THE POTENTIAL OF CHITOSAN FOR PULMONARY DRUG DELIVERY

Ana Grenha[1], Sonia Al-Qadi[2], Begoña Seijo[2], Carmen Remuñán-López[2*]

[1]CBME-Center for Molecular and Structural Biomedicine, IBB-Institute for Biotechnology and Bioengineering, Univ. Algarve, Campus de Gambelas, 8005-139 Faro, Portugal**;** [2]Dep. Pharmaceutical Technology, Univ. Santiago de Compostela, Faculty of Pharmacy, 15782-Santiago de Compostela, Spain

***Corresponding author**

Tel.: +34 981563100 – Extn. 15045

Fax.: +34 981547148

E-mail: mdelcarmen.remunan@usc.es

Abstract

The administration of drugs through the pulmonary route offers great advantages, but also requires overcoming many challenges. There is a need to develop appropriate carriers for each active molecule to be delivered to the desired site in the lung, either for a local or a systemic effect. The polysaccharide chitosan is a very promising material for this purpose, given its demonstrated properties of biodegradability and biocompatibility, as well as mucoadhesivity and ability to enhance macromolecules permeation. In this review, the potential of chitosan to develop drug carriers for delivery to the lung will be discussed. The most important features that can support its selection will be explained. Besides, different approaches to increase its performance, especially concerning solubility, permeation-enhancing properties and gene transfection efficiency, will be presented. Special emphasis will be placed on information of different chitosan-based carriers, namely nanoparticles and microparticles, intended for pulmonary drug delivery.

Key words: Chitosan, Gene delivery, Nanoparticles, Microspheres, Pulmonary drug administration.

1. Introduction

The field of drug delivery has been assisting recently to a strong biotechnological revolution, which brings new potential therapeutic molecules and macromolecules every day. However, the application of these substances in therapeutics requires overcoming many challenges related with the effective maintenance of their stability, circumvention of enzymatic degradation and diminution of immune reactions. In this manner, new strategies have arisen concerning their delivery to specific sites of action and both the design of adequate drug delivery systems and the definition of appropriate routes of administration have become critical features. Particularly, the pulmonary route offers great advantages for the administration of drugs, such as large surface area available for absorption in the alveoli, very thin diffusion path to the blood stream, extensive vascularisation, relatively low metabolic activity compared to other routes and avoidance of first pass hepatic metabolism (1,2). Anyhow, there is still the need to develop appropriate carriers for each active molecule to be delivered to the desired lung site, either for a local or a systemic effect. In fact, in order to succeed in the pulmonary delivery of any therapeutic molecule, many obstacles and lung defence mechanisms, which could hinder the path of foreign substances, must be overcome, such as the effect of the airways' structure, the mucociliary clearance and the phagocytosis by the alveolar macrophages (2,3) and, therefore, the applied carrier should comply with specific characteristics. In this manner, if a systemic effect is expected, the drug should reach the alveolar region, where systemic absorption mostly takes place; while in the case of a local action, the drug should arrive at the specific site of action, which could be for instance the bronchial region in the case of asthma therapy or again the alveoli in the case of tuberculosis (4). Considering the specific anatomy of the airways, it is traditionally believed that droplets and/or particles with aerodynamic diameter within the range of 1 – 3 μm will present appreciable deposition in

the alveolar region, while those with higher aerodynamic diameter will be mainly deposited in the tracheobronchial area (1). Therefore, it becomes obvious that size and density of the delivery systems will both play a critical role in obtaining adequate therapeutic effect.

The selection of suitable drug carriers is a crucial factor to succeed in lung delivery and, hence, materials (polymers, lipids, sugars) used for their preparation are an essential parameter to consider. Amongst the polymers that have been proposed as valuable alternatives for this purpose, polyesters, acrylates, cellulose derivatives and polysaccharides have been mentioned in many occasions (5-7). Amid these polymers, chitosan, a natural polysaccharide derived from chitin, has been presented as a very promising excipient, given its demonstrated properties of biodegradability and biocompatibility (8), as well as mucoadhesivity and enhancement of macromolecules permeation, which facilitate its application in the development of mucosal drug delivery systems (9,10).

In this review, the potential of chitosan as suitable excipient to develop drug carriers for delivery to the lung will be discussed. The most important features that can support its selection will be explained, while different approaches to increase its performance, especially concerning solubility, permeation-enhancing properties and gene transfection efficiency, will be presented. Special emphasis will be placed on information of different chitosan-based carriers, namely nanoparticles and microparticles, intended for pulmonary drug delivery. In this manner, indications on the *in vitro* as well as *in vivo* studies will be provided, and methods applied for their production will also be addressed.

2. Chitosan: a naturally occurring polymer

Chitosan [α-(1-4)-2-amino-2-deoxy-β-D-glucan] is a cationic polysaccharide which is composed of repeating units of N-acetylglucosamine and D-glucosamine (Figure 1). Being the second most abundant polysaccharide in nature, only preceded by cellulose (11), it is obtained by the deacetylation of chitin, the main component of crustaceans exoskeleton and insects and, more recently, it has been obtained by biotechnological processes (12,13). The β-(1-4) glycosidic bonds linking the glucosamine units of chitosan can be destroyed by lysosyme, an enzyme that exists in all the mucosal surfaces of the organism, including the pulmonary (14).

Chitosan possesses structural characteristics similar to those displayed by glycosaminoglycans (GAGs), which are an important component of connective tissues. Owing to that feature, it has been investigated for rather different biomedical applications, such as wound-healing, tissue engineering, dentistry and orthopaedics (13). Furthermore, chitosan presents the ability to attach to fat, preventing its absorption and, thus, is included in marketed dietary supplements, because it can induce weight loss (15).

As it has been previously mentioned, chitosan presents well-documented favourable biological properties such as biocompatibility, biodegradability and low toxicity (8,16), which render this molecule very attractive for drug delivery. Moreover, increased attention has been paid to this polymer because of its reported mucoadhesive properties (9) and the ability to promote the permeation of macromolecules through well-organised epithelia (nasal, intestinal, ocular, buccal, pulmonary) (17-24), features which will be described in detail in a specific section of this review (section 4).

Bearing in mind that this review focuses on the application of chitosan in drug delivery to the lung, its physicochemical properties, which might affect this specific use, will be addressed in the present section. Chitosan comprises a series of polymers with different molecular weight and percentage of acetyl groups and, hence, degree of

acetylation, characteristics which have been proving determinant in its behaviour (11). Actually, the main difference between chitin and chitosan is the number of acetyl groups, although this small difference turns to important features in their physicochemical properties, such as solubility (chitin is insoluble in water and in the most common organic solvents and, hence, not useful for pharmaceutical purposes, whereas chitosan is soluble in acidic solutions), biodegradability and mucoadhesivity (23). Displaying a pK_a of approximately 6.5, in neutral or basic pH, chitosan is insoluble in water, while in acidic pH it becomes water-soluble, due to the protonation of most amino functions (13). Although it has been sometimes claimed that this behaviour strongly interferes with the biomedical application of the polymer, because it is insoluble at physiological pH (7.4), it also has been proven many times that chitosan-based formulations improved the therapeutic effect of macromolecules associated to the formulations, upon *in vivo* administration through physiological routes (19,21). This demonstrates that the presence of chitosan in the site of action as a dissolved form is not a critical issue to improve the therapeutic action of the carried molecules, although it might be decisive in the preparation of formulations. The degree of deacetylation of chitosan is a characteristic that affects its solubility (25), highly deacetylated chitosans (85%) being readily soluble in solutions with pH up to 6.5. It also plays a prominent role in its mucoadhesion properties. In this respect, highly deacetylated chitosans display increased mucoadhesivity, due to the presence of more positively charged amino groups available to mediate the interaction with the negatively charged mucus components (23). This explains that most scientific research reporting the pharmaceutical application of chitosan is performed with highly deacetylated chitosans. Apart from pH, ionic strength interferes with chitosan solubility too and, in this respect, higher ionic strength corresponds to lower solubility (23).

Chitosans administered as solutions by the pulmonary route are actually expected to precipitate, since the pH of the airway surface liquid is known to be approximately 7 (26). This has justified the proposal of a number of optimised chitosan presentations; either in the form of devices (nanoparticles, microparticles), which display better stability themselves and also provide increased stability to the carried therapeutic molecule, or as chitosan molecular derivatives, developed in order to achieve enhanced properties related to absorption promotion, transfection efficiency and better solubility at lung pH. These alternatives will be addressed in subsequent sections of this review.

3. Chitosan derivatives: an approach to increase the potential for drug delivery

Despite all the useful and promising properties displayed by chitosan, in certain applications it sometimes presents a few limitations related, for instance, to its solubility. As mentioned before, due to a pK_a around 6.5, chitosan is insoluble in water, although it is soluble in acidic solutions (13). Over the recent years, this limitation has led to the development of several chitosan derivatives which are water-soluble over a wider pH range, including physiological pH. These chemically-modified chitosans include, among others, N-trimethyl chitosan (27) and *N*-carboxymethyl chitosan (28), which present, respectively, trimethyl and carboxymethyl groups bonded to the amino function, and, less commonly, N-succinyl chitosan (29) and thiolated chitosan (30), displaying, respectively, succinyl and thiol groups attached to the chitosan amino groups. Many other derivatives have been developed in order to specifically improve chitosan performance in applications such as gene delivery, achieved by hydrophilic, hydrophobic, pH-sensitive, temperature-sensitive and specific ligand modifications (31). Hydrophilic modifications of the polymer, which include *grafting* of dextran, polyethylenglycol (PEG) or polyvinylpyrrolidone (PVP)

to chitosan, have rendered increased stability and longer circulation time to chitosan/DNA complexes due to the prevention of aggregation. Hydrophobic derivatives produced by binding N-dodecyl, deoxycholic acid, alkyl groups and stearic acid to the polymer or binding 5β-cholanic acid to glycol chitosan, have demonstrated to increase transfection efficiency, while N-dodecylated chitosan also improved the thermal stability of DNA (31). Urocanic acid-modified chitosan (32), chitosan-*graft*-polyethyleneimine and polypropyl acrylic acid-modified chitosan have all been described as approaches for pH-sensitive chitosan modification to enhance its transfection efficiency (31). Increased transfection efficiency was also achieved by coupling chitosan to a carboxyl-terminated N-isopropylacrylamide/vinyl laurate, derived from N-isopropylacrylamide which is a well known temperature-sensitive polymer (33). Finally, the same effect on transfection was described as a result of the interaction of chitosan with surfaces through specific ligands which were previously immobilised on the molecule, such as galactose, transferrin, folate and mannose, which interact with specific cell receptors (33).

