# Insulin-loaded polymeric mucoadhesive nanoparticles: development, characterization and cytotoxicity evaluation

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Mucoadhesive nanoparticles are particularly interesting for delivery through nasal or pulmonary routes, as an approach to overcome the mucociliary clearance. Moreover, these nanoparticles are attractive for peptide and protein delivery, particularly for insulin to treat diabetes, as an alternative to conventional parenteral administration. Thus, chitosan, a cationic mucoadhesive polysaccharide found in shells of crustaceans, and the negatively-charged dextran sulfate are able to form nanoparticles through ionic condensation, representing a potential insulin carrier. Herein, chitosan/dextran sulfate nanoparticles at various ratios were prepared for insulin loading. Formulations were characterized for particle size, zeta potential, encapsulation efficiency, scanning electron microscopy, differential scanning calorimetry, and *in vitro* drug release. Moreover, the interaction with mucin and the cytotoxicity against a lung cell line were studied, which altogether have not been addressed before. Results evidenced that a proper selection of polyelectrolytes is necessary for smaller particle size formation and also the composition and zeta potential impact encapsulation efficiency, which is benefited by the positive charge of chitosan. Insulin remained stable after encapsulation as evidenced by calorimetric assays, and was released in a sustained manner in the first 10 h. Positively-charged nanoparticles based on chitosan/dextran-sulfate at the ratio of 6:4 successfully interacted with mucin, which is a prerequisite for delivery to mucus-containing tissues. Finally, insulin-loaded nanoparticles displayed no cytotoxicity effect against lung cells at tested concentrations, suggesting the potential for further *in vivo* studies.

**Keywords**: Insulin. Chitosan. Dextran-Sulfate. Nanoparticles. Mucoadhesion.

## INTRODUCTION

Diabetes mellitus represents the most prevalent metabolic disorder nowadays, with 345 million people affected worldwide (Sah *et al.,* 2016). Furthermore, it is believed that in 2030 the number of patients will raise up to 552 million, which can be considered a threat for public health (Whiting *et al.,* 2011). A major concern is that life expectancy is reduced by many years in patients with type 1 or 2 diabetes. The therapy involves different approaches, including diet, physical exercise and hypoglycemic drugs.

For Type 1 diabetes patients, due to insufficient insulin production, exogenous hormone is needed (Salvioni *et al.,* 2016). The peptide insulin is the most effective drug for diabetes treatment, with high specificity and activity (Fonte *et al.,* 2014). However, the most common route for insulin administration, the parenteral route, faces many hurdles, such as the difficulty of achieving a normal pattern of nutrient-related and basal insulin. Furthermore, the subcutaneous injection, which must pass through the skin for systemic effect, results in considerable tissue trauma and pain (Sintov, Levy, Botner 2010; Li *et al.,* 2017).

Considering the instability of peptides, for an effective therapeutic outcome, they should be protected against degradation. Therefore, the use of appropriate carriers is needed for effective delivery (Huang *et al.,* 2009). Strategies have been directed to improve insulin delivery by the use of colloidal systems, possessing diameter less than 1 μm (Diop

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*et al.*, 2015). Among these colloidal carriers, nanoparticles are able to prevent peptide degradation and promote sustained release, with consequent better therapeutic response and patient compliance (Zheng *et al.*, 2013; Giovino *et al.*, 2012). Thus, nanoparticles have been used for nasal or pulmonary routes for delivery of insulin, avoiding the problems associated with the parenteral administration. For instance, the nasal route of delivery has been exploited for needle-free systemic delivery of a wide range of drugs, including small molecules, proteins and peptides, such as insulin. Noteworthy, intranasal drug delivery offers many advantages, such as the large absorptive area with high vascularization, avoiding the first-pass liver metabolism (Casettari, Illum 2014; Zhang *et al.*, 2008). Furthermore, the pulmonary route is also interesting for drug delivery, such as insulin, due to the large surface area for drug absorption, which can also benefit from mucoadhesive formulations, especially those capable of overcoming the mucociliary clearance (Jain *et al.*, 2008; Alpar *et al.*, 2005).

