of hyaluronic acid was also able to modulate the BSA release profile, by reducing the diffusion rate of BSA in PLGA matrix. Additionally, this polymer seemed to protect the protein integrity during the spray-drying used in the production process.

Although the previous referred polymers are the most commonly used to prepare biopharmaceuticals-loaded microparticles, there are studies referring the employ of others, such as the chemically incorporation of salicylic acid into the poly(anhydride-esters) backbone, which was used to prepared microspheres for insulin delivery. These microspheres presented high protein encapsulation efficiency and were able to sustain the simultaneous deliver of insulin and salicylic acid, both retaining bioactivity following processing [71].

Polymeric microparticles are also promising vaccine adjuvants [23, 56, 70, 72, 73] due to their ability for enhance phagocytic uptake of encapsulated antigen and trigger of inflammatory mechanisms [74, 75]. San Roman *et al*. [72] evaluated the resulting immune response, in mice, generated by CpG oligodeoxynucleotides (ODN) co-encapsulated with the antigen ovalbumin within PLGA microparticles. The microparticles were formulated by blending 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) with two types of PLGA that differ in terms of molecular weight and hydrophilicity. Ovalbumin and ODN sequences maintained their integrity during the manufacturing procedure. However, the release of both encapsulated biopharmaceuticals was slow and incomplete irrespectively of the polymer; the immune response was dependent of the PLGA polymer. Chen *et al*. [23] demonstrated that PLA microspheres coated with cationic polymers – chitosan, chitosan chloride and polyetylenimine – and adsorbed HBsAg (i.e. surface antigen of the hepatitis B virus) may be a promising recombinant antigen delivery system to induce strong cell and humoral immune response.

Multifunctional tissue engineering scaffolds, formed from particles with micrometer size that release tissue-regeneration substances (e.g. growth factors and hormones) in a pre-programmed platform, can generate appropriate niche for cells grow and facilitate tissue regeneration, acting as both prosthetic structural support and biomolecules delivery system. This approach was explored by Qodratnama *et al*. [61], which demonstrated that lysozyme release rate can be programmed by blending "in-house" synthesized PLGA-PEG-PLGA triblock co-polymers microparticles. The incorporation of triblock co-polymer increased lysozyme release rate. Authors suggested the use of these systems, with modulated biomolecules release properties, to produce microparticles-based tissue engineering constructors that allow sequential therapeutic protein release prior to polymer degradation; thus, supporting cellular response to released biomolecule(s) and acting as adequate anchorage for cell to grow and differentiate. The same research team [62] proposed an identical technological strategy to control both the spatial-temporal release kinetics of the recombinant human BMP-2 (bone morphogenetic protein-2) and the degradation polymer rate, by adjusting copolymer compositions, in order to diminish current clinical use of high doses. Authors confirmed that lysozyme can be used as model protein for study the BMP-2, since they have similarity of isoelectric points and weights [76]. These results were based on the comparison between release profiles obtained from human serum albumin (HSA)/lysozyme and HSA/BMP-2. Good encapsulation efficiencies and retention of protein activity were demonstrated after co-encapsulated HSA with lysozyme. Agbay *et al.* [65] encapsulated glial cell line-derived neurotrophic factor (GDNF), a growth factor expressed in the central nervous system, into PLC microspheres using BSA as a carrier protein to preserve GDNF activity during the production process in the presence of organic solvents. The bioactivity of GDNF was accessed in *in vitro* cell culture, where the study demonstrated the ability of microspheres to control GDNF release for 25 days.

Encapsulation of living cells (i.e. bioencapsulation) within polymeric microparticles is an expanding area for tissue engineering and regenerative medicine purposes that can be used to target release for specific body sites, protect live cells and prevent adverse immune response [77, 78]. Cells can be embed into the scaffold or can be seed into a preformed scaffold. Generally, spherical microparticles are used, imitating the morphology and physiology of cells in living tissues and organs [79]. Microparticles present an optimal surface area that can support a quick perfusion of the cellular payload, improving cell viability and function, and offering an ease modulation for the implant degradation [80]. Microencapsulation of different cells types (primary, stem and bioengineered cells) has been investigated for the treatment of different disorders, such as: neurodegenerative diseases (e.g. encapsulated porcine choroid plexus cells in alginate-poly-L-ornithine-alginate microparticles for the Parkinson`s disease [46], encapsulated VEGF (vascular endothelial secreting cells) in microcapsules for Alzheimer`s disease [81]); metabolic diseases (e.g. encapsulated pancreatic islets in alginate microcapsules for diabetes [44]); reconstruction of missing or damaged bone (e.g. co-encapsulation murine anti-BMP-2 monoclonal antibody and human bone marrow mesenchymal stem cells in alginate microspheres [40]). One of the most common approaches in this topic is the entrap-

ment of cell into microdevices using polymer hydrogel particles [82, 83]. This is mainly related to their advantages, namely, excellent biocompatibility, minimal inflammatory reactions and tissue damage [40]. Tzouanas *et al.* [83] studied the viability and osteogenic differentiation of mesenchymal stem cells encapsulated in gelatin microparticles, in a chemically and thermally gelling hydrogel system. Authors reported a long-term viability and *in vitro* promoting mineralization, depending on the microparticles size and loading ratios.