In spite of the great number of chitosan derivatives described in the literature, only a few have been reported specifically in the field of pulmonary delivery and, along this section, we will focus on the properties of those derivatives reported to this end. The narrow list of applications might be explained by the fact that most chitosan derivatives have not been subject to extensive study with regard to their benefits and limitations in drug delivery so far and, possibly, it will be a matter of time until works with other derivatives for lung administration application become available.

N-trimethyl chitosan is a partially quarternised derivative of chitosan, prepared by reductive methylation of chitosan with methyl iodide in a strong basic environment at high temperature (27). This chitosan derivative provides higher water solubility over a broader pH range (11,27), since it presents trimethylated amino groups, the degree of methylation

depending on the reaction time and number of reaction steps (34). Moreover, it displays mucoadhesive character, as does the parent molecule, but a study demonstrated that it has lower intrinsic mucoadhesivity than chitosan hydrochloride or glutamate, due to conformational changes, which affect interpenetration in the mucus layer (35). *Polyplexes* (complexes of polymers with DNA), elaborated with this modified chitosan, have further proven to enhance gene transfection efficiency in A549 cells, an alveolar epithelial cell line, although inducing an increase in cytotoxicity, which can be overcome by using polyethyleneglycol-*graft*-trimethyl chitosan block copolymers (36). A hydrophobic derivative obtained by coupling stearic acid with a chitosan oligosaccharide, synthesised by an 1-ethyl-3-(3-simethylaminopropyl) carbodiimide-mediated coupling reaction, has also demonstrated ability to increase transfection efficiency in the same alveolar cell line, as a result of improved capacity for endosomal escaping. Moreover, in aqueous solution, this derivative forms micelle-like structures by self-aggregation and is used to form micelle/DNA complexes nanoparticles, which protect DNA from enzymatic degradation (37).

Trisaccharide-substituted chitosan oligomers were obtained by substituting the oligomers with the trisaccharide branch at their primary amines. *Polyplexes* formed with this derivative have shown increased transfection efficiency both *in vitro*, in the bronchial epithelial cell line 16HBE14o-, and *in vivo* upon lung administration to rats. This effect was attributed to the targeting of this derivative to cell-surface lectins (38).

Urocanic acid-modified chitosan is obtained by coupling water soluble chitosan with urocanic acid, via an active ester intermediate. Urocanic acid is composed of an imidazole ring and it binds to the chitosan amino groups through its carboxylic group (32, 39). This chitosan conjugate provides enhanced transfection efficiency because the imidazole ring of urocanic acid plays an important role in endosomal rupture, a limiting

step for DNA delivery into the nucleus. It was reported that urocanic acid displays buffering capacity in the endosomal compartment, leading to the entrapment of positively charged ions by the amines of imidazole rings, which, consequently, enhances osmolarity and, subsequently, results in endosomal rupture and escape to cytoplasm (39).

As can be easily understood, many efforts are being directed to the chemical modification of the original molecule of chitosan in order to obtain a polymer with enhanced properties. In fact, by means of chitosan modifications, its mucoadhesive properties can be enhanced, as can be improved its capacity to act as carrier for gene delivery, for instance, leading to improved results and better *in vivo* outcomes concerning therapeutic effect of the carried molecule.

The most important objective of researchers is still to design appropriate vehicles for specific therapeutic molecules, enabling their intact release as close as possible to the site of action, provided their protection from harmful conditions. However, safety issues of the developed derivatives should be considered and the risk-benefit of their application, as compared to the original molecules, remains to be seriously evaluated.

4. Benefits and limitations of chitosan for drug delivery to the lung

As previously mentioned in this review, biotechnological advances are responsible for the development of a large number of therapeutic macromolecules, which pose many challenges related to their administration. Most of these molecules are hydrophilic and have high molecular weight as compared to conventional drugs, features that limit their permeation across biological barriers and render them suitable to degradation in the body fluids.

Chitosan has been gaining increasing interest for the development of drug delivery systems that circumvent the limitations associated to the administration of these

macromolecules. In addition to the already cited biocompatibility, biodegradability and low toxicity of the polymer (8,16), which are all crucial in the design of drug carriers, chitosan also displays mucoadhesive properties (9) and the capacity to enhance absorption of macromolecules through mucosal barriers such as the nasal, intestinal, ocular, buccal and pulmonary (23). In fact, owing to its cationic character, chitosan is able to interact with the negatively charged sialic acid residues of membrane-bound glycoproteins, by means of electrostatic forces (40). Although this phenomenon takes place at the level of all the mucosal surfaces, this property is of particular importance in pulmonary administration, because it means that the administered drug delivery system will remain in the lung region until the turnover of the mucus layer, if it binds to the mucus layer, or it can even stay for a longer period of time, if it crosses the mucus layer and attaches to the epithelium cell membranes, thus attaining a prolonged drug effect.

In a study performed by Grenha et al., upon *in vitro* incubation of fluorescein isothiocyanate bovine serum albumin (FITC-BSA)-loaded chitosan nanoparticles with Calu-3 and A549 cells, models of the bronchial and alveolar epithelia, respectively, it was demonstrated that the nanoparticles adhered to the cells surface, even after thorough washing, therefore evidencing the biodhesive properties of nanoparticles (41).

One of the most prominent mechanisms of defence of the lung and, thus, one of the major concerns of scientists working in lung drug delivery, is the capture by alveolar macrophages, which has been reported to be strongly influenced by the particle size and material composition. However, as chitosan displays bioadhesion capacity, phagocytosis is delayed and this will, hence, allow the release of the carried therapeutic molecule (42). As easily understood from the mechanism of adhesion described above, the mucoadhesive character of chitosan will depend on its deacetylation degree. In this respect, a higher

deacetylation degree corresponds to the presence of more free amino groups and, thus, positive charges, which naturally enable greater adhesiveness (43).

Although the lung route is recognised as providing macromolecules permeation, the mean relative bioavailability of macromolecules as compared to subcutaneous injection does not usually exceed 25%, maintaining the challenge of improving pharmacokinetics (2). In fact, several agents such as bacitracin (protease inhibitor), sodium taurocholate (bile salt) and unsaturated fatty acids of oleic acid, among others, have been proposed to promote the lung permeation of buserelin, insulin and calcitonin, respectively (44). However, the use of these substances has raised safety concerns regarding long-term effects, because some of them were demonstrated to induce irreversible alterations on the alveolar epithelial cell layer (45). Chitosan has been proving a valuable approach to solve this limitation. Due to its bioadhesive character, chitosan has the ability to adhere to mucus and cell membranes, therefore remaining concentrated in the area of absorption and allowing drug release in the lung over a prolonged period of time (40,46). Moreover, it has been shown that chitosan transiently opens cells' tight junctions upon incubation with cell lines representative of the bronchial epithelium, such as Calu-3 and 16HBE14o- (47,48). This leads to a decrease of cells' transepithelial electrical resistance (TER), which is mediated by the interaction of the positively charged amino groups of chitosan with the negatively charged sialic acid groups of membrane-bound glycoproteins (40). This reversible disruption of the tight junctions enables an increased permeation of the released macromolecule, and is dependent on several factors such as chitosan concentration, molecular weight and deacetylation degree (23).

Chitosan has been described in the literature as biocompatible, biodegradable and non-toxic. However, few studies have focused on these subjects. In the same study in which a chitosan solution (pH 5.5) was found to induce a decrease of 30% in Calu-3 cell

viability upon 3h of incubation and to cause a strong inflammatory response (high levels of neutrophil infiltration in the lungs after *in vivo* administration), the 60% trimethylated chitosan derivative (TMC60) (pH 7.4) revealed mild or no effect in both cell viability and histological studies (48). In contrast, chitosan oligomer did not cause any membrane damage to lung tissue (as determined by leakage of lactate dehydrogenase in bronchoalveolar lavage) (49). According to *Huang et al.*, chitosan (213 KDa, 88%) solutions and nanoparticles exhibit similar cytotoxicity (50). However, we have demonstrated that chitosan in the form of nanoparticles (prepared by ionic gelation technique) has short-term biocompatibility with the respiratory cells Calu-3 and A549, not inducing significant toxicity at concentrations as high as 1 mg/mL, for up to 48 h (41).

The biological effects related to inflammation of rat lung upon contact with chitosan microparticles were examined (51), demonstrating that chitosan could induce proinflammatory responses in rat lung tissues in a dose-dependent manner, which is probably related to its cationic character. These effects were mild relative to those obtained with lipopolysaccharides and the doses tested were within the upper range of levels previously used in some therapeutic applications in which chitosan was used for pulmonary DNA delivery in mice (52). However, relatively higher doses of chitosan might be needed for delivery of other non-DNA therapeutic agents. Therefore, the main conclusion of this study is that these effects need to be considered in the context of therapeutic application via pulmonary delivery, especially if relatively high concentrations of chitosan are used.

Furthermore, the type of chitosan chosen is of high importance, since the factors referred to greatly influence chitosan toxicity are the type of salt, the molecular weight and, mainly, the deacetylation degree. In this respect, a higher degree of deacetylation of chitosan, which represents a higher positive zeta potential of the particles, was related to a

higher *in vitro* cytotoxicity (50). Accordingly, in this context, the use of less deacetylated chitosan could be desirable. With this idea in mind, we have recently tested the Calu-3 cells sensitivity to chitosans of different deacetylation degree (> 80%, 47% and 38%) in both proliferating and well-differentiated cells, observing significant differences. For instance, for a chitosan concentration of 1 mg/mL, cell viability in proliferating cells remained around 100% with respect to buffer control after 4 hours incubation, for both less deacetylated chitosans, whereas it was only 20% for the high deacetylated polymer. However, it must be mentioned that this marked difference was less pronounced in differentiated cells (41).

Toxicity can be decreased by combining chitosan with other excipients suitable for lung delivery, like cyclodextrins. In fact, chitosan-β-cyclodextrin microspheres constitute a biocompatible and safe pulmonary carrier, taking into account that they do not produce neither hemolysis nor toxicity to rat implantation, apart from no genetic toxicity (53).

In a comparative study, the lung toxicity of microspheres was compared to that of other materials by impinging the particles on Calu-3 monolayers and assessing the cytotoxicity, induction of cytokine release, changes in transepithelial permeability and electrical resistance. It was found that chitosan shows low toxicity, hence being particularly promising for systemic delivery in the lungs. Interestingly, one of the most commonly studied excipients for controlled release in the lungs, PLGA, has the highest toxicity of the polymers studied (54).

Therefore, concerns still remain regarding chitosan long-term safety, as well as its clearance from the lungs, and future studies are required to elucidate the factors that may influence chitosan biocompatibility. Nevertheless, it should be reminded that lysozyme mediates the hydrolysis of chitosan into non-toxic amino sugars, which are, subsequently, processed by the organism (55). This enzyme is widespread in the lung, being secreted 10-

20 mg/day (56), therefore ensuring the cleavage of chitosan units and enabling its elimination.