Bioadhesive polymers, for example chitosan and alginate, are polysaccharides employed in nanoparticle formation owing to their mucoadhesive properties, through the interaction with the negatively charged mucin (Andreani *et al.*, 2015). Chitosan, a cationic polysaccharide with glucosamine and *N*-acetylglucosamine, is derived from the deacetylation of chitin, found in shells of crustaceans and is soluble in mildly acidic aqueous solutions. Chitosan presents many advantages, such as its availability and biocompatibility. Owing to its many advantages, chitosan has been used as a drug delivery carrier, including for controlled release of insulin. Very interestingly, chitosan hydration and gel formation allow to prolong release of the drug at the administration site (Huang *et al.*, 2009; Ravindranathan *et al.*, 2016; Szekalska *et al.*, 2016). Moreover, chitosan is able to disrupt epithelium tight junctions, due to interaction of protein Kinase C (Smith, Dornish, Wood, 2005). Although polyelectrolyte nanoparticles based on chitosan have been previously reported for insulin loading, to our knowledge, our paper is the first to address the *in vitro* mucoadhesion or cytotoxicity on lung fibroblast cells of chitosan/sulfate dextran nanoparticles loaded with insulin. Altogether, these are important preliminary parameters regarding pulmonary or nasal delivery of insulin from nanoparticles (Sarmento, Veiga, Ferreira, 2006; Mao *et al.*, 2006; Lopes *et al.*, 2016). Recently, the effect of albumin coating on chitosan/dextran-sulfate nanoparticles was investigated. The authors aimed to protect insulin from degradation in the acidic or intestinal environments considering the oral drug delivery. Unlike our study, the authors employed the nanoemulsion method for nanoparticle preparation, using

surfactants for stabilization, such as sorbitane monooleate and Poloxamer 188 (Lopes *et al.*, 2016).

Thus, the purpose of this work was to develop and characterize formulations based on chitosan/dextran-sulfate, intended for mucoadhesion of insulin, potentially applied for nasal or pulmonary delivery. We investigated the chitosan/dextran-sulfate ratio influence on encapsulation efficiency, zeta potential and particularly on particle size. Additionally, we studied the *in vitro* drug release using dialysis membrane and the interaction between the formulation and insulin through thermal analysis employing the NanoDSC equipment. Noteworthy, the complexation with mucin for *in vitro* mucoadhesive evaluation and the cytotoxicity of formulations against MCR-5 fibroblast lung cells, both important parameters prior to *in vivo* application, were addressed.

## MATERIAL AND METHODS

### Material

Dextran sulfate sodium salt (average MW>500,000 Da), chitosan (low molecular weight. MW, ranging from 50,000 to190,000 Da), resazurin, porcine stomach mucin, type II, and doxorubicin were purchased from Sigma-Aldrich. Novolin® R human insulin was obtained from Novo Nordisk. Glacial acetic acid was supplied by QUEMIS. 12-14 kDa MWCO (molecular weight cut-off) cellulose dialysis membranes were obtained from Fisherbrand. MRC-5 cells were supplied by American Type Collection. DMEM medium, fetal bovine serum (FBS) and antibiotic/antimicotic solution were purchased from Gibco. 0.25% trypsin/EDTA was supplied by Vitrocell. Ultra-purified water was obtained from Milli®Q Plus System (Millipore).

### Methods

*Development of insulin-loaded nanoparticles*

The nanoparticles were synthesized by the combination of two polymers, a polycation, chitosan (low molecular weight. MW, ranging from 50,000 to 190,000 Da), and a polyanion, dextran-sulfate sulfate sodium salt (average MW>500,000 Da) (Sarmento, Veiga, Ferreira, 2006). The total polymer concentration, 0.5, 1.0, and 1.5 mg/mL in aqueous solution, pH 4,6, and the chitosan/dextran-sulfate ratio, 1:9, 2:8, 3:7, 4:6, 1:1, 6:4, 7:3, 8:2 and 9:1, were investigated. Nanoparticles were obtained after dropwise addition of chitosan solution to dextran-sulfate solution under vortex stirring for 15 min. Insulin was loaded in the formulations at 500 µg/mL

concentration. For this purpose, the peptide addition order was studied, i.e, after the formation of the nanoparticle, or onto chitosan solution, or onto dextran sulfate solution, prior to the nanoparticle formation.

## Physicochemical characterization of insulin-loaded nanoparticles

- Particle size

Particle size was measured through the dynamic light scattering (DLS) technique at 633 nm, using the angle of 173°, at room temperature (Zetasizer Nano-Zs, Malvern Instruments). Samples were diluted with water before analysis. Measurements were done in triplicate with 10 determinations for each one.

- Zeta potential

Zeta potential was measured through electrophoretic mobility of particles. Analysis was carried out using the DLS equipment (Zetasizer Nano-Zs, Malvern Instruments). Samples were diluted with water before analysis. Measurements were done in triplicate with 10 determinations for each one.

- Encapsulation efficiency

The encapsulation efficiency (EE) was determined by ultraviolet-visible (UV) spectroscopy, with insulin measurement at 270 nm, following equation 1. For separation of insulin-loaded nanoparticle and free insulin, centrifugation was performed at 14.000 rpm for 10 min. Free insulin corresponded to the insulin in the supernatant fraction (Sarmento, Veiga, Ferreira 2006).

$$(EE\%) = \frac{\text{Total insulin (µg)} - \text{Free insulin (µg)}}{\text{Total insulin (µg)}} \quad \text{(Equation 1)}$$

- Differential Scanning Calorimetry (DSC)

DSC analysis was carried out using the Nano DSC equipment, TA Instruments. Samples were degassed under vacuum before loading into the capillary cells composed by platinum and heated from 0 to 100°C, at a rate of 2°C/min. The samples scans were subtracted from ultra-purified water reference scan. Data analyses were carried out using the Nanoanalyse software (Andreani et al., 2015).

