**Preventing gastric enzymatic degradation of nanoencapsulated insulin**

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## Introduction

Insulin instability has been regarded as a major obstacle to the development of oral programmable insulin dosing devices aimed at attaining optimal diabetic control. A promising strategy for oral insulin delivery is the use of multifunctional polymers. However, additional properties are required to prevent enzyme attack. Several strategies were tested to prevent pepsin attack including multilayer systems, gastroresistant polymers and protein-coating strategies. Several parameters were analyzed such as mean size, zeta potential, presence of agglomerates and finally, insulin resistance to enzymatic attack.

## Materials and methods

Nanospheres were prepared by emulsification/internal gelation (Reis et al., 2006). An aqueous solution of sodium alginate 2% (w/v) and 0.75 % (w/v) of dextran sulfate was prepared by suspending the polymer and adjuvant in distilled water followed by overnight stirring on an orbital shaker. Insulin was then admixed. An aqueous suspension of calcium carbonate was sonicated and added to alginate-insulin solution. The resulting mixture was emulsified within paraffin oil containing an emulsifier (1.5% v/v, Span 80) using a mixing impeller at 1600 rpm. After emulsification, gelation was triggered by addition of 20 mL paraffin oil containing glacial acetic acid. An acetate buffer solution (USP XXVIII) with dehydrating solvents was added to the oil-particle suspension and nanospheres were recovered by centrifugation. Nanospheres were coated with several polymers and/or protein as shown table 1. Then, nanopheres were frozen and lyophilized at 0ºC for 48 h and stored at 4ºC. Insulin-free nanospheres were prepared as controls.

## Size, agglomerates and zeta potential measurements

Size distribution analysis was performed by laser diffraction spectrometry using a Coulter LS130 (Beckman Coulter Inc., CA). Mean diameters of aqueous suspensions of nanospheres were determined in triplicate. Photographs of nanospheres were taken in order to evaluate the presence of agglomerates. Surface charge was determined by zeta potential measurement on a Malvern Zetasizer 5000 (Malvern, UK). Measurements were carried out at pH 4.5 at 25ºC. Each sample was measured 3 times.

## Insulin resistance to pepsin attack

Insulin content (µg insulin per mg nanospheres) was quantified by HPLC (HP1100 series, Hewlett Packard, Germany; mobile phase water (A): acetonitrile (B) with 0.04% TFA (linear gradient B 30% to 40% over 5 min, with a flow rate 1.2 mL/min at 25ºC) after alginate matrix dissolution with citrate solution (55 mM). Insulin resistance was then quantified after 2 h incubation in simulated gastric fluid with pepsin (pH 1.2) in a shaking water bath 37ºC at 100 rpm. Nanospheres were recovered by centrifugation and transferred to citrate solution under magnetic stirring 100 rpm during 1 h. Aliquots (2 mL) were collected and ethanol (2 mL) was added to the suspension and this mixture was stirred on an orbital shaker. 1.5 mL aliquot was collected, centrifuged and analyzed by HPLC. Insulin non-encapsulated was also tested (reference solution).

## Results and Discussion

Mean particle size was affected by type of coating polymer. Small mean particle was obtained for uncoated and coated chitosan, chitosan-pectin and chitosan-albumin nanospheres. Poly-L-lysine, poly(hexamethylene biguanide) and chitosan-alginate coating led to particle size increase and significant agglomeration as shown table 1. To increase enzymatic resistance, free reaction area allowing interaction both species (enzyme-substrate) should be as small as possible. Larger particles should provide better protein protection since they have a larger path for enzyme diffusion. The specific surface area (usually expressed as $m^2/g$) increases rapidly as particles become smaller. However, agglomerates or larger particles such as formulations C to E are not favorable for intestinal absorption seeing as mean particle size has been described as a crucial factor for intestinal uptake (Norris et al., 1998). In regard to particle charge, some formulations had negative and some had a positive surface charge. The mechanism of insulin

enzymatic resistance is not clear. Pepsin has an isoelectric point around 3.3 and is positively charged at pH 1.2. At low pH, electrostatic interactions or repulsion reactions between pepsin-coating polymer can occur, preventing pepsin uptake and consequent protein degradation. This fact can also increase the probability of exclusion of insulin from the site of enzyme action.

| Formulations | Coating strategies | Size ± SD (µm) | Aggl. | Zeta P. (mV) |
|---|---|---|---|---|
| A | Uncoated | 1.5 ± 1.2 | - | -16.3 ± 1.8 |
| B | Chitosan | 2.6 ± 1.3 | + | +14.5 ± 0.7 |
| C | Coating poly-L-lysine | 54.5 ± 31.6 | +++ | -1.3 ± 2.1 |
| D | Poly(hexamethylene biguanide) hydrochloride | 39.2 ± 21.8 | ++ | -6 ± 2.7 |
| E | Chitosan-alginate | 28.9 ± 14.9 | ++ | 3.0 ± 2.1 |
| F | Chitosan-pectin | 9.3 ± 6.9 | + | 2.0 ± 0.7 |
| G | Chitosan-albumin | 5.9 ± 3.6 | + | -8.8 ± 5.5 |

