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Design, characterisation, and bioefficiency of insulin–chitosan nanoparticles after stabilisation by freeze-drying or cross-linking

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ABSTRACT

Insulin delivery by oral route would be ideal, but has no effect, due to the harsh conditions of the gastrointestinal tract. Protection of insulin using encapsulation in self-assembled particles is a promising approach. However, the lack of stability of this kind of particles in biological environments induces a low bioavailability of encapsulated insulin after oral administration. The objective of this work was to evaluate the effect of two stabilisation strategies alone or combined, freeze-drying and cross-linking, on insulin-loaded chitosan NPs, and to determine their bioefficiency *in vitro* and *in vivo*. NPs were prepared by complex coacervation between insulin and chitosan, stabilised either by cross linking with sodium tripolyphosphate solution (TPP), by freeze-drying or both treatments. *In vitro* bioefficiency NP uptake was evaluated by flow cytometry on epithelial models (Caco-2/RevHT29MTX (mucus secreting cells)). *In vivo*, NPs were injected *via* catheter in the peritoneum or duodenum on insulinopenic rats. Freeze-drying increased in size and charge (+15% *vs* control 412 ± 7 nm; +36 ± 0.3 mV) in comparison with cross linking which decreased NP size (−25%) without impacting the NP charge. When combined the consecutive treatments reduced NPs size and increased charges as compared to standard level. Freeze drying is necessary to prevent the destruction of NP in intestinal environment in comparison with no freeze dryed one where 60% of NP were destroyed after 2 h. Additionally freeze drying combined with cross linking treatments improved bioefficiency of NP with uptake in cell increased when mucus is present. Combination of both treatment showed a protection of insulin *in vivo*, with a reduction of glycemia when NPs were administrated. This work showed that the combination of freeze drying and cross linking treatment is necessary to stabilize (freeze-drying) and increase bioefficiency (cross-linking) of self assembled NP in the delivery of insulin *in vitro* and *in vivo*.

1. Introduction

Subcutaneous insulin injections are widely used for diabetes management. However, injections are often painful, leading to low patient compliance (Al-Tabakha and Arida, 2008). By minimising injections, patient compliance could be increased, thereby improving treatment efficacy. Oral drug administration is a convenient and comfortable method of delivering drugs because it eliminates pain and trauma associated to injections. However, oral insulin administration[1] has low pharmacological efficacy, due to the harsh conditions of the gastrointestinal tract, which may denature orally administered proteins before reaching the blood stream. For example, less than 0.1% of orally administered insulin reaches the blood stream intact (Foss and Peppas, 2004). Furthermore, low pH and protease-mediated hydrolysis limit intestinal absorption of intact insulin (Aoki et al., 2005) and transport into systemic circulation. Thus, to develop an effective oral diabetes therapeutic, it is essential to protect insulin against the gastrointestinal environment. Strategies have been developed to improve insulin uptake, including absorption promoting agents, chemical modifications, and encapsulation in nanocapsules or nanospheres. Polymeric colloidal systems have shown a degree of success in oral delivery of therapeutic proteins. Numerous efforts

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are underway to improve the oral bioavailability of bioactive macromolecules using delivery systems. Nanoparticles (NPs) formulated from natural polymers, such as chitosan, are of interest as protein carriers (Sarmento et al., 2007). Chitosan exhibits many advantages for NP development, including biocompatibility, biodegradability, and low immunogenicity and toxicity (Agnihotri et al., 2004; Pandey et al., 2005). The mucoadhesive properties of chitosan are related to its high positive charge density (Plapied et al., 2010). Therefore, chitosan is an ideal candidate for drug delivery to mucosal tissues (Sayin et al., 2009). The numerous free amine groups on the chitosan backbone result in interesting properties, allowing for extensive use in drug delivery applications. In an acidic environment, NPs form spontaneously, due to intra- and intermolecular linkages between the positively and negatively charged species (Veis, 2011), which form polyelectrolyte complexes (complex coacervation). This process, based on self-assembled chitosan and insulin NPs, appears promising to prepare NPs without the use of harsh organic chemicals. NPs formed through ion-pairing and electrostatic interactions retain polymer integrity. Gastrointestinal uptake of proteins, including insulin, can be improved by entrapment in NPs, which protects insulin from degradation. These carriers have improved oral peptide delivery, due to their prolonged retention in the gastrointestinal tract and excellent penetration into the mucus layer (Renukuntla et al., 2013) mediated by the free amino groups. The positive charge can react with many negatively charged surfaces, including cell membranes, sialic acid in the mucus lining, and anionic polymers. These particulate systems also show a high affinity for negatively charged macromolecules (Mohammadpourdounighi et al., 2010), such as mucin on the mucosal surface. Furthermore, they are taken up by intestinal cells, represent a major absorption pathway. Nevertheless, chitosan is a non-enteric polymer. Consequently, chitosan NPs cannot protect insulin in the gastric environment. A solution was proposed in patent CA2522868 (Belcourt et al., 2004), which described a double encapsulation system based on a gastro-resistant formulation containing NPs that protect the active component in the intestines. In a previous study, we developed enteric-coated capsules adapted for duodenum-specific drug delivery in rats (Reix et al., 2012), which could be filled with insulin-chitosan NPs. In the present study, we assessed the ability of chitosan NPs to protect insulin in intestinal conditions (Chaudhury and Das, 2010). However, it is well-known that complex coacervation are unstable in environments containing phosphate. Thus, these complexes must be stabilised for use as drug carriers. Cross-linking with sodium tripolyphosphate (TPP) and freeze-drying may be an interesting solution. TPP is a polyvalent anion with three negatively charged phosphate groups. This property enables it to cross-link chitosan Rekha and Sharma, (2009). Freeze-drying is a well-established method to preserve unstable molecules over long periods of time, including biotechnological drugs, such as proteins and peptides (Sameti et al., 2003). NPs could form spontaneously in a mixture of TPP, chitosan, and insulin *via* inter- and intra-molecular linkages between TPP phosphates and chitosan amino groups (Wu et al., 2005). This study sought to prepare stable insulin-loaded chitosan NPs using cross-linking and freeze-drying for *in vitro* and *in vivo* validation of biological efficiency.