- Morphology by Scanning Electron Microscopy (SEM)

SEM morphology was evaluated using a JEOL JSM-7500F microscope. For that, a drop of nanoparticle dispersion was applied to the carbon grid, dried and gold coated under vacuum. Photomicrographs were obtained using 2.00 kV electron beam.

## In vitro insulin release

Experiments were done in sextuplicate at 32 °C using a Franz diffusion cell system, at 400 rpm. Cellulose acetate membranes (12-14 kDa MWCO) were placed between the donor and the receptor compartments, the latter filled with deionized water (7 mL), following a method previously described, with modifications (Liu et al., 2012). Samples in the receptor compartments were collected in predetermined intervals, with replacement of fresh medium. Released insulin was quantified in the receptor compartment, using a spectrophotometric method at 270 nm, using an insulin standard curve.

## In vitro mucin/ nanoparticle interaction

Blank nanoparticles were incubated with mucin solution (2 mg/mL) at different mucin/nanoparticles ratios, vortexed and incubated under stirring at 37 °C for 30 min (Andreani et al., 2015). Afterwards, the dispersions were centrifuged for 10 min at 10000 rpm and the supernatant was used for mucin UV spectrophotometric quantification at 280 nm (Boya et al., 2017). Mucin adsorbed on samples was determined by the difference between the final and initial mucin concentration after incubation and centrifugation.

## Cell culture

MRC-5 (normal lung fibroblast cells) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and incubated in DMEM medium supplemented with 10% FBS and 1% penicillin (100 U/mL)–streptomycin (100 µg/mL). Cells were maintained in a humidified environment at 37 °C with 5% $CO_2$ and sub-cultured twice per week.

## Cytotoxicity tests

A resazurin reduction assay was used to investigate cytotoxicity on MRC-5 cells. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Nonviable cells rapidly lose the metabolic capacity to reduce resazurin and thus do not produce a fluorescent signal. Briefly, the cells were detached by treatment with 0.25% trypsin/EDTA (VitroCell, Brazil) and $2.5 \times 10^4$ cells were placed on each well of a 96-well cell culture plate (Costar, USA) in a total volume of 100 µL. Cells were allowed to adhere overnight and then were treated with different

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concentrations of drugs. After 24 h incubation in the presence of the compounds, the medium was removed and 50 μL resazurin (Sigma-Aldrich, Germany) 0.01% w/v in DMEM, was added to each well and the plates were incubated at 37 °C for 3 h.

The fluorescence was measured on Biotek Synergy H1 plate reader (Biotek, Winooski, VT) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Untreated cells constituted the negative control (viable cells), and cells treated with doxorubicin at 100 nmol (Sigma-Aldrich, St. Louis, MO, USA) constituted the positive control (dead cells). All the tests were performed in three independent assays. Graphs were expressed as lethality (%). The $IC_{50}$ values represent the samples concentrations required to inhibit 50% of cell proliferation and were calculated *using* the *GraphPad Prism*® 5 (*Version 5.01*, GraphPad Software, Inc., USA)

## RESULTS AND DISCUSSION

Polyelectrolyte complexes can be synthesized in aqueous solutions through ionic interactions between polyelectrolyte compounds with opposite charges, particularly in low ionic strength media. Critical coagulation concentrations of 0.12 and 0.09 M (with sodium chloride) for cationic and anionic particles has evidenced a mostly electrostatic stabilization (Schatz *et al.,* 2004a). Furthermore, dissociation of complexes decreases with increased salt concentration, which is likely due to the result of reduced attraction between the oppositely charged polyelectrolytes (Mao *et al.,* 2006). For complexation with chitosan, dextran sulfate is the most widely employed polysaccharide, due to many advantages, such as the low price, ease availability and also because of the strong ionic interaction between its sulfate group and the ammonium groups of chitosan (Delair, 2011). Nanoparticles have been shown to be formed through the coacervation between chitosan and dextran sulfate, with application for the loading of proteins and peptides, such as insulin (Sarmento, Veiga, Ferreira, 2006). Furthermore, the ionic coacervation is environment friendly and the results obtained over the years with nanoparticles based on chitosan and dextran sulfate underline the high potential of this strategy for development of human medicine (Delair, 2011).