5. Chitosan-based formulations for pulmonary drug delivery

Recently, innovative delivery systems based on chitosan have been proposed for either local administration of i.e. anti-asthmatic drugs or systemic delivery of peptides and proteins, as well as for gene delivery (consult Table I). However, the application of the concepts of controlled and targeted delivery to the lungs is new and many concerns still remain regarding the safety and clearance of the polymeric carriers from the lungs.

Furthermore, the successful administration of many drugs and macromolecules is seriously compromised by the lack of specificity of the drug carrier towards a given target and, consequently, a portion of the administered dose will not be localised at the target site but remain therapeutically unavailable, while increasing the occurrence of side effects. Although the pulmonary epithelium is highly permeable, there is still the need to design drug delivery systems which provide the protection of the active molecule from the lung defence mechanisms, thus enabling stronger therapeutic effects *in vivo*.

Based on the previously commented advantages of chitosan for lung drug delivery, several chitosan-based liquid and powdered formulations (solutions, nanoparticles, microparticles) were developed, aimed at improving the lung performance of several macromolecules. Among them, the incorporation of drugs in polymeric particles, either nano- or micronsized, has been shown to provide effective delivery to the target site, while simultaneously preventing instability issues and, many times, enabling their controlled release, leading to an increased therapeutic benefit and reduction of side effects (45,57). The characterisation of these chitosan-based formulations in terms of their *in vitro*

performance, biocompatibility and pharmacological effect, will be addressed in this section.

5.1. *Chitosan solutions*

Chitosan solutions were rarely proposed as drug vehicles to the lung. A chitosan (93% deacetylation degree, MW 100-500 kDa) solution containing 190 μM of the peptide octreotide has proven to decrease the TER of Calu-3 cells *in vitro* about 50% and to enhance octreotide permeation by 21 fold, comparing to a control of the peptide in buffer. Upon its *in vivo* administration (0.97 mM) with the chitosan solution to rats, the protein bioavailability increased 2.4 fold comparing to the administration without chitosan and the authors described a linear *in vitro/in vivo* correlation (48). Since chitosan has a pK_a around 6.5, its stability is seriously compromised in media with pH above that value. The lung lining fluid is assumed to have a pH of approximately 7 (26) and, thus, it would not be surprising that chitosan precipitates upon contact with the lung environment. In order to circumvent this limitation, the chemical modification of chitosan is becoming common and *N*-trimethyl chitosan is one of the most used and studied chitosan derivatives (27), with free solubility at neutral and basic pH (please consult section 3 for a more detailed description).

TMC60 (60% trimethylated chitosan derivative) performs better than chitosan, reducing the cells TER by 65% and enhancing the permeation and in vivo bioavailability (30 fold and 3.9 fold, respectively) of the protein octreotide compared to the controls. Moreover, as it was previously indicated, TMC60 (pH 7.4) induced mild or no effects in both cell viability and histopathological studies (48).

In this section, it is necessary to mention the use of chitosan oligomers, which are obtained from the parent molecule by hydrolysis and shows great potential for improving

the lung absorption of bioactive peptides. In fact, a solution of chitosan hexamer (985 Da) containing interferon-α (IFN-α) (2×10^7 IU/mL) has proven more effective in enhancing the pulmonary absorption of the peptide than the chitosan polymer (MW = 96 kDa), achieving a maximum serum concentration (C_{max}) of 1006 IU/mL (hexamer) against 513 IU/mL of the parent polymer. Moreover, IFN-α relative bioavailability as compared to intramuscular injection was 17.8% for the hexamer and 8.5% for the original polymer. Furthermore, the oligomer did not cause any membrane damage to lung tissue, as determined by leakage of lactate dehydrogenase in bronchoalveolar lavage (49).

Stability issues, either concerning shelf-life or administration, are a major drawback in formulating drugs in solutions. When administering a solution of a drug, it should be considered that the molecule itself, unless otherwise mentioned, is completely exposed to the action of the physiological conditions of the route of administration and, in this particular case, of the lung environment. Solutions do not usually provide any protection of the molecule and do not allow for a controlled release. Therefore, other administration devices, such as microparticles and nanoparticles are gaining much popularity and have been proving over the last years that they are the more valuable candidates for the future of drug delivery.

5.2. *Chitosan microspheres*

The majority of the dry powder inhalation systems currently available consist in a micronized form of the active drug by itself or together with an excipient like lactose. However, recently, research work in this field has focused on the design and formulation of microspheres that can be tailored with the desired morphology (shape and porosity) and aerodynamic properties (size and density) for lung delivery (5). As it has been previously mentioned, these microspheres could be prepared using a wide range of synthetic and

naturally occurring polymers and materials and can offer efficient and controlled delivery, as well as protection of the encapsulated molecules. Microspheres produced from chitosan arise with a great potential as protein lung carriers. Besides, microspheres can allow the whole dose of loaded drug to reach the desired area, thus exhibiting increased therapeutic efficacy while reducing the number of needed doses and undesirable side effects. Traditionally, particles within 1 and 3 μm geometric diameter and density around 1 g/cm^3 were thought to be the most suitable for lung delivery, since significant loss due to impact and exhalation of large and small particles, respectively, were avoided (45). Later, in an attempt to overcome limitations of particle aggregation and rapid phagocytosis by alveolar macrophages, it was introduced a new and promising concept based on the design of large and light porous particles (58, 59), with a mass density of approximately 0.1 g/cm^3 and geometric diameter of > 5 μm, exhibiting an aerodynamic diameter which is smaller (< 5 μm) than their geometric diameter. Because of these features, particles can be aerosolized more efficiently, resulting in higher respirable fractions of the formulation, and can evade alveolar phagocytosis (60-62). Nevertheless, the fate of microspheres entering the lungs is dependent on the manufacturing material and technique and on the delivery device. Spray-drying is a very valuable technique for producing dry powders suitable for pulmonary delivery of drugs (63). Spray-drying of compounds of different compositions resulted in a change of particle morphology (64), which is of potential importance in the development of therapeutic powder aerosol formulation. The particle size of the obtained microparticles usually ranges from a few microns to several tens of microns and has a relatively narrow distribution. Recently, a number of articles have been published describing the preparation of chitosan microparticles intended for lung delivery by such spray-drying methods (58-62, 65-71).

We have produced chitosan microspheres with different morphological characteristics and adequate aerodynamic properties (aerodynamic diameter less than 5 μm and tap densities as low as 0.23 ± 0.02 g/cm^3) to reach and deposit in the deep lung (alveolar region), where the loaded protein is intended to be delivered and absorbed. Morphology and surface appearance of the chitosan microspheres as well as their densities and aerodynamic diameters were easily modulated by varying their composition (chitosan type and chitosan-glucomannan ratio) and polymer degree of deacetylation. These particles have a spherical shape and there is an evolution from the spherical shape to a convoluted surface as the chitosan deacetylation degree decreases or the content of the polysaccharide glucomannan increases. The application of this micron-sized system as lung protein carrier was investigated using insulin and fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) (protein association efficiencies of 90% - 100%) as model compounds. Preliminary *in vivo* results of the insulin-loaded microspheres are very encouraging, demonstrating that they induce a significant decrease and prolonged reduction of glucose levels (5). Our hypothesis was that, once in the absorption site, chitosan would improve pulmonary protein absorption by interacting with the epithelial cells, as had been previously reported for chitosan nanoparticles (19,20,22). Interestingly, crosslinked chitosan microspheres have been shown to be compatible with the hydrofluoralkane propellant P134a and, therefore, they are good candidates for lung delivery via pressurized metered dose inhalers (pMDI) (65).

However, when aerosolized alone, the forces generated within the dry powders can be insufficient to entrain the microparticles as a result of poor flowability and particle aggregation. Entrainment can be aided by the addition of a coarse carrier, such as lactose or mannitol (66). In addition to powder entrainment, one of the key factors that determine the fine particle faction is the ability of the inhaler to produce forces sufficient to

deagglomerate the microparticles from the coarse carrier. Therefore, Sivadas et al. produced by spray-drying similar FITC-BSA-loaded chitosan microparticles, with a geometric diameter of 5.49 μm, theoretical mass mean aerodynamic diameter of 2.91 μm, density of 0.28 g/cm^3 and with an encapsulation efficiency of 59.2%. Then, these microparticles were mixed with mannitol at a ratio of 24:1 (mannitol:microparticles) and the mannitol-blended microparticles were aerosolized through a multi-dose dry powder inhaler (DPI) using a multi-stage cascade impactor (Andersen cascade impactor) to investigate their *in vitro* deposition. Results showed that the fine particle fraction is approximately 15% and the mass mean aerodynamic diameter is 3.9 μm (54). The higher experimental mass mean aerodynamic diameter of the microparticles, compared to the theoretical one, could be explained by the incomplete powder deaggregation in the dry powder inhaler (67). Besides, chitosan microspheres display low toxicity, a controlled release pattern and improved systemic delivery and, hence, show a particular promise for systemic delivery to the lungs (54).

The spray-drying technique was also used to produce microspheres loaded with drugs for local effects, such as the glucocorticoid steroids betamethasone (68,69) and beclometasone dipropionate (70)**,** and the β_2-adrenergic agonists salbutamol (71) and terbutaline sulphate (70), used in the treatment of bronchial asthma. Bethametasone-loaded chitosan microspheres (encapsulation efficiencies up to 95%) containing type-A gelatine and ethylene oxide-propylene oxide block copolymer (Pluronic F68) as modifiers, were spherical and smooth and had a size distribution of 1-5 μm and a density of < 0.4 g/cm^3 (68,69). By properly choosing excipient type and concentration, a high degree of control was achieved over their physical properties (shape, size and surface morphology) and *in vitro* drug release. Microspheres had good stability, high entrapment efficiency and a positive surface charge (+37.5 mV) (68,69). As it was already mentioned in section 4,

betamethasone-loaded chitosan microspheres induced pro-inflammatory responses in rat lung tissues in a dose-dependent manner, which demonstrated to be mild as compared to other materials proposed for lung delivery, such as lipopolysaccharides (51) or poly(lactide-co-glycolide) (PLGA), this later being one of the most commonly studied excipients for controlled release to the lungs (54).

Chitosan microspheres containing β-cyclodextrin (chitosan/β-cyclodextrin microspheres) and loaded with theophylline (drug entrapment between 13 and 36%, yields and encapsulation efficiencies higher than 45%), have demonstrated to constitute a biocompatible and safe sustained release carrier for pulmonary delivery. The microspheres are spherical, with smooth or wrinkled surfaces, and hold low tap densities (0.34 - 0.48 g/cm^3) and appropriate aerodynamic diameters (2.20 - 3.04 μm) for pulmonary delivery. Besides, they have the ability to provide a sustained release of the drug, releasing 72% in 12 hours (53,72).

