Encapsulation and controlled release of human growth hormone using dextran sulfate-chitosan nanoparticles

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Introduction

The use of polyelectrolyte complexes composed of oppositely charged natural polymers has been employed under mild conditions to carry proteins (Dumitriu et al.,1998, Sarmento et al.,2006, Tiyaboonchai et al.,2003). Colloidal carriers made by polymer complexation represent a very promising vehicle to protect proteins against harsh gastrointestinal conditions and to control the rate of protein release.

Particular attention has been paid to protein-loaded chitosan-based nanoparticles (Calvo et al.,1997, Chen et al.,2004). Chitosan, an unbranched polymer of D-glucosamine and N-acetyl glucosamine, is characterized for its biodegradable, non-toxic and biocompatible properties (Ilium,1998) providing several biomedical, pharmaceutical and food applications. Additional coincorporation of high charge-density polyanions onto chitosan nanoparticles can improve nanoparticle properties concerning protein association efficiency and modulation of drug release (Janes et al.,2001). Thus, dextran sulfate (DS), a biodegradable and biocompatible branched polyion, negatively charged with approximately 2.3 sulfate groups per glucosyl residue, was chosen to formulate DS/chitosan nanoparticles and encapsulate human growth hormone (hGH).

Human growth hormone (hGH, somatropin), also manufactured by recombinant DNA technology, is a 191 amino acid polypeptide (MW 22 kDa) with two internal disulphide bridges. The isoelectric point is 5.0 (Gellerfors et al.,1989) and it is very soluble in water and most aqueous buffers. Therapeutically, hGH is used in children to treat growth retardation, Turner's syndrome or chronic renal insufficiency. In adults, it is used as a treatment for growth hormone deficiency and for management of HIV-related wasting and cachexia (Cheng et al.,2005).

Some studies have demonstrated the ability to improve the nasal (Cheng et al.,2005), pulmonary (Bosquillon et al.,2004) and intestinal (Mlynek et al.,2000) hGH bioavailability by multiparticulate delivery systems and permeability enhancers. These alternatives employing nonparenteral routes of administration offer considerable advantages in patient compliance due to the exclusion of injection pain and easier administration. Furthermore, the normal endogenous hGH secretion may be mimicked more closely than with s.c. injections due to rapid absorption and a correspondingly high peak amplitude.

In the present work, the physical and morphological properties of nanoparticles were investigated in accordance with formulation parameters and the release profile of hGH was also determined regarding its potential for oral delivery.

Materials and methods

Nanoparticle complexation between DS and chitosan was performed employing aqueous solutions of oppositely charged polymers in a final volume of 20 mL. Complexes were obtained after dropwise addition of chitosan (MW ≈ 50 kDa, Aldrich®) solution at pH 5.0 to DS (MW ≈ 500KDa, PKC ® Denmark and MW ≈ 8 kDa, Sigma®) solution at pH 3.5 containing hGH under magnetic stirring followed by additional mixing for 15 min at 600 rpm. Particles were collected by centrifugation at 20000xg for 25 minutes in a glycerol bed. Supernatant was used for hGH determination. Nanoparticles were kept at 4 ^{o}C and ressuspended before release studies.

Measurements of particle size and zeta potential were performed by photon correlation spectroscopy (PCS) and laser doppler anemometry (LDA) using a Zetasizer 5000 (Malvern Instruments). Nanoparticle morphology was observed by transmission electron microscopy (TEM).

The association efficiency (AE) was determined indirectly. The amount of hGH associated with the particles was calculated by the difference between the total amount used to prepare the particles and the amount of hGH present in the aqueous phase after centrifugation.

To evaluate the in vitro release of hGH, nanoparticles were placed into 10 mL of different pH buffers, namely HCl pH 1.2 acetate pH 4.5, acetate pH 5.2 and phosphate pH 6.8 USP XXVI buffers and incubated at 37ºC. At determined times, samples were taken for hGH determination and replaced by fresh medium. hGH concentration was determined using the Coomassie PlusTM Bradford Assay (Pierce, Rockford, USA) modified Bradford assay (Bradford,1976).

Results and Discussion

Complex coacervation between chitosan and DS may occur by electrostatic attraction between the two oppositely charged polymers. This mild process can be used to prepare nanoparticles at ambient temperature while stirring without using sonication or organic solvents. Such properties are suitable to produce protein-loaded nanoparticles and preserve protein stability since proteins such as hGH are labile and sensitive to different stress factors (van de Weert et al.,2000).