Figure 1 summarized the results of particle size obtained with different nanoparticle compositions, containing decreasing total excipient concentrations, prepared at varied chitosan/dextran sulfate ratios. It became evident that unless prepared with only chitosan or dextran sulfate, nanoparticles composed by the mixture of the two polyelectrolytes were not strongly affected by

the ratio between the components. Conversely, Shatz and co-workers(2004b) observed that higher dextran sulfate concentrations led to increased particle size, whereas higher concentration of chitosan favored the formation of more particles, without influencing particle size. However, the nanoparticles were prepared differently compared to the protocol employed herein, which could potentially influence the particle size results obtained. It was reported the use of a purification step of nanoparticles by centrifugation for 30 min at 8000-12000 rpm, with resuspension in a minimum volume of deionized water, in order to avoid the adsorption of free polymer onto particle surface. Also, we need to consider that the insulin presence in the nanoparticles reported in our paper could have changed the behavior observed in the paper by Schatz *et al.*(2004b), who did not encapsulate the peptide.
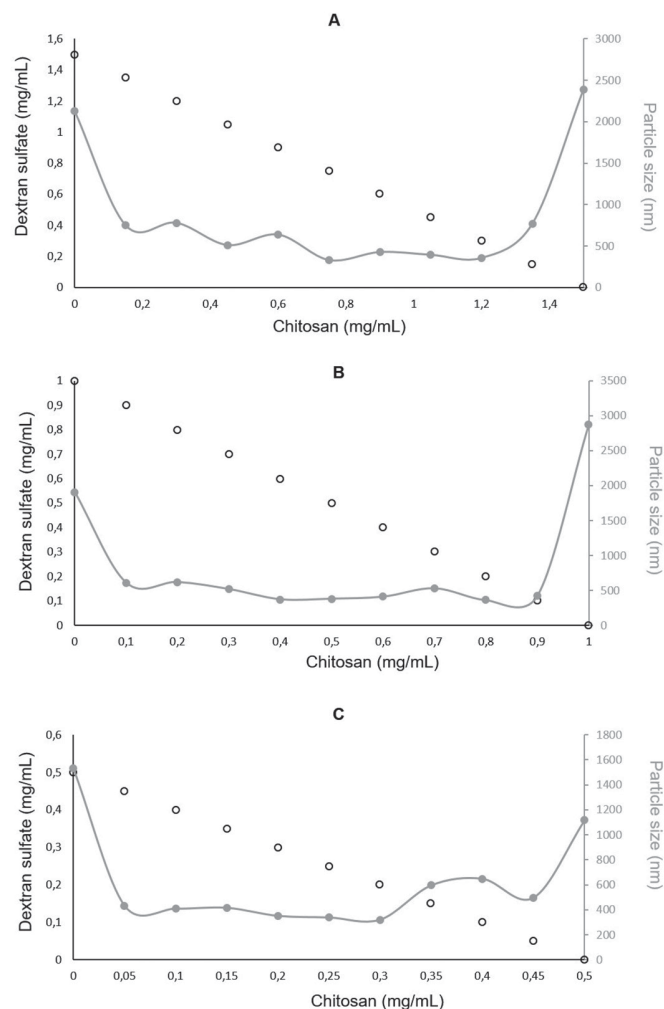


**FIGURE 1 -** Study of the influence of total excipient concentration (A = 1.5 mg/mL, B = 1.0 mg/mL and C = 0.5 mg/mL) and the ratio of dextran sulfate/chitosan (open circle) on particle size (gray closed circle).

Noteworthy, herein we found that the total polymer concentration affected the particle size. Chitosan and dextran sulfate concentrations equivalent to 0.5, 1.0 and 1.5 mg/mL resulted in mean particle size values of all formulations at 605.06, 816.30 and 859 nm, respectively. Therefore, the total polymer concentration chosen for further studies was 0.5 mg/mL and the selected formulations were prepared employing chiton/dextran sulfate at the ratios of 1:9, with particle size of 432.2 nm and zeta potential of -54.7±0.458 mV, due to the negatively charged dextran sulfate in excess, and another composition with chitosan in excess, at the ratio of 6:4, generating particle size of 320.55 nm and zeta potential of 38.6±0.1 mV. Interestingly, particle size achieved with this latter formulation developed herein is smaller than the chitosan/dextran sulfate particles previously reported for insulin loading, which ranged from 489 ± 11 to 1612 ± 248 (Sarmento, Veiga, Ferreira, 2006). The reduction of particle size is important for further applications of the nanoparticles developed herein. For instance, several types of nanoparticles have been employed for lung drug delivery with improved uptake and action and it is known that smaller nanoparticles traverse the surfactant layer more efficiently than larger nanoparticles probably due to minimal steric hindrance (Iyer, Hsia, Nguyen, 2015).