For clinical application of stem cells, cryopreservation is an alternative approach for long-term storage, with maintenance of the structure and function of encapsulated cells, compared to liquid nitrogen freezing, which can damage the live cells during ice formation. Recently, Gurruchaga *et al*. [39] demonstrated that dimethyl sulfoxide (DMSO) 10% was a suitable cryoprotectant solution for the slow cooling cryopreservation of immobilized mesenchymal stem cells into alginate-poly-L-lysine-alginate microcapsules. The processing method is also a critical step that could attend the stringent needs of biotechnology and biomedical applications [84]. Therefore, preparation methods that avoid harsh conditions (e.g. use of toxic solvents, high temperatures and pressures) and optimize the properties of microparticles (e.g. adequate polydispersity index and particle size and small aggregation phenomena) are required to encapsulate cells [85]. For example, Oliveira *et al*. [86] proposed the preparation of PEGylated fibrinogen cell-laden microparticles, by a continuous and rapid method totally compatible with cell encapsulation (i.e. solvent and oil-free, and controlled particle size and polydispersity index of the microparticles). This method was based on the precise control of the rheological properties of the biomaterial precursor (solution of cells in photoreactive PEG - fibrinogen polymer) during the extrusion performed before atomization in a jet-in-air spraying.

A promising area in the topic of target biopharmaceuticals delivery is the use of responsive polymer materials that suffer a phase transition in response to some changes in environmental stimuli, such as pH, temperature and magnetic fields [87, 88]. Li *et al.* [88] integrated the use of responsive polymers with the molecular imprinting technique to prepare thermosensitivity surface BSA-imprinted magnetic microspheres, by a surface grafting copolymerization method on the surface of vinyl modified biocompatible iron oxide (Fe_3O_4) microspheres coated with silicon dioxide (SiO_2) film. Poly(N-isopropylacrylamide) was chosen as the temperature-sensitive component, which allowed for swelling and shrinking in response to temperature changes. The results showed that the adsorption and desorption capacity and specific recognition of BSA molecules could be regulated by system temperature indirectly, which benefited from the presence of a thermosensitivity imprinting layer. In the area of stimuli-responsive delivery systems, the use of magneto-responsive polyelectrolyte microcapsules with the shell structure comprising of polyallylamine hydrochloride and Fe_3O_4 nanoparticles was also used for *in vitro* insulin controlled release [89].

Using cationic and anionic polyelectrolytes, Amancha *et al*. [31] formulated layer-by-layer insulin microparticles. Intrapulmonary administration in rats resulted in sustained serum insulin levels and simultaneous decrease in serum glucose levels.

2.1.2. Lipid Microparticles

Solid lipids (e.g. fatty alcohols, fatty acid esters, polyol esters) have been used to prepare microparticles for the delivery of biopharmaceuticals, showing a sustained-release profile. For example, in the patent US 8729015 B2, the inventors prepared lipid microspheres using different lipids or a combination of them (e.g. Gelucire®, Precirol®, Dynasan®) containing human growth hormone or a functional derivative encapsulated in the inner solid core of the microparticles [90].

2.1.3. Inorganic Microparticles

Concerning inorganic carriers, biopharmaceuticals microencapsulation has been prepared with biocompatible porous materials, such as mesoporous carbon [91], mesoporous silica [45, 92] and calcium carbonate ($CaCO_3$) [93], based on adsorption phenomena. For example, magnetic dual-mesoporous carbon microspheres were synthesized using various amounts of ferric nitrate nonahydrate ($Fe(NO_3)_3.9H_2O$), which influence the structural parameters and magnetic properties of the delivery system [91]. In this study, BSA was selected as the adsorption protein model. Mesoporous carbon materials present large surface area, high pore volume, concentrated pore size distribution and good biocompatibility which make them promising adsorption materials [94-96]. The inclusion of magnetic nanoparticles (e.g. Fe, Ni and Co nanoparticles) in the mesoporous material facilitates the absorption and separation of protein and could be used as targeted delivery systems [91, 97]. Horák *et al*. [98] used multistep swelling polymerization methods to produce monodisperse magnetic poly(glycidyl methacrylate) (PGMA)-based microspheres, followed by precipitation of iron oxide inside the microspheres pores. In order to minimize the nonspecific protein adsorption, microspheres were coated with albumin. The system was developed for the capture of circulating tumor cells (CTCs); therefore, antibodies of epithelial cell adhesion molecule were immobilized on the microspheres. The authors demonstrated the success of the microspheres to capture human breast cancer MCF7 cells, used as a model of CTCs, and a very good rejection of lymphocytes, suggesting their promising use in a microfluidic immunomagnetic cell sorting device.

