Encapsulated insulin in layer-by-layer assembly for effective per oral delivery

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Introduction

Administering drugs orally is by far the most widely used route of administration, although it is generally not feasible for peptide and protein drugs. The main reasons for the low oral bioavailability of biologicals are presystemic enzymatic degradation and poor penetration of the intestinal membrane [Mahato et al, 2003; Hamann et al. 2005]. Much has been learned in the past few decades about macromolecular drug absorption from the gastrointestinal (GI) tract, including the barriers that restrict GI absorption. Various strategies have been pursued to overcome such barriers and to develop safe and effective oral delivery systems for proteins and peptides [Shah et al. 2002]. The oral route for peptide and protein administration continues to present a significant challenge and represents a focus for many pharmaceutical researchers. In this paper, we investigated the microencapsulation of insulin as macromolecules in polymeric matrix assembled using layer-by-layer (LBL) technique and surface was modified with lipid (PE-PEG 2000, Lipoid, AG, Germany) for improved absorption through GIT.

Material and methods

Sodium alginate (SA), Glycol chitosan (GC), Pluronic F-68 (PF-68) and human recombinant insulin was purchased from Sigma. Lipid (PE-PEG 2000) was purchased from Lipoid AG Germany. $CaCl_2.2H_2O$ and Na_2CO_3 (Hi media, Mumbai) were used without further purification. The water used throughout the experiment was purified with a Milli Q system from Millipore Co., USA.

Encapsulation of insulin in layer-by-layer assembly

Alternate deposition of polyelectrolytes (PEs, viz. SA and GC) was done over preformed porous $CaCO_3$ core particles to get five bilayer of each using LBL technique [F-1,$CaCO_3$ $(SA/GC)_5$]followed by surface pegylation with PE-PEG 2000 in equimolar concentration [F-2, $CaCO_3$ $(SA/GC)_5$PE-PEG]. One ml capsular suspension was centrifuged at 3000 rpm for 10 min. and the supernatant was removed. The capsule pellets thus obtained was re-dispersed/incubated in one ml of insulin solution (1-6mg/ml in 0.01N HCl, pH=3-4) for 2h. After desired incubation the capsular suspension was centrifuged and washed thoroughly and the supernatant thus obtained was used to estimate free insulin by Micro-BCA assay.

In-vitro characterization

Powder X-ray diffraction (XRD) patterns of porous $CaCO_3$ core particle synthesized by both conventional as well as modified co-precipitation method were recorded using a Rigaku D/max- 3A instrument (monochromatic Cu-K radiation).Typically; the diffractogram was recorded in a 2θ range of 5–25 °C. The morphology of the prepared $CaCO_3$ core particles and $CaCO_3$ $(SA/GC)_5$ PE-PEG were examined. Atomic force microscopy (AFM, Digital Instrument Inc., Veeco, USA) was carried out to check the surface topography of the developed system. Layer-by-layer growth was determine by the ζ-potential of each adsorbing layer on the $CaCO_3$ dispersed in milli Q water was using Zetasizer Nano ZS (UK). The ζ-potential value was the average of three successive measurements. The pay load efficiency was determined as mentioned above. For in-vitro release study the ultra sink condition was maintained. Briefly, both insulin loaded formulations (1ml) was taken from stock in eppendrof and pelletd/decanted by centrifugation and finally dispersed using a

vortex in one ml different physiological buffers (Simulated gastric fluid for first 2h followed by shifting into simulated intestinal fluid). The sample was maintained under horizontal agitation at 37°C. At specified time intervals the dispersion was centrifuged at 18,000 rpm for 3 minutes and the supernatant was assayed by Micro-BCA method. After each sampling the medium was replaced with fresh buffer. Integrity of the released insulin was determined by circular dichroism (CD) spectra using (CD spectrophotometer, J-715, Jasco, Japan). The degradative action of digestive enzymes viz. pepsin, trypsin and α-chymotrypsin was studied in-vitro on the insulin loaded formulation as reported by M.A. Radwan and H.Y. Aboul-Enein [2001] with slight modification.