The encapsulation efficiency of insulin in nanoparticles was low, equivalent to 9.73 and 16.67%, for the chitosan:dextran sulfate (1:9) and chitosan:dextran sulfate (6:4) formulations, respectively, in disagreement with previous observations of high encapsulation efficiency of insulin in formulations prepared with excess dextran sulfate (Sarmento, Veiga, Ferreira, 2006). In order to circumvent this drawback, it was attempted to add insulin in the chitosan solution or in the dextran sulfate solution, prior to the nanoparticle coacervation, albeit even lower encapsulation efficiency values were achieved (data not shown). Within this context, Mao and co-workers proved that the complexation between chitosan and insulin is pH dependent, and it is favored above the critical pH of 6.0 (Mao *et al.*, 2006). On the other hand, in that paper, the formulation was different than the one reported herein, because it was not used dextran sulfate, a counterion commonly employed to form nanoparticles though ionic complexation (Chaiyasan, Srinivas, Tiyaboonchai 2013). Furthermore, the pH requirement for nanoparticle formation when dextran-sulfate is used in combination with chitosan is different, considering that Sarmento, Veiga and Ferreira (2006) prepared insulin-loaded nanoparticles in pH below 5.0 with excellent encapsulation efficiency, higher than 85%. However, we need to consider that while Sarmento, Veiga and Ferreira (2006) used insulin at a concentration of 58.33 µg/mL, whereas we employed a higher drug concentration of 500 µg/mL, which could have affected the encapsulation efficiency due to insulin excess. Hence, the insulin concentration of our nanoparticles was almost 10 times higher, which could compensate the low encapsulation efficiency of insulin. Finally, the low encapsulation of hydrophilic drugs in polymeric nanoparticles has been commonly reported and remains a challenge during formulation development (Vrignaud, Benoit, Saulnier, 2011). Therefore, the low encapsulation efficiency shortcoming should be addressed in more details in future studies.

The evaluation of the morphology properties of the formulations was conducted by SEM. Photomicrographs shown in Figure 2 evidenced the aggregate nature of spherical chitosan/dextran sulfate (1:9) nanoparticles, unlike with the chitosan/dextran sulfate (6:4) formulation, which did not present defined structure, probably to excess adhesive chitosan which resulted and highly aggregated formulation.
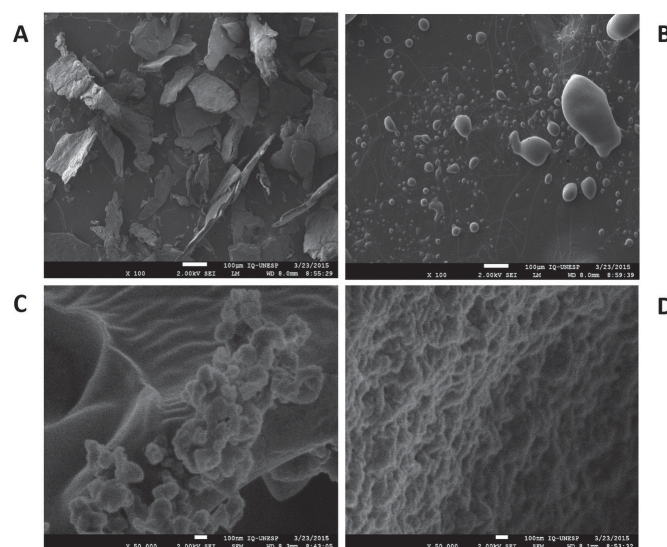


**FIGURE 2** - Scanning electron microscopy (SEM) of A (dextran sulfate), B (chitosan), C (chitosan/dextran sulfate (1:9) nanoparticles) and D (chitosan/dextran sulfate (6:4) nanoparticles).

DSC experiments can reveal structural changes of proteins when incorporated into delivery systems and was therefore carried out to elucidate the thermal events of insulin-loaded nanoparticles (Jorgensen *et al.,* 2004). All the endothermic and exothermic insulin peaks were indicated with arrows. Figure 03 revealed a minor endothermic peak centered at 77°C ($\Delta H = 0.04$ kJ mol$^{-1}$) followed by a major exothermic peak at 85.30 °C ($\Delta H = 0.4$ kJ mol$^{-1}$) for insulin, which has been already reported

for dendrimer-loaded insulin (Nowacka *et al.*, 2016). The sequence of endothermic followed by exothermic peak could be correlated with protein denaturation followed by aggregation (Sarmento, Veiga, Ferreira, 2006). It is possible that insulin once denatured can expose interaction sites able to produce insulin-insulin linkages and, therefore, promote the formation of peptide aggregates (Gibson, Murphy, 2006).