Chitosan was used as an emulsion stabilizer in the preparation of the PLGA, poly-ε-caprolactone (PCL) and combined PLGA + PCL microspheres. The polysaccharide not only imparts a positive charge to the surface of the microspheres, which is an important characteristic for mucosal absorption and translocation of particles through the mucosa, but is also able to increase their loading efficiency. Combined PLGA+PCL microspheres display substantially altered release kinetics, which may be useful in the delivery of the antitubercular drug rifampicin and other agents that may benefit from sustained and controlled delivery profiles (73). It is worthy to mention that several advantages have been proposed for administering antitubercular drugs in particulate lung carriers, including a reduction in the dose and frequency of administration, increasing patient compliance, as well as drug targeting to macrophages that harbor Mycobacterium tuberculosis. This enables to address the disease locally, thus allowing the attainment of high drug

concentrations and controlled release at the site of action, and reducing side effects usually accompanied with their systemic administration.

With the same purpose, rifampicin-loaded PLGA, chitosan and PLGA/chitosan microparticles were prepared using the technique of emulsion or precipitation, being compared for their stability during nebulisation. It was shown that the proper combination of both polymers enabled the formation of very stable PLGA/chitosan microspheres with high drug loading capacity and nebulisation ability. Moreover, encapsulation efficiency, percentage of drug collected in nebulised microparticles and nebulisation efficiencies were found to increase proportionally with the chitosan content. Cytotoxicity assays performed with these carriers, demonstrated lower cytotoxicity towards alveolar epithelial cells as compared to plain PLGA microspheres, and mucoadhesive properties similar to those found for chitosan microspheres were reported (74,75). Results generated by the referred study should be considered with particular attention in the design of colloidal formulations of other drugs, especially if increased mucoadhesion is desired. In fact, if not of particular interest for rifampicin-loaded microspheres, which are aimed for macrophage uptake, enhancement of the mucoadhesiveness of particles intended for delivery to the lungs through surface modification, may be required in other different cases. In line with this, the fact that PLGA/chitosan microspheres, which display the best stability profile during nebulization, have a positive surface charge and very good mucoadhesive properties, is indeed a considerable advantage for many therapeutic applications.

5.3. *Chitosan-based nanoparticles*

The administration of nanoparticles through the lung route presents a severe challenge owing to their small dimensions. In this sense, and taking into account previous

considerations made on particles/droplets size for lung delivery (see introduction), nanoparticles have to be administered as droplets or powdered particles incorporating a number of nanostructures. In both cases, apart from the nanoparticle development itself, there is a need to endow efforts as well on administration technologies and/or devices and all the stability/compatibility issues derived from that process. Maybe this is a valuable justification for the fact that there is a much fewer number of chitosan-based nanoparticles proposed for pulmonary delivery compared to microparticles.

We have reported the preparation of chitosan / tripolyphosphate (CS/TPP) nanoparticles via an extremely mild and rapid ionotropic gelation procedure with the counter-ion sodium TPP (76,77). These nanoparticles showed an excellent capacity for protein entrapment and an improvement of peptide absorption across mucosal surfaces, such as the nasal and ocular (19-21). Later on, the composition and structure of these nanopacrticles was adapted for different applications and administration routes. Hence, a new generation of safe hybrid nanoparticles consisting of chitosan and either anionic hyaluronic acid or cyclodextrin derivatives were developed intended for pulmonary and nasal delivery of peptides (78,79) and gene therapy (80). TEM microphotographs of the chitosan nanoparticles formulations are shown in Figure 2.

The rational behind the design of the chitosan/β-cyclodextrin (sulfobutylether-β-cyclodextrin and carboximethyl-β-cyclodextrin) nanocarriers was to combine the advantageous behaviour of chitosan nanoparticles with the excellent biopharmaceutical properties of cyclodextrins. Indeed, cyclodextrins are very well known in the pharmaceutical field because of their ability to protect drugs from physical, chemical and enzymatic degradation, and to enhance membrane permeability (81,82). Besides, they have been conjugated with polycationic polymers (polyamidine, polyethylenimine) and dendrimers, to form *polyplexes*, which elicit an increased transfection efficiency and

stability against enzymatic degradation with low *in vitro* and *in vivo* toxicity (82-85). The mechanism of formation of the chitosan/β-cyclodextrin nanoparticles nanosystems combines the electrostatic interaction between chitosan and cyclodextrins, which are oppositely charged, with the ability of chitosan to undergo a liquid–gel transition due to its ionic interaction with TPP.

Bearing all this information in mind, we hypothesised that the incorporation of cyclodextrins to the already effective chitosan-based gene delivery-nanocarriers could positively contribute to promote cellular uptake and decrease the cytotoxicity of the systems. In fact, in a Calu-3 cell culture model, chitosan/cyclodextrin nanoparticles were shown to cause a reversible reduction in the transepithelial resistance of the cell monolayer, thus increasing the membrane permeability (79) and to have the ability to enter epithelial cells and promote gene expression (80). These nanoparticles presented an adequate size range (100–200 nm, depending on chitosan molecular weight, MW), a positive surface charge (+22 to +35 mV) and very high DNA association efficiency (>90%). The ability of the nanoparticles to entrap pDNA was in agreement with the previous results obtained for other chitosan-based nanometric systems, and it can be easily explained by the high affinity of chitosan for the DNA (86, 87). Indeed, it is known that the strong electrostatic interaction exists between the phosphate groups of DNA and the amino groups of CS, as well as hydrophobic and hydrogen bonds (88).

The transfection efficiency of the different formulations, measured by the concentration of secreted gene product (SEAP) (plasmid DNA model that encodes the expression of secreted alkaline phosphatase) indicated that all the nanoparticles were able to elicit a significantly higher response than the naked DNA (control), the transfection efficiency being more important for low MW chitosan nanoparticles than for those composed of medium MW chitosan (Figure 3). Furthermore, these nanoparticles exhibited

a low toxicity. Considering all these observations, these nanocarriers represent a promising approach for gene therapy at the level of mucosal surfaces and, in particular, the respiratory mucosa (79, 80).

A mucoadhesive nanocarrier made from chitosan and hyaluronic acid and loaded with the macromolecular drug heparin was produced which was suitable for pulmonary delivery and use in antiasthmatic therapy (78). Hyaluronic acid is a naturally occurring hydrogel based on a linear polysaccharide comprised of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine, linked by β-1,4 and β-1,3 glycosidic bonds. Applications using hyaluronic acid for pulmonary controlled drug delivery are reported in literature (89). Chitosan and mixtures of hyaluronic acid with two unfractionated or low-molecular-weight heparins were combined to form nanoparticles by the ionotropic gelation technique. The resulting heparin-loaded nanoparticles were between 152 and 217 nm in size, their zeta potential ranged from +28.1 to +34.6 mV, and association efficiency was up to 70%. The nanosystems were stable in phosphate buffered saline pH 7.4 for at least 24 hours and were effectively internalized by rat mast cells. *Ex vivo* experiments aimed at evaluating the capacity of heparin to prevent histamine release in rat mast cells indicated that the free or encapsulated drug exhibited the same dose–response behaviour (78).

Using the same technique of ionic gelation, Huang et al. produced FITC-labelled CS/TPP nanoparticles of 200 nm with a positive zeta potential of approximately 36 mV, and investigated their uptake by the A549 cells. Results demonstrated that the nanoparticles uptake was concentration- and temperature-dependent, increasing with higher concentration and decreasing with lower temperatures (inhibition at 4 ºC compared to 37 ºC). Uptake of chitosan nanoparticles was 1.8 fold higher than that of chitosan solution (68). In a more recent study, the same authors demonstrated that chitosan MW and deacetylation degree (DD) affected the uptake efficiency by modulating the particles zeta

potential. In this respect, it was reported that the binding affinity and the uptake capacity decreased when decreasing chitosan MW and DD, the best performance being shown by the nanoparticles produced with chitosan 213 KDa and 88% deacetylated. Moreover, the authors found that chitosan solution and nanoparticles exhibit similar cytotoxicity (50).

Following a different approach**,** Yamamoto et al. produced PLGA nanospheres by nanoprecipitation (emulsion solvent diffusion) and included chitosan afterwards as a surface modifier by simply incubating the preformed nanospheres in a solution of the polymer. The developed nanoparticles, modified with chitosan, presented a size of approximately 650 nm and efficiently encapsulated elcatonin. Nanoparticles were assessed for their aerosolisation pattern using a nebuliser, resulting in aerosol droplets with a geometrical diameter of 6.5 μm, which led to a respirable fraction of 51%. The *in vivo* performance of these nanospheres was also evaluated upon nebulisation to guinea pigs. They evidenced a slower elimination rate from the lung than the unmodified particles and the presence of chitosan resulted in a prolonged and stronger hypocalcaemic effect of elcatonin, which was attributed to the mucoadhesive properties of chitosan, as well as to its ability to transiently open tight junctions (22).

It has already been established that spray drying constitutes a very valuable technique for producing dry powders adequate for pulmonary delivery of drugs (63,64). Therefore, we believed that the severe limitation of the pulmonary administration of chitosan nanoparticles due to their small size could be circumvented by using a spray-drying process to encapsulate them in microspheres, thus obtaining a dry powder which possesses adequate properties to reach the deep lung. In fact, protein-loaded chitosan nanoparticles were microencapsulated using the carbohydrate mannitol (90), which is a typical aerosol excipient approved by the Food and Drug Administration (FDA) and other regulatory organisms for inhalation purposes (67). SEM microphotographs shown in

Figure 4 reveal that the obtained microspheres are mostly spherical and possess appropriate aerodynamic properties for pulmonary delivery (aerodynamic diameters between 2 and 3 μm, apparent density lower than 0.45 g/cm^3). Moreover, their morphology was strongly affected by the nanoparticles content. Chitosan nanoparticles show a good protein loading capacity (65–80%), providing the release of 75–80% insulin within 15 min, and can be easily recovered from microspheres after contact with an aqueous medium with no significant changes in their size and zeta potential values.

Based on the previous considerations and acquired knowledge about the chitosan/TPP nanoparticles benefits when administered by other routes, we hypothesized that these microspheres, with an aerodynamic diameter between 1 and 5 μm, once inhaled, are expected to reach and deposit in the deep lung where the carrier carbohydrate will dissolve, delivering the nanoparticles, which will then promote the absorption of the associated therapeutic macromolecule. Importantly, mannitol was shown to act simply as inert carrier of the nanoparticles to the lung, as it completely dissolves upon *in vitro* incubation with an aqueous medium, as that found in the lung environment, releasing the nanoparticles without any significant changes in their physicochemical characteristics (300 nm and a positive zeta potential of + 34 mV) or the protein release profile (90).