Biochemical evaluation of intestinal damage

The fresh ileal loop of small intestine of Wistar rat was treated with 20 ml of Krebs ringer oxygenated buffer (warmed to 37°C) and then flushed out with air. One milliliter of various formulations in various segments was placed for 2h and intestinal damage was assessed for both with and without surface modified formulations. The Positive and negative controls used were 1% triton x-100 and plain saline respectively. Finally, the ileal loop was washed with 1.0 ml of PBS, and the intestinal fluid was collected. The concentration of lactate dehydrogenase (LDH) in the fluid was determined using protocol as provided in LDH-UV kit (Sigma).

Results and Discussion

Micro/nano-encapsulation of various substances into different micro- and nanoparticles such as capsules, polymer spheres, liposomes, and so forth, has received considerable attention due to increased interest in biotechnology, medicine, catalysis, ecology, nutrition etc. Monodisperse porous $CaCO_3$ core particles (3-5μm) doped with PF-68 with porous inner structure was prepared as reported previously [Gupta et al. 2008] and used as template to fabricate assembly. Modification of conventional procedure by incorporating appropriate concentration of PF-68 during controlled co-precipitation lead to the formation of vaterite (spherical) polymorph in abundance as shown in XRD data shown in Fig. 1 and 2, as this polymorph is desired for uniform and amplified surface area to get uniform layering of PEs and high payload of insulin. Multilayer assembly was fabricated by sequential recharging of SA and GC applying LBL technique followed by surface pegylation. AFM images (3 dimensional view, 3D) of bare core particles and fabricated assembly provides clear illustration of striated surface with bulged capping of PEs (dense network) after adsorption procedure as shown in Fig. 3 and 4, which remains stable even after core removal. Surface electrical potential (zeta potential) at each stage of the layering process are shown in Fig. 5. The bared $CaCO_3$ core particles are positively charged with ζ-potential + 3.8 mV when suspended in milli Q water. The first SA layer reversed the charge to -26.75 mV. The first GC coating changed the charge to +26.54 mV. The reversal in charge followed the same trend with additional layers and the magnitude of the charge remained constant up to five complete bilayers.

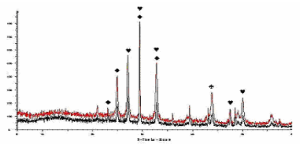

Fig. 1. XRD of $CaCO_3$ synthesized by conventional method.

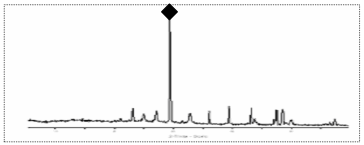

Fig. 2. XRD of $CaCO_3$ synthesized by modified method (◆ >90% vaterite).

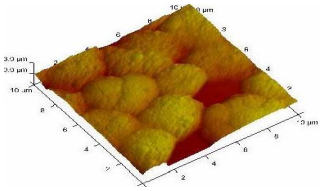

Fig. 3. AFM picture of bare $CaCO_3$ core (3Dimensional view)

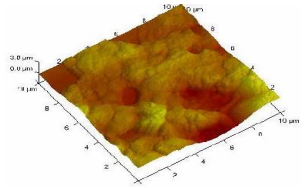

Fig. 4. AFM picture of fabricated assembly (3Dimensional view)

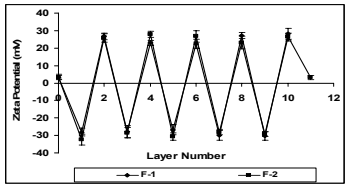

Fig. 5. Zeta potential study at different layering

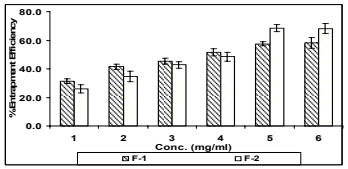

Fig. 6. The encapsulation efficiency of fabricated assembly as a function of insulin conc. in the bulk solution at acidic pH.