Factors affecting characteristics of DS/chitosan nanoparticles and the optimal conditions for their preparation were studied. The screening made with empty nanoparticles led to the assumption that particles prepared with chitosan at final concentration of 0.1% and DS (MW of 500 kDa) resulted in a nanorange size distribution. Thus, in order to further study the effect of DS:chitosan mass ratio on the size, zeta potential and hGH AE, nanoparticles were formulated with DS:chitosan mass ratio from 1:2 to 2.5:1. Results are plotted in Table 1.

Table 1. Influence of DS:chitosan mass ratio on nanoparticle properties ($n \geq 3$)

DS : Chitosan mass ratio	Size (nm)	Zeta potential (mV)	AE (%)
1.5:1	572 ± 21	-8.3 ± 1.5*	-
1:2	669 ± 29	2.6 ± 0.1*	~ 0*
1:1	n.d.	1.5 ± 2.8*	61.6 ± 9.6*
1.5:1	673 ± 254	-5.2 ± 0.4*	86.9 ± 3.3
2:1	842 ± 73	-10.0 ± 0.5	87.8 ± 1.0
2.5:1	1140 ± 93*	-11.3 ± 0.4	~ 100*

*The mean difference is significant at the 0.05 level. n.d. Not determined due to aggregation

No significant differences were found in the mean particle size of formulations prepared with DS:chitosan mass ratio ranging from 1:2 to 2.1. However, when this ratio was reduced to 1:1, agglomeration resulted, making it difficult to determining particle size since PCS is suitable for particles ranging from 5 nm to approximately 5 µm. Higher mean size was obtained when particles were formulated with mass ratio of 2.5:1, presumably due the thicker deposition of DS on the surface of the nanoparticle core. This finding indicates that the presence of one polymer in excess possibly acts as a colloidal protector preventing particle aggregation and simultaneously enhancing particle strength. Excess polymer is likely placed on the particle surface, as indicated by the significantly different zeta potential values, positive when chitosan is the main component and negative when DS is presented in higher concentration. The zeta potential of particles formulated with DS:chitosan mass ratio of 1.1 was approximately zero, which can indicate the absence of charge repulsions between particles promoting their agglomeration.

The hGH AE was observed to be dependent on DS:chitosan mass ratio. It significantly increased when DS:chitosan mass ratio was varied from 1:2 to 2.5:1. hGH as an amphiphilic molecule is able to be electrostatically attached to both DS and chitosan with a high avidity, allowing the formation

of a stable colloidal drug carrier. One can induce from theses results that the affinity of hGH to DS is higher than for chitosan due the stronger electrostatic interaction of the protein to negative sulfate groups. Under the conditions used in the assay, DS:chitosan mass ratio of 2:1 appeared to be adequate to entrap most of the hGH while maintaining the nanorange size of particles.

Table 2. Influence of DS MW on nanoparticle properties (n ≥3)

DS Mw	Size (nm)	Zeta potential (mV)	AE (%)
500	673 ± 254	-5.2 ± 0.4	86.9 ± 3.3
8	3289 ± 845*	-0.6 ± 0.2*	~ 100*

*The mean difference is significant at the 0.05 level.

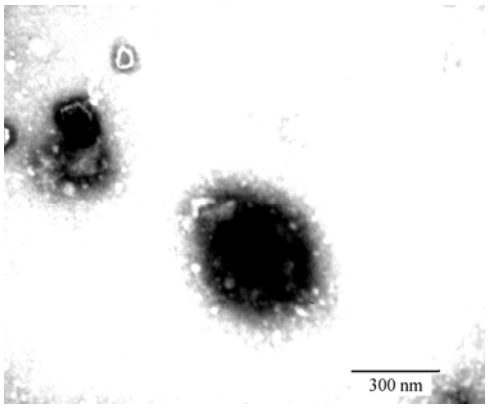

Figure 1. TEM photomicrograph of DS/chitosan nanoparticles produced with DS:chitosan mass ratio of 1.5:1

As it was deduced above, DS is the major component responsible for the association of hGH to nanoparticles and therefore its MW can be important for determining the degree of association to hGH. Although particles produced with low MW DS entrapped practically all the hGH, they also presented a mean size significantly higher that those made by high MW DS as well as lower zeta potential.