Kovalainen *et al*. [45] loaded peptide YY3-36 into mesoporous silicon microparticles and evaluated the effects of different surface chemistries (thermally oxidized, thermally hydrocarbonized and undecylenic acid treated) in the delivery of this peptide. The results demonstrated improvement on *in vivo* bioavailability of the peptide when using thermally oxidized mesoporous silicon microparticles, and also a sustained release.

$CaCO_3$ microparticles present high loading capacity and have been used to encapsulate DNA [99], enzymes and proteins [93]. Tran *et al.* [93] used lysozyme as a model protein and prepared lysozyme-loaded $CaCO_3$ microparticles in an aqueous solution in CO_2 media, avoiding the use of any volatile organic solvents in the encapsulation process, which are considered toxic for the body and the environment. The en-

capsulation yields reached 60%. The lysozyme release profile revealed to be pH-dependent. The authors purposed the incorporation of these particles into an implantable hydrogel system for bone and cartilage tissue engineering, in order to slow the release of therapeutic proteins.

In the patent US 8808747 B2, the inventors prepared microparticles comprising one or more nucleic acid (e.g. antisense oligonucleotide or siRNA) and one or more solubilized inorganic cations (e.g. Ba^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Na^{+}, K^{+}, Li^{+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Fe^{3+} or Al^{3+}), for local target of the nucleic acids (e.g. lung tissues) [41].

Synthetic biological ceramic substances, such as calcium silicilate, were also used to prepare microparticles carriers. Li *et al.* [100] produced hollow calcium silicate microspheres modified with simulated body fluid to develop a micro-porous structure and interconnected channels that increased the protein adsorption amount as well as controlled local delivery of the BSA and lysozyme, which were used as model substances. The authors suggested the potential of developed microspheres to be used as osteoconductive graft materials as well as for controlled local substances delivery in bone regeneration.

2.2. Nanoparticles

Concerning their extensive and different applications, several definitions have been used for nanoparticles. Some researchers classify as nanoparticles the particles with sizes up to 100 nm, while others limit the upper size to 1000 nm. Nonetheless, according to the scientific articles data that have been published in this area, we consider the definition of J. Kreuter for nanoparticles as the more complete [101]: "Nanoparticles for pharmaceutical purposes are solid colloidal particles ranging in size from 1 to 1000 nm (1 μm) consisting of macromolecular materials in which the active principle (drug or biologically active material) is dissolved, entrapped, or encapsulated, or to which the active principle is adsorbed or attached."

Regarding biopharmaceuticals and, more precisely, protein delivery, insulin is the most accepted model to study oral protein delivery by means of nanoparticles. In this area, a variety of materials have been tested to prepare nanoparticles, enabling novel approaches and bringing important therapeutic advances [102]. However, despite the benefits that oral insulin administration could represent for patients suffering from type 1 diabetes, so far there are none oral insulin formulations in the market. Reasons for this are the low stability of insulin in the gastrointestinal tract and its poor permeation in the intestinal cells due to its high molecular size [102, 103].

As mentioned, several materials can be used to prepare nanoparticles, such as polymers, lipids, proteins, metals and minerals. Among these, polymers and lipids are the most explored for drug delivery. Therefore, in this article we will only refer to them.

2.2.1. Polymeric Nanoparticles

Polymeric nanoparticles are colloidal carriers ranging from 1 to 1000 nm, although sizes over 100-200 nm are desirable for drug delivery purposes. Usually these nanoparticles have spherical shape and are composed by natural (e.g. chitosan, alginate), semi-synthetic or synthetic (e.g. PLGA; PLA; poly(alkyl cyanoacrylate), PACA; poly(methyl methacrylate), PMMA) polymers. The carried molecules may be in the solid or liquid state, and can be encapsulated within the nanoparticles, or adsorbed or linked to their surface. The use of polymeric nanoparticles for drug delivery presents several advantages, such as: biocompatibility, biodegradability, sustained release mechanisms, molecule protection from degradations and easiness of production. Concerning their disadvantages as carrier systems, the production processes, which frequently require the use of organic solvents that can originate a residual toxicity in the final formulations, and the difficulties on transfer these processes to the large scale, are the main pointed limitations. Different methods have been employed to prepare polymeric nanoparticles, such as coacervation, nanoprecipitation, solvent emulsification-evaporation, solvent emulsification-diffusion, ionotropic gelation, supercritical fluid technology and polymerization of monomers [104-107].