**Table 1. Different strategies to prevent enzymatic attack and particle mean size, zeta potential and presence of agglomerates.** $^{-}$No agglomeration; $^{+}$ Slight, $^{++}$Moderate and $^{+++}$Strong agglomeration

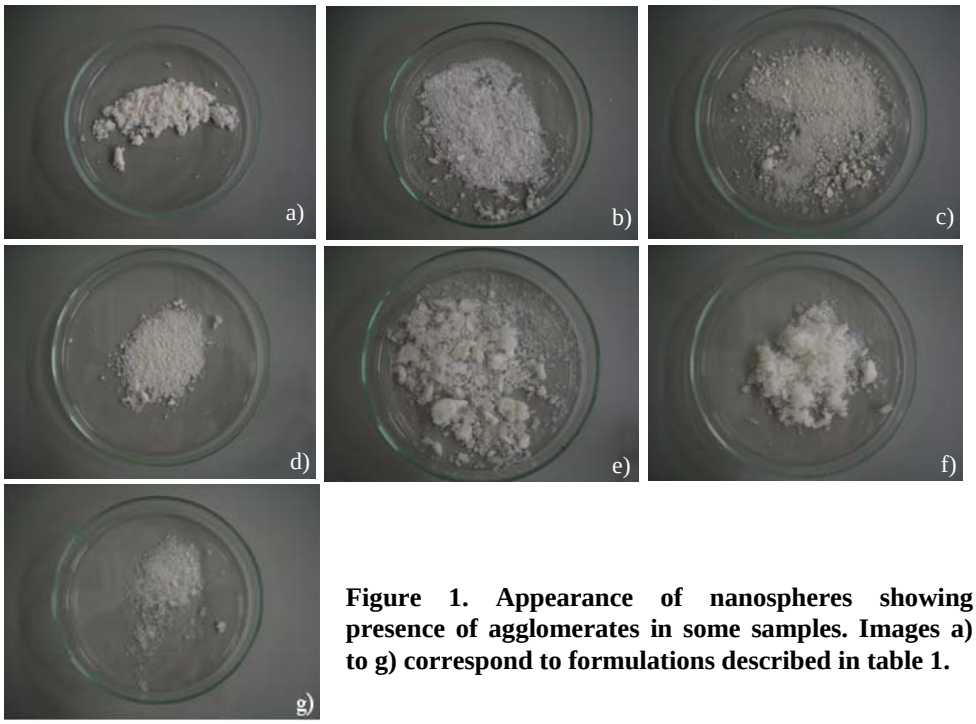


**Figure 1. Appearance of nanospheres showing presence of agglomerates in some samples. Images a) to g) correspond to formulations described in table 1.**

After pepsin incubation, insulin only appeared in chromatograms of nanospheres coated with pectin- and mainly with albumin. Insulin retention

in these formulations was similar to that of reference solution. A shift of the retention time of insulin on the chromatogram is an indication of insulin aggregation or denaturation. Chromatograms of insulin released from pectin and albumin-coated alginate particles highlighted a single peak identical to a reference solution peak. Moreover, concentration of insulin in albumin-coated nanospheres following pepsin exposure was the same as insulin concentration prior to pepsin incubation. Insulin transformation products were not detected which suggested the maintenance of insulin stability after enzyme incubation. All remaining formulations did not protect nanoencapsulated insulin since insulin was not detected by HPLC, suggesting that insulin was fully hydrolyzed.

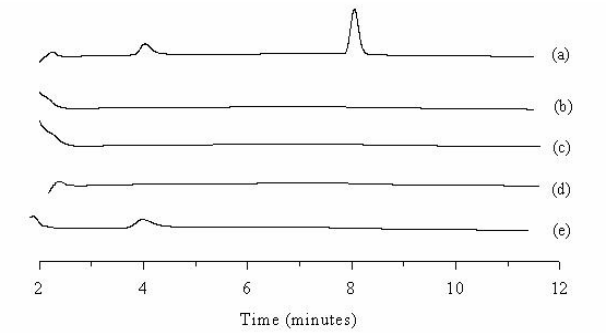


**Figure 2. Insulin HPLC chromatograms, a) Reference solution, b) uncoated, c) formulations B to E, d) pectin and e) albumin nanospheres. In addition, reference solution showed additional peak (8.3 min). This peak corresponds to meta-cresol, additive, and it is usually eliminated after nanospheres production and recovery.**

## Conclusions

Albumin and pectin coated nanospheres provided a protective effect for insulin against pepsin attack. This novel carrier matrix is expected to become an important tool toward future peroral delivery of insulin.

## References

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