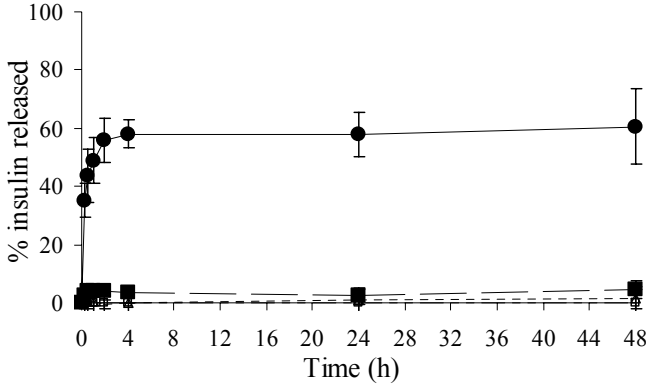

Figure 2. hGH cumulative release profile from DS/Chitosan nanoparticles produced with DS:chitosan mass ratio of 2:1 at pH 1.2 (□), 4.5 (■), 5.2 (Δ) and 6.8 (●). (n=3)

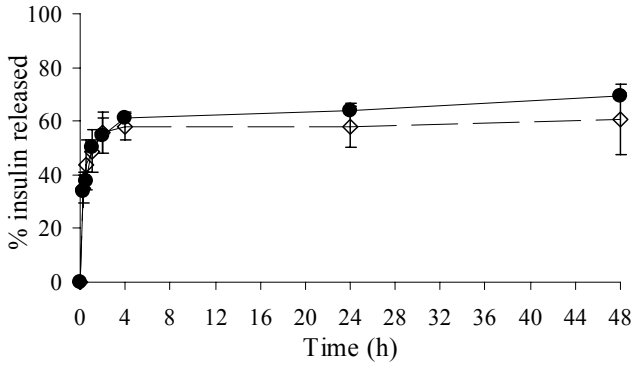

Figure 3 hGH cumulative release profile at pH 6.8 from DS/Chitosan nanoparticles produced with DS:chitosan mass ratio of 1.5:1 (●) and 2:1 (◊).(n=3)

Nanoparticle morphology was assessed by electronic microscopy, confirming obtained size results. Although nanoparticles were collected by centrifugation, they were easily ressuspended in aqueous media.

DS/chitosan nanoparticles produced by dropwise addition of chitosan, at a final pH of 4.8, with a DS:chitosan mass ratio of 2:1 and a theorical hGH loading of 1% (final hGH 0.0025%) were used to assess the cumulative release behaviour of hGH at different aqueous pH buffer environments.At pH of 1.2, 4.5 and 5.2, the release of hGH was completely avoided but at pH 6.8 the release of hGH was controlled. After 48 hours of the assay time, the amount of hGH released at pH 1.2, 4.5 and 5.2 was less that 4 %. At pH lower than the p*I* of hGH, the protein presents an overall positive charge that is able to strongly interact with the high anionic charge density of nanoparticles provided by DS. The strong electrostatic interactions between hGH and polyelectrolytes at this pH range may be responsible for the permanent hGH retention as also observed for insulin encapsulated in

DS/chitosan nanoparticles (Sarmento et al.,2006). Additionally, DS/chitosan nanoparticles appear to preserve their structure without erosion, which also contributes to the absence of hGH release.

The amount of hGH released at pH 6.8, during 48 hours presented a controlled release profile in which 35% of hGH release was observed in the first 15 minutes of assay following a lower rate of release. After 240 minutes of assay, 58% of hGH was released and reached 61% after 48 hours (Figure 2). The initial stage of release is attributed to the hGH located at the surface of the nanoparticles and the remainder of the unreleased hGH is most probably entrapped within the nanoparticles, ionically associated with dextran sulfate and chitosan.

The effect of DS:chitosan mass ratio on the hGH cumulative release at pH 6.8 was then studied. As depicted in Figure 3, the increase of DS:chitosan mass ratio from 1.5:1 to 2:1 was responsible for decreasing the hGH cumulative release. After 48 hours of release, 70 and 60 % of hGH was released from nanoparticles prepared with DS:chitosan mass ratio of 1.5:1 and 2:1, respectively. This finding suggested once more the main role of DS on hGH entrapment. Also, the dissociation mechanism described above to explain hGH release, appears to be slowed down by the presence of the higher negative net charge of the nanoparticle matrix.

Conclusion

DS/chitosan nanoparticles produced by a mild complex coacervation method had been suggested as a valid alternative to encapsulate hGH. The physical properties and release patterns confirm retention of hGH under pH values up to 5.2, and a controlled release profile at higher pH values. This release behaviour is desirable regarding the oral delivery applications.

Acknowledges

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