Accurate analysis of the surface and inner structure of the system using confocal microscopy, X-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectrometry, revealed an even distribution of the nanoparticles through the whole microparticle (91). These chitosan nanoparticle-loaded microspheres were further demonstrated to be biocompatible with two different pulmonary cell lines of human origin, Calu-3 and A549 cells, which are representative of the bronchial and alveolar epithelium, respectively (Figure 5). In that study, nanoparticle concentration corresponding to amounts of chitosan as high as 1 mg/mL did not induce overt cell toxicity for up to 48 hours.

Furthermore, as mentioned in section 4, uptake studies suggested that nanoparticles were not internalised by the cells, but evidenced adhesion to the cell membranes (41).

In vivo studies (microencapsulated insulin-loaded chitosan nanoparticles containing 20% insulin and 80% mannitol) further indicated the suitability of the developed system. Upon intratracheal administration to anesthetised rats (16.7 IU insulin/kg) using a Harvard® ventilator that allows to simulate the physiological breathing mode of rats, the hypoglycemic response was compared with that of an insulin solution of the same doses. Curves depicted in Figure 6 demonstrate that microencapsulated insulin-loaded chitosan nanoparticles induce a strong and prolonged (up to 240 min.) hypoglycemic effect, and that the glucose level decreases to a greater extent as compared with that of control insulin (70% against 40% of the basal blood glucose at 60 min. post-administration). The novel dry powder carrier incorporating nanoparticles into a micron-scale structure has the advantage, when compared to liquid formulations, of improved stability and easiness of administration. In definitive, it allows overcoming the issues of storage and pulmonary administration of nanoparticles, and constitutes a very promising carrier for lung delivery of therapeutic proteins (92).

Encouraged by these results and given the favourable properties already commented of the hybrid nanocarriers (78-80), we are developing similar carriers composed of mannitol microspheres encapsulating chitosan-hyaluronic or chitosan-cyclodextrin nanoparticles. Nanoparticles and microspheres were produced using the same methods as before, ionic gelation and spray-drying, respectively. Both types of nanoparticles were spherical and compact structures (Figure 4) and displayed a size in the nanometric range and a positive zeta potential.

6. Main remarks

According to the aforementioned comments, it is clear that chitosan and its derivatives represent excellent materials to produce lung delivery carriers. Techniques such as ionotropic gelation and spray-drying are very useful in the preparation of chitosan nanoparticles and microspheres for this purpose. Special attention should be paid to the encouraging results related to the absorption of peptides and proteins using microencapsulated nanoparticles. However, many challenges have to be faced from now on biocompatibility and chitosan carriers will only be successfully administered through the lung when they have such properties that enable them to overcome the distinct barriers, including difficult accessibility, aerodynamic specificities and the mucociliary clearance. Whilst encouraging results may be achieved in cell culture and animal models, promising agents such as chitosan needs to be proven in clinical trial.

Acknowledgements:

Authors acknowledge funding from the European Union for the Nanobiosaccharides project (Ref. No.013882 FP6), Spanish Government (ISCIII, Acción Estratégica de Salud, PS09/00816), Xunta de Galicia (PGIDIT, 09CSA022203PR, NANOPULMOGENIC) and IBB/CBME, LA, FEDER/POCI 2010, and the predoctoral fellowship to S. Al-Qadi from the Spanish Agency of International cooperation for Development (MAEC-AECID).

References:

(1) TAYLOR G., KELLAWAY I. - Pulmonary drug delivery. - In: Drug delivery and targeting. For pharmacists and pharmaceutical scientists, A. Hillery, A. Lloyd A., J. Swarbrick Ed., Taylor & Francis, New York, 2001, pp. 269-300.

(2) COURRIER H.M., BUTZ N., VANDAMME T.F. - Pulmonary drug delivery systems: recent developments and prospects. - Critical Reviews in Therapeutic Drug Carrier Systems, **19**, 425-498, 2002.

(3) CLARK A. - Formulation of proteins and peptides for inhalation. - Drug Delivery Systems & Science, **2**, 73-77, 2002.

(4) PANDEY R., KHULLER G.K. - Antitubercular inhaled therapy: opportunities, progress and challenges. - Journal of Antimicrobial Chemotherapy, **55**, 430-435, 2005.

(5) GRENHA A., CARRIÓN-RECIO D., TEIJEIRO-OSORIO D., SEIJO B., REMUÑÁN-LÓPEZ C. - Nano- and microparticulate carriers for pulmonary drug delivery. - In: Handbook of Particulate Drug Delivery, Vol. 2 (Applications), M.N.V. Kumar Ed., American Scientific Publishers, Valencia, CA, 2008, pp.165-192.

(6) SUNG J.C, PULLIAM B.L, EDWARDS D.A. - Nanoparticles for drug delivery to the lungs. - Trends in Biotechnology, **25**, 563-570, 2007.

(7) JIANG W., KIM B.Y., RUTKA J.T., CHAN W.C. - Advances and challenges of nanotechnology-based drug delivery systems. - Expert Opinion in Drug Delivery, **4**, 621-633, 2007.

(8) HIRANO S., SEINO H., AKIYAMA Y., NONAKA I. - Biocompatibility of chitosan by oral and intravenous administrations. - Polymer Materials and Science Engineering, **59**, 897-901, 1988.

(9) LEHR C.M., BOUWSTRA J.A., SCHACHT E.H., JUNGINGER H.E. - In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. - International Journal of Pharmaceutics, **78**, 43-48, 1992.

(10) ISSA M., KOPING-HOGGARD M., ARTURSSON P. - Chitosan and the mucosal delivery of biotechnology drugs. - Drug Discovery Today: Technologies, **2**, 1-6, 2005.

(11) KURITA K. - Chitin and chitosan: functional biopolymers from marine crustaceans. - Marine Biotechnology, **8**, 203-226, 2006.

(12) MUZZARELLI R.A.A. - Chitin. - In: The polysaccharides, Aspinall G.O. Ed., Academic Press, Orlando, 1985, pp. 417-450.

(13) SINGH D.K., RAY A.R. - Biomedical applications of chitin, chitosan and their derivatives. - Reviews in Macromolecular Chemistry and Physics, **C40**, 69-83, 2000.

(14) MUZZARELLI R.A.A. - Human enzymatic activities related to the therapeutic administration of chitin derivatives. - Cellular and Molecular Life Sciences, **53**, 131-140, 1997.

(15) THARANATHAN R.N., KITTUR F.S. - Chitin - the undisputed biomolecule of great potential. - Critical reviews in food science and nutrition, **43**, 61-87, 2003.

(16) DORNISH M., HAGEN A., HANSSON E., PEUCHEUR C., VEDIER F., SKAUGRUD O. - Safety of Protasan™: Ultrapure chitosan salts for biomedical and pharmaceutical use. - In: Advances in chitin science., A. Domard, G.A.F. Roberts, K.M., Varum Ed., Jacques Andre publisher, Lyon, 1997, pp. 664-670.

(17) ARTURSSON P., LINDMARK T., DAVIS S.S., ILLUM L. - Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). - Pharmaceutical Research, **11**, 1358-1361, 1994.

(18) BORCHARD G., LUEβEN H.L., DE BOER G.A., VERHOEF J.C., LEHR C.M., JUNGINGER H.E. - The potential of mucoadhesive polymers in enhancing intestinal

peptide drug absorption III: Effects of chitosan glutamate and carbomer on epithelial tight junctions in vitro. - Journal of Controlled Release, **39**, 131-138, 1996.

(19) FERNÁNDEZ-URRUSUNO R., CALVO P., REMUÑÁN-LÓPEZ C., VILA-JATO J.L., ALONSO M.J. - Enhancement of nasal absorption of insulin using chitosan nanoparticles. - Pharmaceutical Research, **16**, 1576-1581, 1999.

(20) FERNÁNDEZ-URRUSUNO R., ROMANÍ D., CALVO P., VILA-JATO J.L., ALONSO M.J. - Development of a freeze-dried formulation of insulin-loaded chitosan nanoparticles intended for nasal administration. - S T P Pharma Sciences, **9**, 429-436, 1999.

(21) DE CAMPOS A., SÁNCHEZ A., ALONSO M.J. - Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. - International Journal of Pharmaceutics, **224**, 159-168, 2001.

(22) YAMAMOTO H., KUNO Y., SUGIMOTO S., TAKEUCHI H., KAWASHIMA Y. - Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. - Journal of Controlled Release. - Journal of Controlled Release, **102**, 373-381, 2005.

(23) PREGO C., GARCÍA-FUENTES M., TORRES D., ALONSO M.J. - Transmucosal molecular drug delivery. - Journal of Controlled Release, **101**, 151-162, 2005.

(24) PREGO C., TORRES D., ALONSO M.J. The potential of chitosan for the oral administration of peptides. - Expert Opinion in Drug Delivery, **2**, 843-854 (2005)**.**

(25) CHO Y.W., JANG J., PARK C.R., KO S.W. Preparation and solubility in acid and water of partially deacetylated chitins. - Biomacromolecules, **1**, 609-614, 2000.

(26) WALTERS D.V. Lung lining liquid - The hidden depths. - Biology of the Neonate, **81** (Supp. 1), 2-5, 2002.

(27) SIEVAL A.B., THANOU M., KOTZÉ A.F., VERHOEF J.C., BRUSSEE J.C., JUNGINGER H.E. - Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride. - Carbohydrate Polymers, **36**, 157-165, 1998.

(28) LE DUNG P., MILAS P., RINAUDO M., DESBRIÈRES J. - Water soluble derivatives obtained by controlled chemical modifications of chitosan. - Carbohydrate Polymers, **24**, 209-214, 1994.

(29) KATO Y., ONISHI H., MACHIDA Y. - N-succinyl-chitosan as a drug carrier: water-insoluble and water-soluble conjugates. - Biomaterials, **25**, 907-915, 2004.

(30) BERNKOP-SCHNÜRCH A., GUGGI D., PINTER Y. - Thiolated chitosan: development and in vitro evaluation of a mucoadhesive, permeation enhancing oral drug delivery system. - Journal of Controlled Release, **94**, 177-186, 2004.

(31) KIM T. H., JIANG H.L., JERE D., PARK I.K., CHO M.H., NAH J.W., CHOI, Y.J., AKAIKE T., CHO C.S. - Chemical modification of chitosan as a gene carrier *in vitro* and *in vivo*. - Progress in Polymer Science, **32**, 726-753, 2007.

