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Evaluation of pharmacological efficacy of 'insulin–surfoplex' encapsulated polymer vesicles

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ABSTRACT

The present study has been designed to study whether formation of ion-pair complex or 'surfoplex' can enhance the pharmacological efficacy of protein-loaded PCL–PEG-based polymerosomes. Insulin was selected as the model protein and was complexed with sodium deoxycholate, a naturally occurring bile salt. The surfoplexes were characterized for extent and site of complexation, stability, mass and partition coefficient. The lipophilicity of insulin was enhanced 5-fold upon complexation resulting in an increase in entrapment efficiency by 10–50% for all formulations compared to free insulin. The release of insulin from the systems was also modulated with reduction in burst release by 30%. The surfoplex was found to be therapeutically active for 8 h duration (C_{max} serum insulin = 64.15 ± 13.28 mIU/mL) in diabetic rat model. However, pharmacological efficacy of the complex-loaded nanoparticles (Nps) did not show significant enhancement with respect to insulin-loaded systems. The study therefore suggests that while ion-pair complexes may improve the in vitro kinetics of protein-loaded carriers, their therapeutic potential is dependent on the intensity of interactions between the peptide chains and polymer matrix.

1. Introduction

Protein–surfactant interactions have extensive applications in biotechnology for protein isolation and crystallization (Garavito et al., 1996) and separation techniques viz. chromatography (Regnier, 1987) and electrophoresis (Righetti et al., 2001). These conjugates aid in increasing the stability of proteins during various processing stages and storage conditions (Kendrick et al., 1997). Being lipophilic in nature, they may enhance the protein loading in nanoparticulate carriers as well as reduce burst release (Choi and Park, 2000). This addresses the common lacunae associated with entrapment of hydrophilic molecules in nanoparticles (Nps). The basic mechanism of this association is the neutralization of the charges present on the protein at a definite pH by that of the surfactant so as to result in the formation of precipitates accounting for ion-pairs. These are analogous to 'surfoplexes' wherein the phosphate groups of DNA are neutralized by the associated surfactant molecules (da Silva et al., 2004). Protein surfoplexes may be hydrophobic in nature depending on the lipophilicity of the conjugating species.

For an effective nanoparticle-based system, an adequate loading of protein is desirable along with protein stability during preparation and release. The w/o/w double emulsion technique has been widely used for encapsulation of hydrophilic macromolecules in micro- and nanoparticles (Bilati et al., 2005). However, apart from the preparation techniques, the polymer composition and degradation also play vital roles in catalyzing protein deterioration after encapsulation. Although the well known, hydrophobic nature of commonly employed Federal Bureau of Drug administration (FDA) approved biodegradable polymers such as poly(lactic acid), PLA; poly(glycolic acid), PGA; and co-polymers; PLGA and poly(caprolactone); PCL result in denaturation and aggregation of proteins (van de Weert et al., 2000). Increasing the hydrophilicity by introduction of poly(ethylene glycol); PEG blocks has been reported to enhance the protein stability (Zhou et al., 2003). In a previous study by our group it has been noted that while the conformational stability and therapeutic potential of insulin was maintained in PCL and PEG co-polymer Nps, the loading and release profiles and duration of action was dependent on the polymer composition (Rastogi et al., submitted for publication).

Based on these considerations, we have undertaken this work to explore the potential of insulin–surfoplex in improving the entrapment efficiency of the protein in Nps. The ultimate goal is to attain a high level of protein loading along with uniform release and full bioactivity. PCL–PEG–PCL (CEC) co-polymers have successfully utilized earlier for preparation of insulin-loaded nanoparticles (Rastogi et al., submitted for publication). Insulin–surfoplex was

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prepared with sodium deoxycholate, a naturally occurring bile salt chosen as the surface-active agent. The complexation behavior and degree of association of insulin in aqueous solution was investigated by turbidity, zeta potential measurements and chromatographic analysis. The complexes were isolated and their molecular weight was determined by electrospray ionization-mass spectrometry (ESI-MS). Lastly surfoplex-loaded nanoparticles were tested for in vitro loading and release kinetics and pharmacological potential in diabetic rat model.

2. Materials and methods

2.1. Complexation of insulin

Poly(ethylene glycol) [PEG; M_n 2000], ε-caprolactone (ε-CL), human recombinant insulin (activity 28.6 IU/mg; expressed in *E. coli*) and streptozocin were purchased from Sigma–Aldrich, USA. Sodium deoxycholate was a product of Loba Chemie, Mumbai, India. All other reagents were of analytical grade and used as such. Ultrapure water (Milli-Q, Millipore Corporation, Bedford, USA) of resistivity 18.2 MΩ was used throughout the study.

Ion-pair complexes were prepared by adding a 1.72×10^{-3} M aqueous solution of sodium deoxycholate to 1 mg/mL of insulin solution in acidified water pH 2.5 till the formation of a precipitate. The solution was centrifuged at 14,000 rpm at 25 °C for 10 min and the supernatant was removed to assess the amount of uncomplexed insulin.