The pattern of endothermic event followed by exothermic event was also observed for insulin loaded in the two compositions of chitosan/dextran sulfate nanoparticles studied. However, we can observe that the two types of nanoparticles show opposite behavior regarding the location and shape of the peaks. While chitosan/ dextran sulfate 1:9 nanoparticles had a shift of the endothermic peak to lower temperature ($\sim 67\ °C$, $\Delta H = 0.7\ kJ\ mol^{-1}$) and a shift and broadening of the exothermic peak to higher temperature ($\sim 88°C$, $\Delta H = 1.0\ kJ\ mol^{-1}$), chitosan/ dextran sulfate 6:4 nanoparticles had a shift of both endothermic and exothermic peaks to higher temperatures, 88 °C ($\Delta H = 0.3\ kJ\ mol^{-1}$) and 94.5 °C ($\Delta H = 0.6\ kJ\ mol^{-1}$) respectively. These results suggested that insulin was, at least partially, protected from degradation in the cationic nanoparticle (chitosan: dextran sulfate, 6:4), which also presented the highest degree of insulin encapsulation.
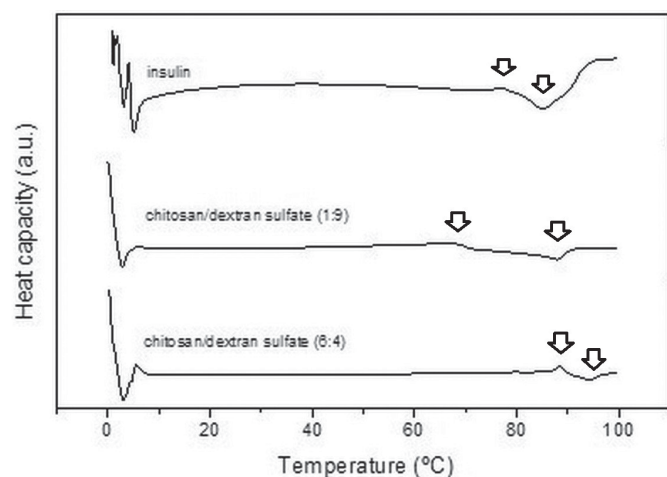


**FIGURE 3** - Dynamic scanning calorimetry (DSC) curves of insulin (A), chitosan/dextran sulfate (1:9) nanoparticle (B) and chitosan/dextran sulfate (6:4) nanoparticle (C), heated from 0 to 100 °C.

*In vitro* drug release has been regarded as a parameter to assess formulation safety and efficacy, being used to reflect the *in vivo* behavior (Souza, 2014). Nanoparticles may serve as a platform for sustained release of drug, achieving a prolonged effect due to slow drug release,

with several advantages, including lesser frequency of administration, reduced side effects, with consequent better patient compliance (Natarajan *et al.*, 2014). Herein, the *in vitro* release studies using a dialysis membrane showed that both formulations presented similar release, with a slow pattern in the first 10 h, then reaching the plateau (Figure 4). Sarmento, Veiga and Ferreira (2006) obtained similar results in their evaluation of chitosan/ dextran sulfate nanoparticles for oral delivery, however plateau was reached around 5 h in pH 6.8 release medium. Therefore, the nanoparticles reported herein seem to present more sustained insulin release.

The process of drug release is complex and influenced by the physicochemical properties of the drug, the characteristics of the matrix and the release environment. Solute diffusion, polymeric material swelling and degradation have been suggested as the main factors explaining drug release. In general, mathematical models give insight into the release mechanism, revealing the process kinetics. Insulin release kinetics from the negatively charged nanoparticle corresponded to the Weibull model and is related to Fick's law diffusion, related to drug diffusion. On the other hand, the positively charged, chitosan/dextran sulfate (6:4) nanoparticle released insulin according to the Peppas model, an anomalous non-Fickian release kinetics, related to polymeric swelling (Grassi, Grassi, 2005). Hence, the chitosan/dextran sulfate ratio affected the kinetics mechanism of insulin release.
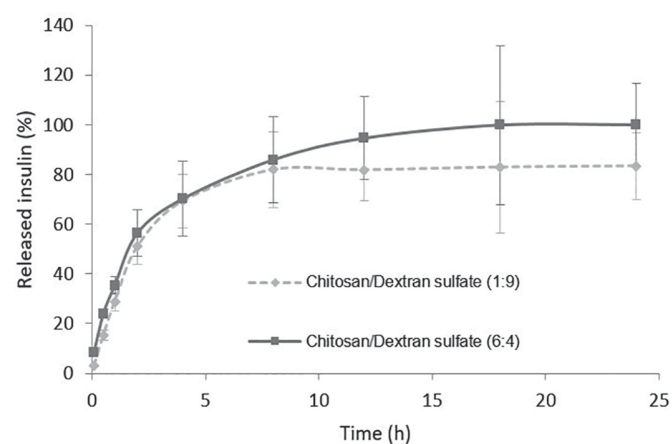


**FIGURE 4 -** *In vitro* release study of insulin-loaded nanoparticles.

Herein, it was shown the successful complexation with mucin, which was better for the chitosan/dextran sulfate (6:4) formulation, with maximum 66.72% interaction, compared to 34.70% interaction achieved with the chitosan/dextran sulfate (1:9) formulation, at 0.5 mucin/nanoparticle ratio (Figure 5). It is important to consider that the formulation prepared with more chitosan

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presents positive zeta potential, and it is known that positive surface has an important role on mucoadhesion, since the positive charge of nanoparticles can interact with the negatively charged sialic groups of mucin (Barbi *et al.,* 2015). The high interaction with mucin presented by the positively charged nanoparticle might be an indicative of promising nasal or pulmonary applications.