(32) JIN H., KIM T.H., HWANG S.K., CHANG S.H., KIM S.K., CHANG S.H., KIM H.W., ANDERSON H.K., LEE H.W., LEE K.H., COLBURN N.H., YANG H.S., CHO M.H., CHO M.H., CHO C.S. - Aerosol delivery of urocanic acid-modified chitosan/programmed cell death 4 complex regulated apoptosis, cell cycle, and aniogenesis in lungs of K-*ras* null mice. - Molecular Cancer Therapy, **5**, 1041-1049, 2006.

(33) SUN S., LIU W., CHENG N., ZHANG B., CAO Z., YAO K., LIANG D., ZUO A., GUO G., ZHANG J. - A thermoresponsive chitosan-NIPAAm/vinyl laurate copolymer vector for gene transfection. - Bioconjugate Chemistry, **16**, 972-980, 2005.

(34) MERWE S.M.V.D., VERHOEF J.C., VERHEIJDEN J.H.M., KOTZÉ A.F., JUNGINGER H.E. - Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs. - European Journal of Pharmaceutics and Biopharmaceutics, **58**, 225-235, 2004.

(35) SNYMAN D., HAMMAN J.H., KOTZÉ A.F. - Evaluation of the mucoadhesive properties of *N*-trimethyl chitosan chloride. - Drug Development and Industrial Pharmacy, **29**, 61-69, 2003.

(36) GERMERSHAUS O., MAO S., SITTERBERG J., BAKOWSKY U., KISSEL T. - Gene delivery using chitosan, trimethyl chitosan or polyethyleneglycol-*graft*-trimethyl chitosan block copolymers: establishment of structure-activity relationships *in vitro*. - Journal of Controlled Release, **125**, 145-154, 2007.

(37) HU F., ZHAO M., YUAN H., YOU J., DU Y., ZENG S. - A novel chitosan oligosaccharide-stearic acid micelles for gene delivery: properties and *in vitro* transfection studies. - International Journal of Pharmaceutics, **315**, 158-166, 2006.

(38) ISSA M.M., KÖPING-HÖGGARD M., TØMMERAAS K., VARUM K.M., CHRISTENSEN B.E., STRAND S.P., ARTURSSON P. - Targeted gene delivery with trisaccharide-substituted chitosan oligomers *in vitro* and after lung administration *in vivo*. - Journal of Controlled Release, **115**, 103-112, 2006.

(39) KIM T.H., IHM J.E., CHOI Y.J., NAH J.W., CHO C.S. - Efficient gene delivery by urocanic acid-modified chitosan. - Journal of Controlled Release, **93**, 389-402, 2003.

(40) WEERS J.G., TARARA T.E., CLARK A.R. - Design of fine particles for pulmonary drug delivery. - Expert Opinion in Drug Delivery, **4**, 297-313, 2007.

(41) GRENHA A., GRAINGER C.I., DAILEY L.A., SEIJO B., MARTIN G.P., REMUÑÁN-LÓPEZ C., FORBES B. - Chitosan nanoparticles are compatible with respiratory epithelial cells *in vitro*. - European Journal of Pharmaceutical Sciences, **31**, 73-84, 2007.

(42) YAMAMOTO H, KUNO Y, SUGIMOTO S, TAKEUCHI H, KAWASHIMA Y. - Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. - Journal of Controlled Release, **102**, 373-381, 2005.

(43) HE P., DAVIS S.S., ILLUM L.. - *In vitro* evaluation of the mucoadhesive properties of chitosan microspheres. - International Journal of Pharmaceutics, **166**, 75-88, 1998.

(44) HUSSAIN A., ARNOLD J.J., KHAN M.A., AHSAN F. - Absorption enhancers in pulmonary protein delivery. - Journal of Controlled Release, **94**, 15-24, 2004.

(45) CRYAN S.A. - Carrier-based strategies for targeting protein and peptide drugs to the lungs. - AAPS Journal, **7**, E20-E41, 2005.

(46) TAKEUCHI H., YAMAMOTO H., KAWASHIMA Y. -Mucoadhesive nanoparticulate systems for peptide drug delivery. - Advanced Drug Delivery Reviews, **47**, 39-54, 2001.

(47) LIM S.T., FORBES B., MARTIN G.P., BROWN M.B. - In vivo and in vitro characterization of novel microparticulates based on hyaluronan and chitosan hydroglutamate. - AAPS PharmsciTech., **2**, 14, 2001.

(48) FLOREA B.I., THANOU M., JUNGINGER H.E., BORCHARD G. - Enhancement of bronchial octreotide absorption by chitosan and *N*-trimethylchitosan shows linear in vitro/in vivo correlation. - Journal of Controlled Release, **110**, 353-361, 2006.

(49) YAMADA K., ODOMI M., OKADA N., FUJITA T., YAMAMOTO A. - Chitosan oligomers as potential and safe absorption enhancers for improving the pulmonary absorption of Interferon-a in rats. - Journal of Pharmaceutical Sciences, **94**, 2432-2440, 2005.

(50) HUANG M., KHOR E., LIM L.Y. - Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. - Pharmaceutical Research, **21**, 344-353, 2004.

(51) HUANG Y.C., VIEIRA A., HUANG K.L., YEH M.K., CHIANG C.H. J. Pulmonary inflammation caused by chitosan microparticles. - Biomedical Materials Research, **75A**, 283 - 287 (2005).

(52) BIVAS-BENITA M., VAN MEIJGAARDEN K.E., FRANKEN K.L.M.C., JUNGINGER H.E., BORCHARD G., OTTENHOFF T.H.M., GELUK A. - Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis. - Vaccine, **22**, 1609-1615, 2004.

(53) ZHANG W. F., ZHOU H.Y., CHEN X.G., TANG S.H., ZHANG J.J. - Biocompatibility study of theophylline-cyclodextrin microspheres as pulmonary delivery carriers. - Journal of Materials Science: Materials in Medicine, **20**, 1321-1330, 2009.

(54) SIVADAS N., O'ROURKE D., TOBIN A., BUCKLEY V., RAMTOOLA Z., KELLY J.G., HICKEY A.J., CRYAN S-A. - A comparative study of a range of polymeric microspheres as potential carriers for the inhalation of proteins. - International Journal of Pharmaceutics, **358**, 159-167 (2008).

(55) SINGH DK, RAY AR.- Biomedical applications of chitin, chitosan and their derivatives. - Reviews in Macromolecular Chemistry and Physics, **C40,** 69-83, 2000.

(56) KONSTAN M.W., CHEN P.W., SHERMAN J.M., THOMASSEN M.J., WOODM R.E., BOAT T.F. - Human lung lysozyme: sources and properties. - American Review of Respiratory Disease, **123**, 120-124, 1981.

(57) DAI L. - Polymer nanostructures. - In: Encyclopedia of nanoscience and nanotechnology, H.S. Nalwa Ed., American Scientific Publishers, 2004, pp. 763-769.

(58) EDWARDS D.A, BEN-JEBRIA A., LANGER R.- Recent advances in pulmonary drug delivery using large, porous inhaled particles. - Journal of Applied Physiology, **84**, 379-385, 1998.

(59) EDWARDS D.A., HANES J., CAPONETTI G., HRKACH J., BEN-JEBRIA A., ESKEW M.L., MINTZES J., DEAVER D., LOTAN N., LANGER R. - Large Porous Particles for Pulmonary Drug Delivery. - Science, **276**, 1868-1871, 1997..

(60) BEN-JEBRIA A., CHEN D., ESKEW M.L., VANBEVER R., LANGER R., EDWARDS D.A. - Large porous particles for sustained protection from carbachol-induced bronchoconstriction in guinea pigs - Pharmaceutical Research, **16**, 555-61, 1999.

(61) VANBEVER R., MINTZES J., WANG J., NICE J., CHEN D., BATYCKY R., LANGER R., EDWARDS, D.A. - Formulation and physical characterization of large porous particles for inhalation. - Pharmaceutical Research, **16**, 1735-1742, 1999.

(62) DUNBAR C., SCHEUCH G., SOMMERER K., DELONG M., VERMA A., BATYCKY, R.- In vitro and in vivo dose delivery characteristics o large porous particles for inhalation. - International Journal of Pharmaceutics, **245**, 179-189, 2002.

(63) SEVILLE P.C., LI H-Y. LEAROYD T.P. - Spray-Dried Powders for Pulmonary Drug Delivery. - Critical Reviews™ in Therapeutic Drug Carrier Systems, **24,** 307-360, 2008.

(64) VEHRING R. - Pharmaceutical Particle Engineering via Spray Drying (Expert Review). - Pharmaceutical Research, **25**, 9999-1022, 2008.

(65) WILLIAMS R.O., BARRON M.K., ALONSO M.J., REMUÑAN-LOPEZ C. - Investigation of a pMDIsystem containing chitosan microspheres and P134a. - International Journal of Pharmaceutics, **174**, 209-222, 1998.

(66) CHAN H.K., CLARK A., GONDA I., MUMENTHALER M., HSU C. - Spray dried powders and powder blends of recombinant human deoxyribonuclease (rhDNase) for aerosol delivery. - Pharm. Respir., **14**, 431-437, 1997.

(67) BOSQUILLON C., LOMBRY C., PREAT V., VANBEVER R. - Influence of formulation excipients and physical characteristics of inhalation dry powders on their aerosolization performance. - Journal of Controlled Release, **70**, 329-339, 2001.

(68) HUANG Y.C., YEH M.K., CHIANG C.H - Formulation factors in preparing BTM-chitosan microspheres by spray drying method. - International Journal of Pharmaceutics, **242**, 239-242, 2002.

(69) HUANG Y.C., YEH M.K., CHENG S.N., CHIANG C.H. - The characteristics of betamethasone-loaded chitosan microparticles by spray-drying method. - Journal of Microencapsulation, **20**, 459-472, 2003.

(70) LEAROYD T.P., BURROWS J.L., FRENCH E., SEVILLE P.C. - Sustained delivery by leucine-modified chitosan spray-dried respirable powders. - International Journal of Pharmaceutics, **372,** 97–104 (2009).

(71) CORRIGAN D.O., HEALY A.M., CORRIGAN O.I. - Preparation and release of salbutamol from chitosan and chitosan co-spray dried compacts and multiparticulates. - European Journal of Pharmaceutics and Biopharmaceutics, **62**, 295-305, 2006.

(72) ZHANG W., CHEN X., LI P., HE Q., ZHOU H., CHA D.- Chitosan and β-cyclodextrin microspheres as pulmonary sustained delivery systems. - Journal of Wuhan University of Technology, Materials Science Edition, **23**, 541-546, 2008.