An improvement in payload efficiency of insulin 68.76±4.8% was achieved with developed assembly which could be ascribed to two mechanisms i.e. (i) acidic pH facilitates electrostatic interaction of insulin with anionic complex at the interior (ii) improvement in the permeability of wall due to pH change. With increasing insulin concentration in the bulk solution from 1.0 to 6.0mg/mL, the loading capacity increases first and then reaches plateau (~ 68.76±4.8%; Fig 6) due to attainment of equilibration [Ye et al. 2006]. The release rate of insulin in pH 7.4 buffers was much faster than that in pH 1.4 buffers, may be due to the charge reversal of insulin induced by pH change. The insulin is released very slowly at pH 1.4 and only ~8% of the loaded insulin can be released after 2h. While at pH 7.4, a much fast release is observed. About ~50% insulin has been released within the first 6h, and then the release rate decreases and the curve gradually levels off as the cumulative release amount reaches ~80%. This significant difference of insulin release with the change in solution pH is also resulted from the insulin ionization. The loaded insulin is positively charged at pH 1.4 as mentioned above, thus strongly attracted with the negatively charged complex inside the assembly, restricting the release. The insulin charge becomes negative at pH 7.4 and preferentially moves into the solution from the matrix due to the electrostatic repulsion, leading to a rapid release.

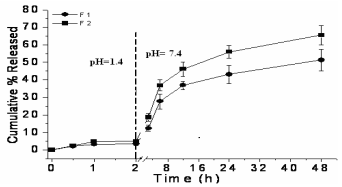

Fig. 7. In-vitro release profile of insulin in physiological milieu.

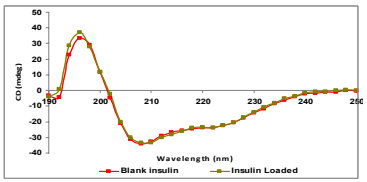

Fig. 8. CD spectra of pure and released insulin (after 6h) in PBS (pH=7.4).

The process of encapsulation at low pH and release altogether did not affect the secondary structure of insulin, ensuring biological activity. Circular dichroism spectra obtained for pure and released insulin (after 6h) in both the fluid indicated similar α-helical conformation as shown in Fig.8. Insulin encapsulated LBL assembly protected 68–90% of the entrapped insulin (Fig. 9) whereas plain insulin solutions were almost degraded under these conditions. The protein protective ability was more in the order of pepsin>trypsin>chymotrypsin. The protective effect was in the order of F-2>F-1. Interestingly, it has been revealed that the LDH release (an indicator of membrane damage) was not significant in any of the developed formulation as compared to positive control (1% Triton-X) and is expected to be a good vehicle for oral administration on insulin (Fig. 10).

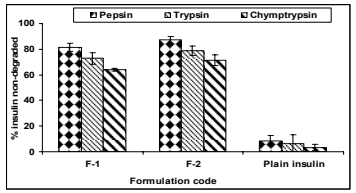

Fig. 9. In-vitro proteolytic enzyme degradation study of the formulation

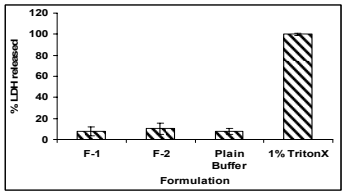

Fig. 10. Effect of different formulations on LDH in ileal loop of rat small intestine.

Conclusions

Layer-by-layer based encapsulation strategy proven its potential for controlled delivery of insulin via non-invasive route. However, we believe that only further research into delivery systems can make it possible for the oral route to represent a viable route of administration for peptide and proteins, improving convenience for, and compliance from patients who would benefit from these enzyme sensitive molecules. Further investigations are still underway to gather toxicity profile and mode of absorption of the proposed formulation.

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