Furthermore, it is interesting to note that the positively charged formulation interaction with mucin decreased in the presence of higher mucin/nanoparticle ratios, whereas the behavior is opposite for the negatively charged formulation, indicating that the electrostatic contribution for interaction decreases when the formulation is present in lower concentrations. On the highest mucin/nanoparticle ratio, the percentage interaction with mucus is similar for the two formulations, around 40%. It should be noted, on the other hand, that other parameters affect interaction between nanoparticles and mucus, for instance, buffer conditions such as pH and ionic strength (Lieleg, Vladescu, Ribbeck, 2010). Nanoparticles not only must be mucoadhesive, but also pass through mucus barrier to reach circulation. For mucus penetration, nanoparticles must be small enough to avoid steric inhibition by dense fiber mesh. In this context, it has been previously demonstrated that nanoparticles as large as 500 nm can rapidly diffuse though physiological human mucus (Lai *et al.,* 2007). Therefore, the nanoparticles developed herein might have potential for mucus penetration.

The nasal or pulmonary routes represent promising alternative delivery sites, offering the possibility to avoid the first-pass metabolism enabling direct blood drug absorption. Noteworthy, the pulmonary route is particularly attractive for protein and peptide delivery owing to the large absorptive surface area with the thin alveolar mucosal membrane (Ahmed, Aljaeid, 2016). Albeit promising for the delivery of several drugs, these routes present the challenge of the mucociliary clearance (Alpar *et al.,* 2005). Thus, mucoadhesive formulations emerged as an alternative to address this issue (Barbi *et al.,* 2015). In this context, chitosan is one of the most employed biomaterials for the purpose of mucoadhesion, due to electrostatic interactions of cationic chitosan with negatively charged mucin (Sogias, Williams, Khutoryanskiy, 2008). Regarding insulin effect following *in vivo* administration, chitosan gels have caused increase in insulin absorption and reduction of glucose level after nasal administration (Varshosaz, Sadrai, Heidari, 2006). Another paper reported the preparation of a dry powder system consisting of microencapsulated insulin-loaded chitosan nanoparticles, which were evaluated *in vivo* in rats and resulted in deep lung deposition with pronounced and prolonged hypoglycemic effect (Ahmed, Aljaeid, 2016). On the other hand, chitosan-sulfate dextran is a novel approach for insulin delivery to the nasal or pulmonary routes and presents potential for future evaluation.
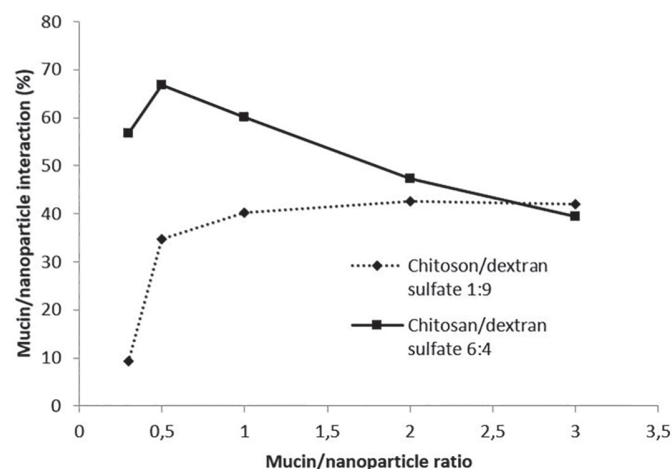


**FIGURE 5** - Mucin and insulin-loaded nanoparticles interaction study.

Toxicology of nanoparticles is an important aspect to be evaluated before clinical application. Indeed, cytotoxicity studies have been widely employed as part of this evaluation (Yildirimer *et al.,* 2011). In our studies, we evaluated the cytotoxicity potential of insulin and insulin-loaded nanoparticles against normal lung fibroblast cells. This cytotoxicity study showed that all nanoparticles had non-inhibitory effect on cells at the tested concentrations, with $IC_{50}$ values greater than 250 µg mL$^{-1}$, as depicted in Figure 6, while doxorubicin, employed as positive control, presented IC50 at 4.5 µgmL$^{-1}$. Hence, the relatively low cytotoxicity of the insulin-loaded nanoparticles could be attributed to the presence of biocompatible polymers, which are responsible for weak interactions with the cell membrane (Lopes *et al.,* 2016).