Different polymers have been used to prepare insulin-loaded polymeric nanoparticles. Examples of these are the synthetic and biodegradable PLGA and PLA, and the natural chitosan and alginate polymers. In addition, some excipients are commonly employed to stabilize insulin and other proteins in polymeric nanoparticles, such as polymers (e.g. PEG and poly(vinyl alcohol), PVA) [108], amphiphilic lipids (e.g. soybean phosphatidylcholine and sodium caprate) [109] and peptides (e.g. poly(arginine) [110] and valine [111]). Therefore, an adequate combination of the components in polymeric nanoparticles should be optimized for the success of drug delivery.

Among the referred polymers, chitosan and alginate have been extensively explored to prepare polymeric nanoparticles, since they have adhesive properties, improving drug absorption. Furthermore, their natural source originates a low toxicity on the final formulations. In this regard, insulin-loaded chitosan and alginate nanoparticles for oral delivery have been widely studied. For example, Mukhopadhyay *et al*. [26] observed a glycemic reduction of 29-33% passing 4h of the oral administration of insulin-loaded chitosan nanoparticles to diabetic rats. Besides, this effect lasted up to 8h. In other study, the same authors tested the benefits on insulin bioavailability, preparing chitosan/alginate nanoparticles for oral delivery to diabetic rats. The *in vivo* experiments showed an improvement of the oral relative bioavailability of insulin (~ 8.11%). From the results, it was concluded that the mucoadhesive effects of both used polymers contributed to prolong the nanoparticles residence time in the intestine. In addition, no systemic toxicity was observed after nanoparticles administration [27].

Nanoparticles composed by mixtures of polymers (e.g. chitosan) and lipids (e.g. decanoic acid) enhanced the intestinal insulin absorption and improved the synergistic effect of adhesion and permeation, respectively. Thereby, the *in vivo* serum glucose level was reduced by 57.18%. Furthermore, the prepared nanoparticles showed low toxicity in the intestinal mucosa tissue of the tested rats [28].

Recently, some researchers carried out studies using chitosan soluble derivatives to prepare polymeric nanoparticles

in order to improve oral insulin delivery. For example, Mansourpour *et al.* [112] observed an insulin permeability of 8.41% with trimethylchitosan nanoparticles across Caco-2 cells, showing a remarkable increase, compared to chitosan nanoparticles.

Other polymers have been tested to improve insulin delivery by means of nanoparticles. For instance, Zhang *et al*. [29] observed a prolonged effect of reduction in blood glucose levels (from 2 up to 8h), after the administration of insulin-loaded Eudragit L100-cysteine/reduced glutathione nanoparticles into the ileum loop of healthy rats, suggesting the potential of this system for the treatment of diabetes. In other study, these authors carried out *in vitro* experiments in Caco-2 and Caco-2/HT29-MTX co-cultured cells models, and suggested the occurrence of paracellular transport of insulin across intestinal cells [30].

Alternative administration routes to improve insulin absorption by means of polymeric nanoparticles have been proposed. Patil *et al.* [25] demonstrated a pharmacological availability of 100% and a bioavailability of 80% after sublingual administration of insulin-loaded alginic acid nanoparticles to diabetic rats. Mortazavian *et al*. [113] reported an enhancement of insulin permeation (~ 97.18%) through rabbit buccal mucosa after encapsulation of this protein in thiolated dimethyl ethyl chitosan nanoparticles, compared to chitosan nanoparticles.

The use of tablets containing leuprolide-loaded nanoparticles of polyacrylic acid was suggested as a promising oral delivery system for this and other peptide drugs. After oral administration to rats, the relative leuprolide bioavailability increased up to 4.2-fold, compared to conventional tablets [36]. Nonetheless, more studies are required to confirm the feasibility of the prepared tablets for oral delivery of leuprolide. In contrast, the nasal route was proposed to improve the delivery of leuprolide by means of thiolated chitosan-thioglycolic acid nanoparticles. Compared to nasal solution, the leuprolide-loaded polymeric nanoparticles increased the drug transport through porcine nasal mucosa by 2.0-5.2 folds. Furthermore, the *in vivo* studies showed a 6.9-fold increase in area under the curve, more than 4-fold increase in elimination half-life, and a ~3.8-fold increase in maximum plasma concentration. The leuprolide relative bioavailability was about 19.6%, compared to 2.8% of subcutaneous leuprolide solution [37].