2.2. Chromatographic analysis of insulin and ion-pair complexes

Reverse phase-HPLC analysis was carried out to determine the amount of uncomplexed insulin. The method was derived from a reported method on Waters 1525 system equipped with a Waters 2487 UV detector (Khaksa et al., 1998). A Symmetry Shield RP18 (300 Å, 4.6 mm × 100 mm; 3.5 μm) column at 35 °C was used for analysis. The mobile phase consisted of 26% acetonitrile and 74% 0.2 M Na_2SO_4 (pH 2.3) with 1 mL/min flow rate and UV wavelength was set at 213 nm.

Size exclusion chromatography was carried out on a Protein Pak 125 (100 Å, 4.6 mm × 250 mm; 5 μm) column using the above method. Elution was monitored at 213 and 280 nm.

The extent of insulin released from the deoxycholate complex was studied in isotonic phosphate buffered saline (PBS, pH 7.4) at 25 °C. Approximately 1 mg of the prepared complex was incubated in 2 mL of the buffer and the amount of free insulin was determined in the supernatant obtained by centrifugation at 14,000 rpm for 10 min at 25 °C.

2.3. Turbidity measurements

The turbidity changes were monitored at 350 nm using a dual-beam spectrophotometer (Cary 100 Bio, Varian, USA). The insulin sample in distilled water, pH 6.5, with concentration of 1 mg/mL (1.72×10^{-3} M) was used in the experiment. The aliquots of 1.72×10^{-3} M deoxycholate were added into a cuvette filled with the protein solution and incubated for 10 min. The final volumes of the mixtures were adjusted to 1 mL. Simultaneously, the charge neutralization was determined in the same samples by zeta-potential measurements (Zetasizer Nano ZS, Malvern, UK). However, only clear samples could be measured by this technique.

2.4. Electrospray ionization-mass spectroscopy

To study the products formed on complexation of deoxycholate with insulin, ESI-MS analysis of the complexed insulin was carried out on a XStar MS/MS system (PerkinElmer, USA). Prior to analysis, the analyte solutions were mixed with 0.2% acetic acid and 30% acetonitrile to give a final concentration of nearly 2 ppm. Approximately 20 μL of each sample was loaded into a quartz microsyringe.

The molecular weights of the formed complexes were calculated from the m/z values. The number of deoxycholate molecules involved in complexation was determined as follows:

$$N_{SDC} = \frac{M_C - M_I}{M_{SDC}} \quad (1)$$

where in N_{SDC} is number of sod. deoxycholate units and M_C, M_I and M_{SDC} are the molecular weights of the complex, insulin and sod. deoxycholate, respectively. The average molecular weight of insulin was found to be 5807 Da (determined from mass analysis) and that of deoxycholate as 414.6, the formulae was written as

$$N_{SDC} = \frac{M_C - 5807}{414.6} \quad (2)$$

2.5. TNBS assay

The assay was modified from the reported method by Habeeb (1966) and carried out for increasing insulin:deoxycholate molar ratios (Habeeb, 1966). To 1 mL of the insulin solution (1 mg/mL) with molar aliquots of sod. deoxycholate, 1 mL of 4% $NaHCO_3$, pH 8.5 and 1 mL of 2,4,6-trinitrobenzene sulphonic acid (TNBS) were added and the solution was allowed to react at 40 °C for 2 h. 1 mL of 10% sodium dodecyl sulphate (SDS) was added to prevent the precipitation of protein on addition of 0.5 mL 1 M HCl and the absorbance was read at 348 nm. Aqueous solution of deoxycholate (1 mg/mL) was taken as a control.

2.6. Partition coefficient

The hydrophobicity of the insulin–deoxycholate complex was determined from 1-octanol/water partition coefficient at 25 °C. 1 mg of the prepared complex was vigorously shaken in equal volumes of 1-octanol and water mixture. The system was allowed to equilibrate for 4 h and the insulin concentration was determined in the aqueous and octanol phase by measuring absorbance at 280 nm.

2.7. Native PAGE characterization of insulin–sodium deoxycholate complexes

Native polyacrylamide gel electrophoresis (PAGE) analysis of the complexes was carried out by loading samples with an average of 10 μg protein content per well and run at constant voltage of 100 V per gel. Discontinuous PAGE was carried out as per Laemmli method (Laemmli, 1970). The gel dimension was 8 cm × 8 cm. The running buffer was Tris–glycine. The stacking gel, resolving gel and the running buffer was devoid of SDS. Since the complexes were not soluble in water, 20% ethanol was added in the sample buffer to assist dissolution. The gels were stained with Coomassie Blue R250 to identify the protein portion.

SDS-PAGE was also carried out under identical conditions in the presence of SDS in the gel, sample and running buffer.

2.8. FTIR analysis

Fourier Transform Infrared (FTIR) spectroscopy analysis of insulin, sodium deoxycholate and the adduct was carried out to study the complex formation. The spectra were collected in the frequency range of 4000–650 cm^{-1} on a Perkin Elmer Fourier Transform Infrared Spectrophotometer equipped with ATR accessory (zinc sulfide IRE with a 60° incident angle). All spectra reported