(73) PANDIT S., MARTIN, C., ALPAR, H.O. - Positively charged rifampicin-based microspheres for lung delivery. - Journal of Drug Delivery Science and Technology, **15**, 281-287, 2005.

(74) MANCA M.L., MOURTAS S., DRACOPOULOS V., FADDA A.M., ANTIMISIARIS S.G. - PLGA, chitosan or chitosan-coated PLGA microparticles for alveolar delivery? A comparative study of particle stability during nebulization. - Colloids and Surfaces B: Biointerfaces, **62**, 220-231, 2008.

(75) MANCA M.L., LOY G., ZARU M., FADDA A.M., ANTIMISIARIS S.G. - Release of rifampicin from chitosan, PLGA and chitosan-coated PLGA microparticles. - Colloids and Surfaces B: Biointerfaces, **67**, 166-170, 2008.

(76) CALVO P., REMUÑAN-LOPEZ C., VILA-JATO J.L., ALONSO M.J. - Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. - Journal of Applied Polymer Science, 63, 125.132, 1998.

(77) CALVO P., REMUÑÁN-LÓPEZ C., VILA-JATO J.L., ALONSO M.J. - Chitosan and Chitosan/Ethylene Oxide-Propylene Oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. - Pharmaceutical Research, **14**, 1431-1436, 1997.

(78) OYARZUN-AMPUERO F.A., BREA J., LOZA M.I., TORRES D., ALONSO M.J. - Chitosan – hyaluronic acid nanoparticles loaded with heparin for the treatment of asthma. - International Journal of Pharmaceutics, **381**, 122-129, 2009.

(79) TEIJEIRO-OSORIO D., REMUNÁN-LÓPEZ C., ALONSO M.J. - New Generation of Hybrid Poly/Oligosaccharide Nanoparticles as Carriers for the Nasal Delivery of Macromolecules. - Biomacromolecules, **10**, 243-249, 2009.

(80) TEIJEIRO-OSORIO D., REMUNÁN-LÓPEZ C., ALONSO M.J. - Chitosan / cyclodextrin nanoparticles can efficiently transfect the airway epithelium in vitro. - European Journal of Pharmaceutics and Biopharmaceutics, **71**, 257-263, 2009.

(81) CRYAN S.A., HOLOHAN A., DONOHUE R., DARCY R., O´ DRISCOLL C.M. - Cell transfection with polycationic cyclodextrin vectors. - European Journal of Pharmaceutical Sciences, **21**, 625-633, 2004.

(82) CHALLA R., AHUJA A., ALI J., KHAR R.K. - Cyclodextrins in drug delivery: an updated review - AAPS PharmSciTech, **6**, E329–E357, 2005

(83) ARIMA H., KIHARA F., HIRAYAMA F., UEKEMA K. - Enhancement of gene expression by polyamidoamine dendrimer conjugates with a-, b-, and c-cyclodextrins. - Bioconjugate Chemistry, **12**, 476–484, 2001.

(84) HWANG S.J., BELLOCQ N.C., DAVIS M.E. - Effects of structure of b-cyclodextrincontaining polymers on gene delivery - Bioconjugate Chemistry, **12**, 280-290, 2001.

(85) FORREST M.L., GABRIELSON N., PACK D.W. - Cyclodextrin-polyethylenimine conjugates for targeted in vitro gene delivery. - Biotechnology and Bioengineering, **89**, 416-423, 2005.

(86) CSABA N., KOPING-HOGGARD M., FERNÁNDEZ-MEJÍA E., NOVOA-CARBALLAL R., RIGUERA R., ALONSO M.J. - Ionically crosslinked chitosan nanoparticles as gene delivery systems: effect of PEGylation on in vitro and in vivo gene transfer.- Journal of Biomedical Nanotechnology, 5, 162-1671, 2009.

(87) DE LA FUENTE M., SEIJO B., ALONSO M.J. - Design of novel polysaccharidic nanostructures for gene delivery. - Nanotechnology, **19**, 0751065 (9 p), 2008.

(88) LI X.W., LEE D.K.L., CHAN A.S.C., ALPAR H.O. - Sustained expression in mammalian cells with DNA complexes and chitosan nanoparticles. - Biochim. Biophys. Acta, **1630,** 7–18 (2003).

(89) SURENDRAKUMAR K., MARTIN G.P., HODGERS E.C.M., JANSEN M., BLAIR, J.A. - Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs. - Journal of Controlled Release, **91**, 385-394, 2003.

(90) GRENHA A., SEIJO B., REMUÑAN-LOPEZ C. - Microencapsulated chitosan nanoparticles for lung protein delivery. - European Journal of Pharmaceutical Sciences, **25**, 427-437, 2005.

(91) GRENHA A., SEIJO B., SERRA C., REMUÑÁN-LÓPEZ C. - Chitosan nanoparticle-loaded mannitol microspheres: structure and surface characterisation. - Biomacromolecules, **8**, 2072-2079, 2006.

(92) AL-QADI S., GRENHA A., SEIJO B. GOYCOOLEA F., ALONSO M.J., REMUÑAN-LÓPEZ C.- Chitosan nanopaticle-based inhalable dry-powders for protein lung delivery: In vivo evaluation of microencapsulated insulin-loaded nanoparticles in rats.- PO2-22 - EUCHIS 2009 - 9th International Conference of the European Chitin Society, Venice, Italy 23-26 May 2009.

Table I – Examples of chitosan-based formulations and molecules under investigation.

Chitosan formulation	Molecule	Ref.
Chitosan solutions		
Chitosan, 60% N-trimethyl chitosan (TMC60)	Octreotide	(48)
Chitosan oligomer (hexamer, 985Da)	Interferon-α	(49)
Chitosan microspheres		
Chitosan	Insulin	(5)
	Fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA)	(5)
Chitosan	Bethametasone	(68,69)
	Beclometasone dipropionate	(70)
	Salbutamol	(71)
	Terbutaline sulphate	(70)
Chitosan/β-cyclodextrin	Theophylline	(53,72)
Chitosan, chitosan/PLGA, chitosan /PCL	Rifampicin	(73)
Chitosan nanoparticles		
Chitosan/tripolyphosphate	Insulin	
Chitosan/hyaluronic acid/tripolyphosphate	Heparin	(78)
Chitosan/-β-cyclodextrin/tripolyphosphate	Plasmid DNA model	(80)
Chitosan-coated PLGA nanoparticles	Elcatonin	(22)
Microencapsulated chitosan nanoparticles	Insulin	(90)

FIGURE CAPTIONS

Figure 1 – Chemical structure of chitosan.

Figure 2 – TEM microphotographs of chitosan nanoparticles produced with different compositions: (a) chitosan/tripolyphosphate, (b) chitosan/hyaluronic acid/ tripolyphosphate and (c) chitosan/carboxymethyl-β-cyclodextrin/tripolyphosphate nanoparticles.

Figure 3 - Secreted alkaline phosphatase (SEAP) concentration in the Calu-3 cell culture model after transfection with two different nanoparticle formulations: (▲) chitosan/sulfobutylether-β-cyclodextrin nanoparticles and (■) chitosan/carboximethyl-β-cyclodextrin/tripolyphosphate nanoparticles encapsulating pDNA; (○) control naked pDNA (means ± SD, n = 5) [Reprinted from (80) (Eur. J. Pharm. Biopharm., 71, Teijeiro-Osorio et al., Chitosan/cyclodextrin nanoparticles can efficiently transfect the airway epithelium in vitro, 257-263, 2009) with permission from Elsevier].

Figure 4 - SEM microphotographs of mannitol microspheres containing: (a) chitosan / tripolyphosphate nanoparticles; (b) chitosan/hyaluronic acid / tripolyphosphate nanoparticles and (c) chitosan / carboxy methyl-β-cyclodextrin nanoparticles.

Figure 5 – A549 cell viability measured by MTT cytotoxicity assay after 24h exposure to increasing concentrations of the (◇) nanoparticle suspension and nanoparticle-containing mannitol microspheres with nanoparticle:mannitol ratios of (♦) 90/10; (■) 80/20 and (▲) 60/40. Data represent mean ± S.E.M. (*n* = 3 experiments, six replicates per experiment at each test concentration) [Adapted from (41) (Eur. J. Pharm. Sci., 31, Grenha et al.,

Chitosan nanoparticles are compatible with respiratory epithelial cells in vitro, 73-84, 2007) with permission from Elsevier].

Figure 6 - Hypoglycemic effect following intratracheal administration to rats of (■) microencapsulated insulin-loaded chitosan nanoparticles; (●) control insulin solution in PBS pH 7.4; and (▲) control microencapsulated unloaded chitosan nanoparticles (insulin doses = 16.7 U/Kg; $n \geq 3$) [Adapted from (92) (PO2-22 - EUCHIS 2009, Al-Qadi et al., Chitosan nanopaticle-based inhalable dry-powders for protein lung delivery, 2009)].