The advantages of salmon calcitonin encapsulation in polymeric nanoparticles for oral administration have been studied more than a decade ago, by Sakuma and co-workers. This research group carried out several studies, evaluating the effects of polymer composition and surface attached molecules in the performance of polystyrene nanoparticles, aiming to enhance calcitonin absorption in the gastrointestinal tract. From the results, authors concluded that calcitonin encapsulation in polymeric nanoparticles improves its oral absorption, which is related with the composition and surface grafted molecules of these nanoparticles [114-116]. In addition, the pulmonary delivery of calcitonin by means of glycol chitosan and glycol chitosan coupled with thioglycolic acid chitosan nanoparticles was investigated. An increase of both lung tissue mucoadhesion and biocompatibility were observed, after intra-tracheal administration of calcitonin-loaded chitosan based nanoparticles to rats. Moreover, a hypocalcemic effect from 12 up to 24h, with a corresponding bioavailability of 27 up to 40%, was observed with the calcitonin-loaded nanoparticles [13].

Glowka *et al*. [14] reported the success of using PLGA nanoparticles to obtain a sustained release of calcitonin, after subcutaneous administration. It was observed an *in vitro* release of 20% of calcitonin, after four weeks. This result was confirmed by *in vivo* studies, where nanoparticles induced a sustained increase of calcitonin plasma level during 3 days, with an increased bioavailability, compared to a peptide solution.

A parathyroid hormone-related protein was encapsulated in N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticles. An *in vitro* slow-continuous-release profile was obtained, suggesting the suitability of the system to manage the treatment of osteoporosis, despite additional animals and humans *in vivo* studies are required [117].

Lysosyme, an antimicrobial protein-based enzyme model, was encapsulated in chitosan nanoparticles and their antibacterial activity against *Staphylococcus epidermidis* was tested *in vitro*. The results showed that lysozyme-loaded nanoparticles are able to maintain the enzyme activity, which was slowly released for 3 weeks, remaining active up to five days of incubation with the tested bacteria. Moreover, the developed nanosystem was compatible with murine fibroblasts [118].

The ability of chitosan nanoparticles for the controlled delivery was tested, using BSA as a model. Shrestha *et al.* [119] evaluated the relevance of molecule release in the regulation of alkaline phosphatase activity (ALP) of stem cells, from apical papilla. For the experiments, BSA-loaded chitosan nanoparticles were prepared by two different methods, in order to obtain encapsulated or surface adsorbed BSA nanoparticles. From the results, two different time-controlled BSA release profiles were observed. Besides, cell viability was significantly enhanced over time in the presence of BSA-loaded chitosan nanoparticles, when compared to chitosan nanoparticles alone. ALP activity was significantly higher in the presence of BSA-loaded chitosan nanoparticles. Accordingly, authors highlighted the potential of timed-controlled molecule release in the differentiation processes of stem cells for dentin pulp regeneration.

According to their multiple therapeutic applications, monoclonal antibodies can be used for different approaches [120-122]: alone as drugs, in clinical diagnosis and grafted to the surface of colloidal carriers for drug targeting. Regarding polymeric nanoparticles, the surface grafting for targeting other drugs has been the most explored application, despite some studies reported the encapsulation of monoclonal antibodies. For example, Chen *et al*. [10] reported the improvement of therapeutic efficacy for an anti-tumor necrosis factor monoclonal antibody, after encapsulation in PLGA nanoparticles. From the experiments, authors observed an induced cytotoxic T lymphocyte proliferation, tumor antigen-specific cytotoxicity and cytokine production more strongly than free monoclonal antibody, using mouse tumor models. These results suggested that PLGA nanoparticles might be effective systems for antibody delivery in cancer immune therapy, although more studies are required to prove it. Varshochian

et al. [12] prepared bevacizumab-loaded albumin-PLGA nanoparticles to improve the treatment of eye posterior segment neovascularization, reducing the frequency of intravitreal injections. The *in vivo* tests were carried out in rabbits and showed an elevated bevacizumab vitreous concentration during 8 weeks. Furthermore, it was confirmed that nanoparticles remained in the ocular tissues during 56 days.