## CONCLUSION

In this paper, we reported the development and characterization of nanoparticles based on chitosan/dextran sulfate formed by polyelectrolytes condensation for insulin loading. Results demonstrated that the proper selection of polyelectrolyte total concentration, along with chitosan/dextran sulfate ratio, affected nanoparticles size and zeta potential. Although herein nanoparticles were able to encapsulate insulin with low efficiency, it was clear that positively charged nanoparticles based on chitosan/dextran sulfate at the ratio of 6:4 better encapsulated the peptide compared to the composition at the ratio of 1:9,
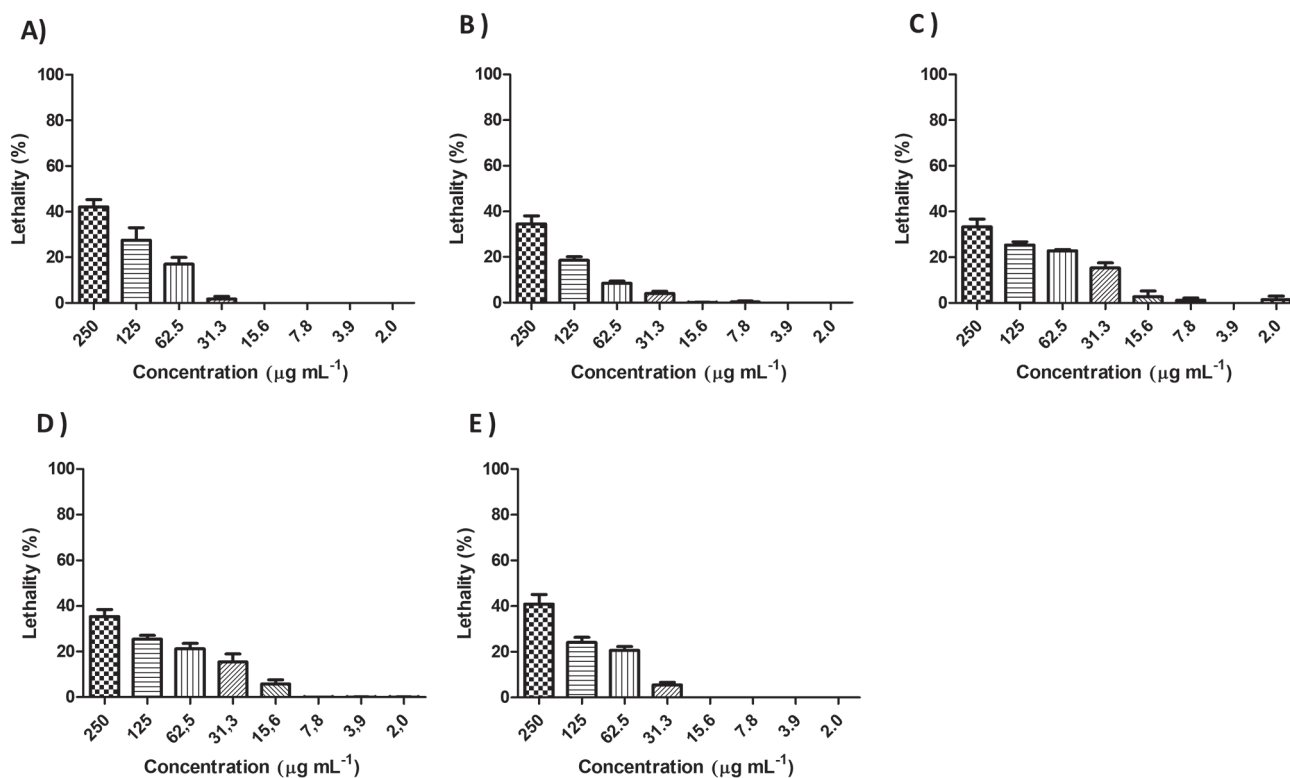
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**FIGURE 6 -** Cytotoxicity evaluation on MRC-5 cells by the resazurin method in different concentrations of insulin (A), blank chitosan:dextran sulfate (1:9) nanoparticle (B), insulin-loaded chitosan:dextran sulfate (1:9) nanoparticle (C), blank chitosan:dextran sulfate (6:4) nanoparticle (D), insulin-loaded chitosan:dextran sulfate (6:4) nanoparticle (E).

due to excess chitosan. Insulin appeared to be partially protected from degradation when encapsulated, according to thermal analysis. Interestingly, insulin release from nanoparticles was sustained, particularly in the first 10h. It was demonstrated the efficient mucus complexation between mucin and nanoparticles, especially with the positively-charged one, which could suggest potential application for nasal or pulmonary delivery. Finally, the insulin-loaded formulations exhibited no cytotoxicity potential against a lung cell line. Taken together, the results shown herein evidenced potential for future studies regarding insulin-loaded chitosan/dextran sulfate nanoparticles for nasal or pulmonary delivery, potentially applied to treat diabetes.

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