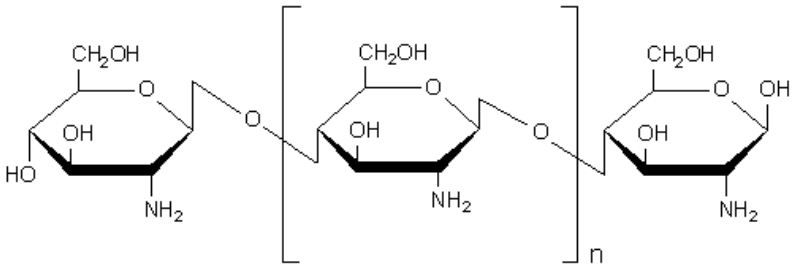

Figure 1

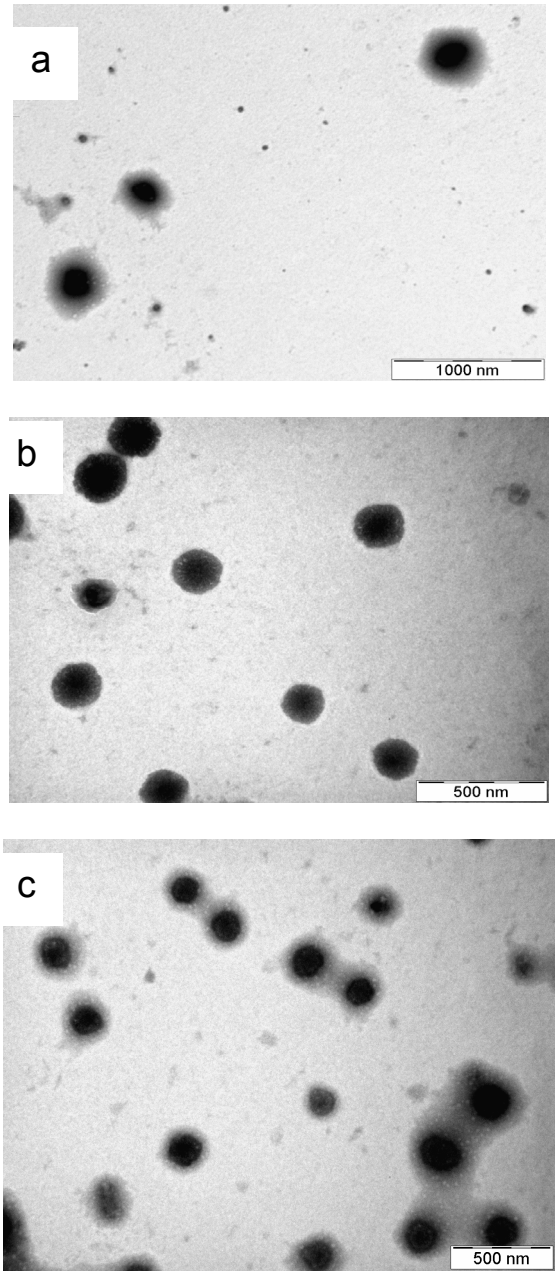

Figure 2

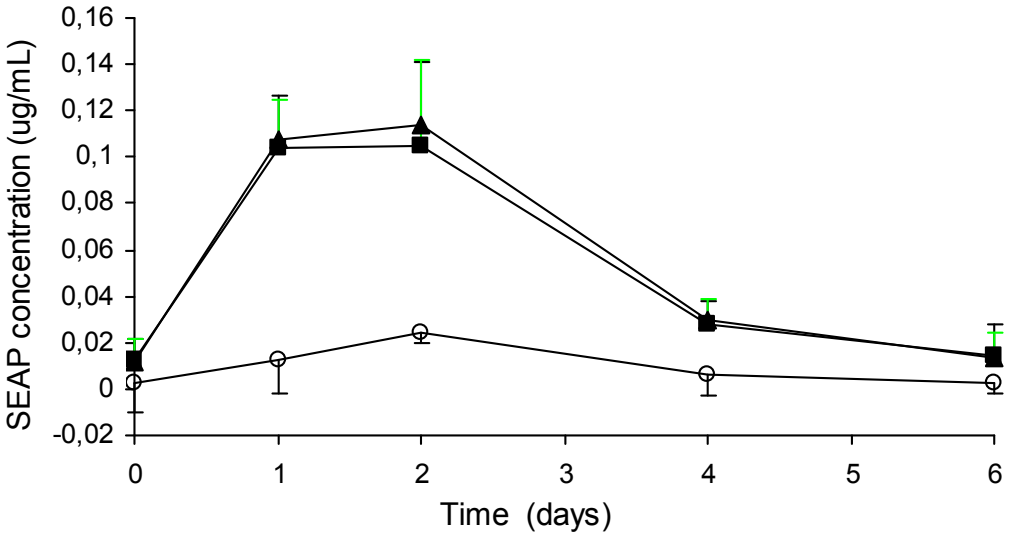
SEAP concentration (ug/mL)
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
-0,02
0
1
2
3
4
5
6
Time (days)

Figure 3

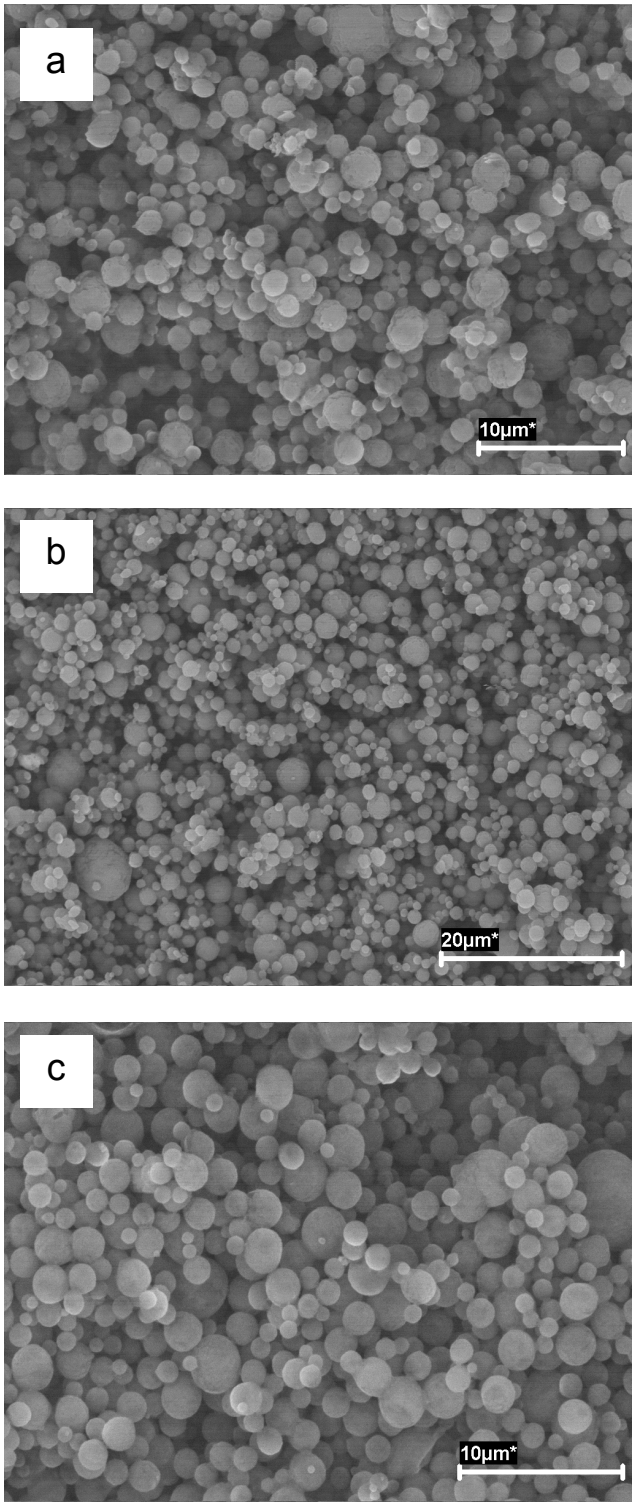

Figure 4

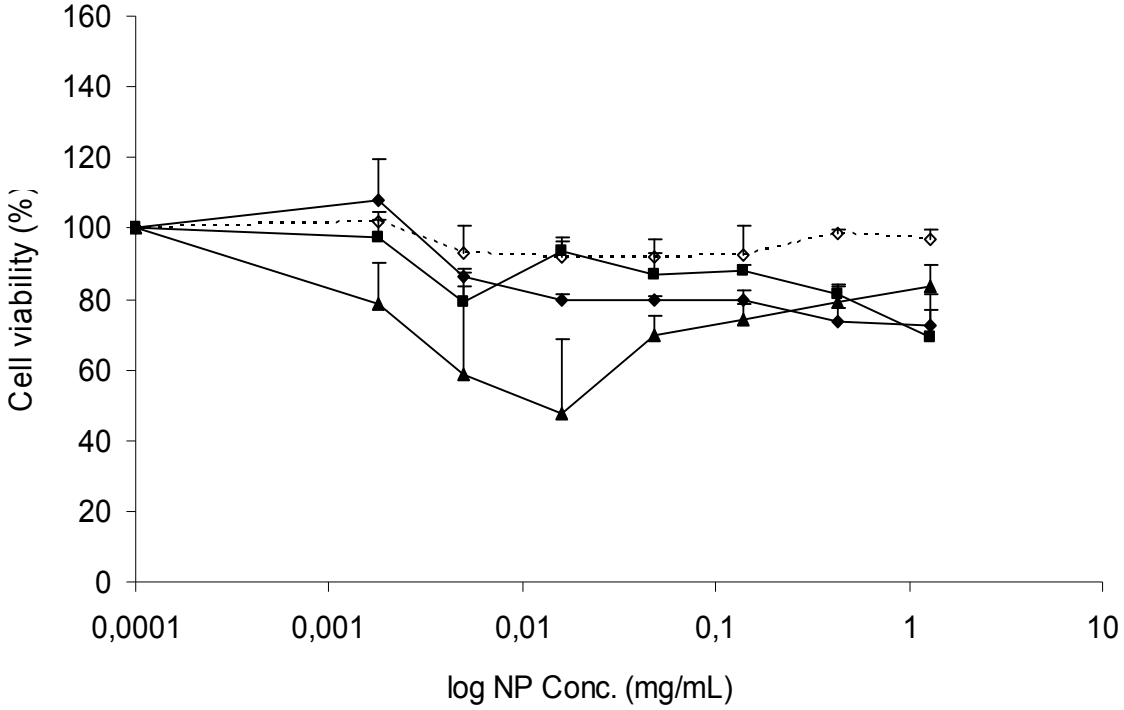
160
140
120
100
80
60
40
20
0
Cell viability (%)
0,0001
0,001
0,01
0,1
1
10
log NP Conc. (mg/mL)

Figure 5

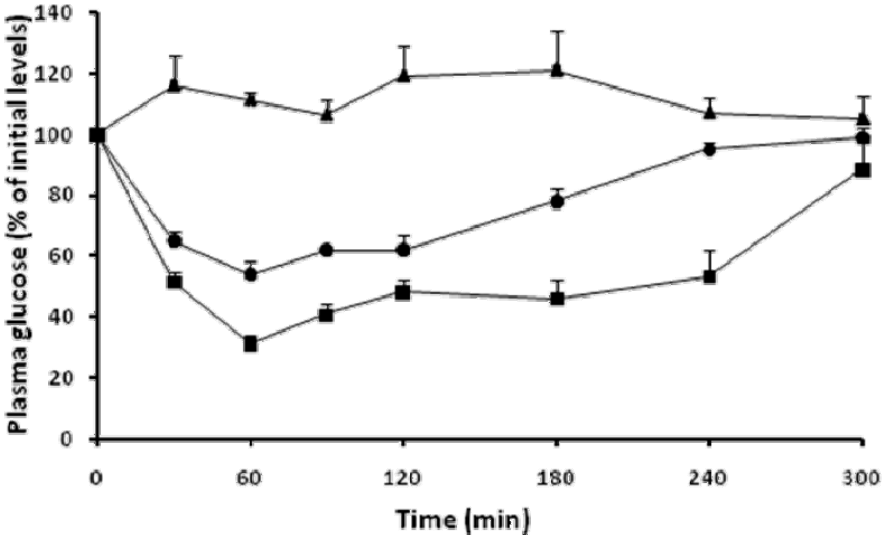
Plasma glucose (% of initial levels)
140
120
100
80
60
40
20
0
0
60
120
180
240
300
Time (min)

Figure 6