Despite being suggested many years ago [123], the use of polymeric nanoparticles to improve vaccination has been increasing last years. Among these, oral and nasal vaccines offer various advantages over the parenteral ones. For example, Biswas *et al*. [38] studied the potential of alginate coated chitosan nanoparticles loaded with measles antigen for oral vaccine delivery. The *in vitro* data showed a protection effect of alginate coating against acid pH degradation of antigen, during 2h. A sustained release kinetic of the antigen from the nanoparticles, a low cytotoxicity and both local and systemic immune responses were observed. Tafaghodi *et al*. [21] prepared chitosan and trimethyl chitosan nanoparticles, loaded with hepatitis B surface antigen (HBsAg), and evaluated their efficacy as antigen carrier systems for nasal and intramuscular administrations. The *in vivo studies* showed that, after administration, the serum and nasal antibody titers were higher, compared with an HBsAg solution. Moreover, after intramuscular administration, both types of nanoparticles induced higher titers of specific immunoglobulins. From this study, authors concluded that HBsAg-loaded chitosan-based nanoparticles are potent mucosal adjuvants for hepatitis B, after nasal and intramuscular administration. In other study, Bal and co-workers showed that the inclusion of additional immunopotentiators into antigen-loaded N-trimethyl chitosan nanoparticles improves the overall immunogenicity of vaccines, despite the success of the response depends on the immunopotentiator (i.e. adjuvant) as well as the route of administration (intranasal or intradermic). For example, the NOD-like receptor 2 ligand muramyl dipeptide is effective only for intranasal administration, whereas the CpG oligonucleotide is intradermally. In contrast, the lipopolysaccharide ligand seems to be suitable for both nasal and intradermal routes [124]. Recently, Bento *et al*. [11] also tested the effects of adjuvants to enhance nasal mucosal immunity. These authors prepared *Bacillus anthracis* antigen-loaded chitosan-based nanoparticles associated to mast cell activator compound, to increase the cellular uptake and prolong the antigen residence time in nasal cavity, while promote a local microenvironment favorable to the devolvement of an immune response. The results of *in vivo* studies, after nasal immunization to mice, showed the production of high levels of serum specific antibodies and cytokines, compared to a solution of the same compound, suggesting that the combination of adjuvants and nanoparticles is a promising strategy to improve the efficacy of nasal vaccination. Stano *et al*. [43] used ovalbumin as an antigen model to study the effect of polypropylene sulfide nanoparticles size (30 or 200 nm) in the magnitude and quality of mucosal immune responses, after intranasal immunization to mice. Mice immunized with nanoparticles of 200 nm showed higher serum levels of specific immunoglobulins (IgG and IgA), and authors concluded that these nanoparticles are promising carriers for nasal antigen delivery of vaccines against pathogens that require multifunctional $CD4^+$ T-cells for protection. These results contributed to the understanding of how the size of antigen-loaded polymeric nanoparticles can influence mucosal immune responses to protein antigens, and can be useful to engineer vaccines that allow for appropriate immune responses. In this context, Galloway *et al*. [24] tested the efficacy of a particle molding technology (Particle Replication in Nonwetting Templates - PRINT®) to prepare influenza antigen (hemagglutinin) loaded-PLGA nanoparticles in a commercial trivalent injectable vaccine formulation. In this study, the authors presented the advantages of PRINT process, such as: independent evaluation of particle composition, size and charge, which enables the test of the impact of these factors on the immune response in future vaccine products; exploration of the strain specificity to immune response; test the extent of antigen source; investigate the viability of the produced antigens; estimate the vaccine dose; possibility of including extra immunostimulatory molecules (i.e. adjuvants). The hemagglutinin-loaded PLGA nanoparticles formulations were tested *in vivo* (in murine models) and showed safety and enhanced immune responses.

Rahimian *et al*. [42] tested both *in vitro* and *in vivo* efficacy of ovalbumin loaded poly(lactide-co-hydroxymethylglycolic acid) nanoparticles as a model vaccine. Nanoparticles were more effective to transport (i.e. provided a sustained release) the antigen from the injection site to the lymph nodes, and induced an antigen cross-presentation to ovalbumin specific $CD8^+$ T-cells superior, when compared to an ovalbumin solution. Thereby, authors concluded that the tested polymeric nanoparticles are promising vehicles for protein antigen delivery, providing an effective induction of cellular immunity.

Dixit and co-workers reported the successful encapsulation of a membrane peptide of *Chlamydia trachomatis* in PLA–PEG nanoparticles. The authors reported an *in vitro* slow release and absence of toxicity to macrophages. In addition, an *in vivo* effective subcutaneous mice immunization with higher production of specific T-cell cytokines and antibodies was observed. This enhanced immune response suggested the application of the developed system as a new vaccination strategy against *Chlamydia trachomatis* infection and other intracellular pathogens [15].