2. Materials and methods

2.1. Materials

Ultra-pure chitosan chloride (CS Protasan UP CL113, 75 to 90% deacetylated, molecular weight 70–150 kDa) was purchased from Nova Matrix (FMC BioPolymer, Drammen, Norway). Commercial human insulin solution (Umuline® 100 IU/mL) was generously supplied by Eli Lilly Pharma (Indianapolis, IN, USA). Human recombinant insulin, FITC-labelled insulin, TPP, D-Mannitol, isopropanol, fetal bovine serum (FBS), trypsin, streptozotocin, 24-well plates (CELLSTAR® organic Greiner) were provided by Sigma–Aldrich (St. Louis, MO, USA). Chemicals used for high performance liquid chromatography (HPLC) were of liquid chromatography grade. The acetonitrile was from VWR (Fontenay-sous-Bois, France) and the anhydrous sodium sulphate was from SDS (Peypin, France). The human adenocarcinoma cell line Caco-2was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and the RevHT29MTX cell line was supplied by Dr. Thécla Lesuffleur (INSERM U505, Villejuif, France). Dulbecco's Modified Eagle's Medium (DMEM), antibiotics and antimycotics, non-essential amino acid solution, glutamine, phosphate buffered saline (PBS), and Hanks buffered salt solution (HBSS) were purchased from Invitrogen (Cergy Pontoise, France). Propidium iodide and the annexin-V kit were purchased from Clinisciences (Nanterre, France).

2.2. NP preparation

Chitosan chloride CL113 was dissolved in redistilled water (1 mg/mL [w/v]). To a chitosan solution (500 μL), an equivalent volume of insulin was added dropwise by syringe under gentle magnetic stirring (300 rpm). The mixture was maintained under gentle stirring at room temperature for 30 min to obtain standard (STD) NPs.

For the cross-linking, a TPP solution (27 mM) was prepared by dissolving the powder in redistilled water. TPP solution (50 μL) was then introduced to the complexing medium and stirred for 30 min (300 rpm) to obtain the cross-linked NPs.

Freeze-drying was performed overnight on native or cross-linked NPs in presence or absence of mannitol used as a lyoprotectant. Mannitol was directly dissolved in the NP preparation (5 mg/mL) before freeze-drying. After freeze-drying, NPs were dispersed in redistilled water at the target concentration for further analysis.

2.3. NP characterisation

The physicochemical parameters of the NPs were determined by dynamic light scattering using a Malvern NanoZS (Malvern

Table 1
Impact of freeze-drying and cross linking process on insulin-loaded chitosan particles physicochemical characteristics.

	STD NPs	Cross-linked NPs	Freeze-dried		Freeze-dried with mannitol	
			STD NPs	Cross-linked NPs	STD NPs	Cross-linked NPs
Size ± SD (nm)	412 ± 7	346 ± 7[a]	490 ± 26	313 ± 3[a,b]	368 ± 6[a,b]	326 ± 19[b,c]
PDI	0.3 ± 0.06	0.31 ± 0.04	0.4 ± 0.08	0.3 ± 0.06	0.3 ± 0.07	0.3 ± 0.05
Zêta potential ± SD (mV)	+36 ± 1	+36 ± 1	+44 ± 1[a]	+42 ± 1[c]	+42 ± 2[a]	+43 ± 1[c]

Dynamic light scattering measurement, data are presented in mean ± SD. ANOVA one-way Tukey was applied to compare all groups.
[a] $p < 0.05$ *vs* STD NPs.
[b] $p < 0.05$ *vs* freeze-dried NPs without mannitol.
[c] $p < 0.05$ *vs* freeze-dried NPs with mannitol.