The use of polymeric nanoparticles for nucleic acids transfection has been reported elsewhere, by several research groups. The interested reader can obtain complete information regarding this issue in a review article recently published by our group [107]. These systems are called polyplexes and consist of nano-sized condensed structures, formed by electrostatic interactions between negative charges of DNA/RNA molecules and positive charges of cationic polymers, which can easily enter to the cells (usually by endocytosis) and reach the nucleus, expressing the encoded protein. Nonetheless, some limitations to the *in vivo* performance of polyplex systems have been reported. To circumvent these problems, PEGylated polymers (i.e. polymers including PEG molecules in their structure) have been successfully employed to improve polyplexes stability [107].

2.1.2. Lipid Nanoparticles

Lipid nanoparticles formulations are aqueous dispersions of solid nanoparticles made by physiological biocompatible

lipids and stabilized by one or two surfactants. The latter are GRAS (generally recognized as safe) substances. Regarding the non-toxicity of the components of lipid nanoparticles, their use for drug delivery is promising and has been much explored since their invention (in the nineties) [106, 125-127].

There are two types of lipid nanoparticles, the solid lipid nanoparticles (SLN), which comprise one lipid that is solid at both body and room temperatures. The second type are the nanostructured lipid carriers (NLC), developed after recognizing the limitations of SLN, namely drug expulsion during storage, low drug loading efficiency and poor long-term stability. In the NLC, the matrix of the nanoparticles consists of a mixture of a solid and a lipid liquid, and it is also solid at both room and body temperatures. The liquid lipid allows for a more disarranged inner structure of the nanoparticles, which increases the drug loading and storage stability. For the reasons previous mentioned, the NLC have been claimed as superior carriers over the SLN [125, 128]. However, several authors have shown that SLN systems are also suitable delivery systems, presenting good long-term stability and high drug loading efficiency [129].

Regarding the production of lipid nanoparticles, the high pressure homogenization and other similar high energy techniques (e.g. ultrasound) are commonly employed. All these techniques require the previous melting of the solid lipid, which means that a relatively high temperature is required, according to the lipid melting point. Moreover, during the production process, the drug is usually added to the lipid phase, which means that it will be submitted to the same temperature [130, 131]. Thereby, according to the sensitive characteristics of biopharmaceuticals molecules, they have the risk of inactivation during the production of lipid nanoparticles. Moreover, the high energy procedures (e.g. high pressure and ultrasound) that must be employed to produce particles with nanometer sizes also increase the temperature and the risk of damage these molecules. Thus, the use of lipid nanoparticles for the delivery of biopharmaceuticals has been little explored, although some studies have been reported elsewhere. To overcome the temperature exposition of molecules, some of the techniques employed in the production of polymeric nanoparticles have been adapted to produce lipid nanoparticles. Nonetheless, all these techniques require the use of organic solvents that are eliminated by evaporation after nanoparticles preparation, which means that some residual toxicity could remain in the final formulation. Besides, if this occurs, one of the major advantages associated to the use of lipid nanoparticles as delivery systems is not realized [4, 132].

Two review articles, reporting the most promising results obtained using lipid nanoparticles for improve the delivery of biopharmaceuticals, have been recently published by our group [4, 107]. Since then, not much relevant results have been published. In the following paragraphs we will summarize the ones previous presented and refer some new data. The interested reader can assess more information in the provided references.

For proteins, therapeutic hormone delivery has been the most explored, particularly the encapsulation of insulin in lipid nanoparticles, protecting the molecule from degradations and increasing its bioavailability. In this area, SLN formulations for oral insulin administration are much studied. Several authors observed improvements in insulin delivery after oral administration to rats, with approximate results of relative bioavailability of 5.0% [32], 7.11% [133] and 13.86% [33]. Furthermore, the pulmonary route has also been considered for improve insulin administration, where was observed an insulin relative bioavailability of 35.62% [134]. However, considerable fluctuations in the plasma levels of insulin could occur after pulmonary administration of this hormone. Additionally, some *in vitro* studies suggest the suitability of SLN systems for the encapsulation of insulin [135, 136]. Nonetheless, this must be confirmed by *in vivo* experiments.

The encapsulation of gonadotrophins in lipid nanoparticles (gonadorelin [137] and leuprolide [138]) has also been suggested, although this application was not fully explored.

The success of oral administration of calcitonin encapsulated in lipid nanoparticles has been suggested by several research groups, after performing *in vitro* and *in vivo* studies. Furthermore, some of these authors observed that a chitosan surface coating on the lipid nanoparticles enhance the oral delivery of calcitonin, suggesting a general application of this strategy to improve oral protein delivery [139-143].

Xie *et al*. [136, 144] reported the effects on protein encapsulation efficiency, stability and activity in lipid nanoparticles systems, using polymeric emulsifiers as PLGA. For these studies bovine serum albumin was used as a model protein. Additionally, the influence of formulation parameters (e.g. type of lipid, time of exposure to different temperatures, pressure and the number of homogenization cycles) on the integrity and activity of peptides, was studied by Almeida *et al.* [145], using lysozyme as a peptide model.

The encapsulation of recombinant cytokines (interferon-α and interleukin-2) in lipid nanoparticle systems was suggested to improve the delivery of veterinary drugs and vaccines. Nonetheless, in this regard few studies were performed and only one report to *in vivo* experiments [35, 146]. Accordingly, this remains a promising open field for the research in veterinary and human cytokines delivery.

In the same manner as previously mentioned for polymeric nanoparticles, the use of monoclonal antibodies in lipid nanoparticles has been employed only for surface grafting for targeting other drugs. For example, the surface coating of SLN for improve blood brain barrier permeation of antiretroviral drugs [147] and the coating of NLC for tumor active targeting [148, 149].

Recently, the use of lipid nanoparticles for topical administration of recombinant human epidermal growth factor (rhEGF) to treat chronic wounds was suggested by Gainza *et al*. [47, 48]. The authors described the effectiveness of SLN and NLC systems for wound healing in both cell culture and diabetic animal models, suggesting that lipid nanoparticles could be used to improve the treatment of chronic wounds. More specifically, the *in vivo* experiments showed that the wound healing process was more effective in animals administered with four topical doses of 10 and 20 μg of SLN-rhEGF or NLCrhEGF, when compared to the animals treated with the same number of intralesional doses of 75 μg of free

rhEGF and as effective as a single intralesional administration of 75 μg of rhEGF-loaded alginate microspheres. Furthermore, among the tested lipid nanoparticles systems, authors suggested that NLC would be more effective for the proposed application, since they showed higher rhEGF encapsulation efficiency over the SLN. Based on these experiments, authors tested the rhEGF-NLC in porcine excisional wound model, using large white pigs. From the results, it was observed that 20 μg of rhEGF-NLC topically administered twice a week increased the wound closure and percentage of healed wounds by day 25, compared with the same number of intralesional administrations of 75 μg free rhEGF and NLC alone. In addition, authors reported that rhEGF-NLC improved several wound healing processes (e.g. arranged microvasculature, fibroblasts, collagen and inflammatory response).

Cationic lipids have been extensively explored for the preparation of lipid nanoparticles systems to deliver nucleic acids, since they establish electrostatic interactions with the phosphate groups of RNA and DNA molecules, forming the so-called lipoplexes. In this area, several research articles have been published reporting *in vitro* and *in vivo* results. Nonetheless, difficulties related to nucleic acids reaching cell's nucleus and the achievement of an appropriate gene expression remain unsolved. Moreover, some cytotoxicity of nucleic acids-based lipid nanoparticles systems has been reported [4, 107].

The improvement in vaccination by antigen encapsulation in lipid nanoparticles has been poorly studied. Only one article reported *in vivo* results of hepatitis B immunization after encapsulation of the respective antigen protein in surface grafted SLN [22]. In addition, vaccination improvements by means of SLN cysteine proteinase type I encapsulation against *Leishmania major* showed promising results after peritoneal administration to mice, although alternative administration routes are needed for human application [16]. Furthermore, this research group reported the effectiveness of SLN-DNA vaccines (i.e. SLN-DNA plasmid) for immunization against *Leishmania* spp. infections [17-20].

3. CONCLUSIONS AND PROSPECTS

Biopharmaceuticals are pharmacological products that have been showing promising results for the treatment of several disorders, particularly severe diseases. Formulating these products is very challenging, according to the sensitive biopharmaceuticals molecular structures. Pharmaceuticals technologists should guarantee biopharmaceuticals stability and biological activity of the final product, in order to achieve an optimal therapeutic efficacy.

Several delivery systems (e.g. microparticles and nanoparticles) composed of different materials (e.g. polymers and lipids) have been explored and demonstrated excellent outcomes, such as: high cellular transfection efficiency for nucleic acids, cell targeting, increased proteins and peptides bioavailability, improved immune response in vaccination, and viability maintenance of microencapsulated cells. However, important issues need to be addressed before they reach clinics. For example, more *in vivo* studies in animals, to access the toxicity potential and predict *in vivo* failure of these delivery systems are required.

CONFLICT OF INTEREST

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

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