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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

are an average of 64 scans (data collection time was 120 s) with a resolution of 2 cm^{-1}, Fourier-transformed and ratioed against a background interferogram.

2.9. Preparation of complex-loaded nanoparticles

PCL–PEG–PCL (CEC1 and CEC2) triblock co-polymers were synthesized and Nps prepared by modified solid-in-oil-water (s/o/w) emulsion method instead of the double emulsion method as shown in Scheme 1 (Rastogi et al., submitted for publication). The use of the s/o/w method avoids the water–organic solvent interface formed during the first emulsification step. Approximately, 1.2 mg of the lyophilized complex (equivalent to 1 mg of insulin) was used for preparation of surfoplex-loaded Nps.

For determination of entrapment efficiency, approximately 5 mg of the prepared Nps were suspended in dichloromethane (DCM) and the solution was shaken with equal volume of 0.1 M HCl to partition insulin in the aqueous phase. The mixture was centrifuged at 5000 rpm for 5 min and the supernatant was separated. The process was repeated twice. Total protein assay was carried out by Bradford method (Bradford, 1976); absorbance at 595 nm was measured using UV–Visible spectrophotometer in comparison with a standard curve obtained by assaying known concentrations of BSA standard solution. Each sample was assessed in triplicate from three different batches.

2.10. Release of complexed insulin from triblock co-polymer

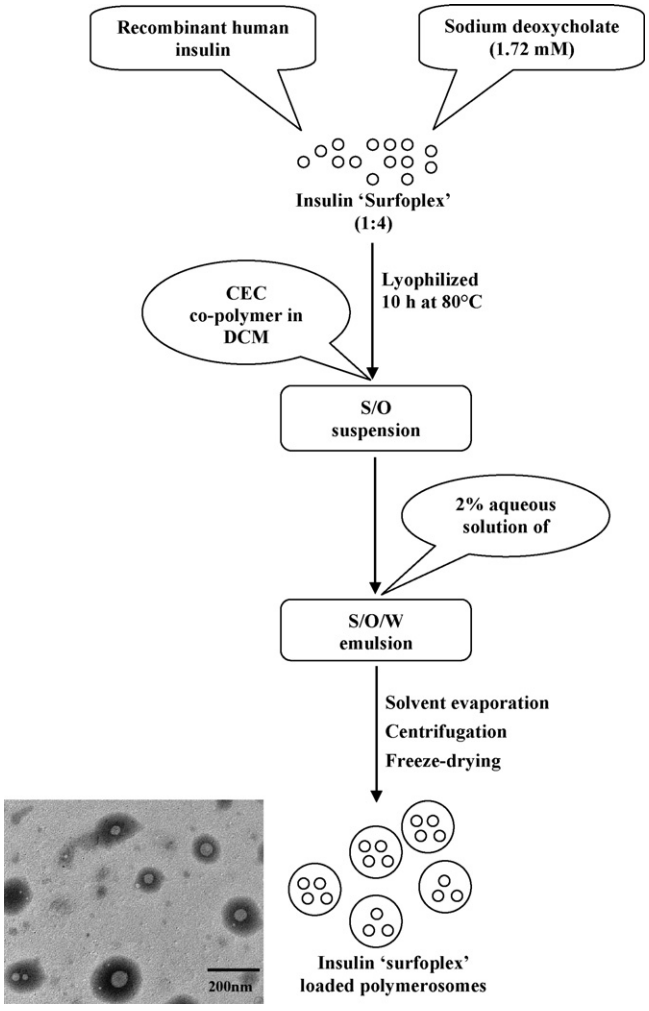
Both free insulin and surfoplex-loaded Nps (5 mg) were incubated in 1.5 mL of isotonic PBS pH 7.4 at 37 °C. At predetermined time intervals, the suspension was centrifuged at 14,000 rpm for 10 min at 25 °C. 0.5 mL of the supernatant was separated and analyzed for the amount of insulin released by Bradford method and replaced with same quantity of fresh buffer medium.

2.11. Pharmacological activity of complexed insulin

The protocols followed for in vivo studies were approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences, New Delhi, India. Adult Wistar rats, either sex with body weight ranging from 150 to 200 g were selected for the study. The animals were fasted for 16 h with free access to water prior to the experiment. Diabetes mellitus was induced by an intra-peritoneal (i.p.) injection of 45 mg/kg of streptozocin (STZ) in citrate buffer pH 4.5 in fasted animals under light ether anesthesia (Junod et al., 1969). The buffer was mixed to STZ just prior to the injection. The body weight and blood glucose levels of the animals were monitored regularly for the complete duration of the study. The diabetic status of the animals was confirmed 48 h after STZ administration. Animals were grouped into 5 sets ($n = 6$ in each group) namely, control, insulin (s.c.), complex (s.c.) and surfoplex-loaded CEC1 and CEC2 vesicles.

Free insulin and surfoplex (equivalent to 30 IU insulin/kg body weight) were administered subcutaneously to diabetic animals. Surfoplex-loaded CEC1 and CEC2 Nps were administered intravenously at insulin doses equivalent to 30 IU free insulin/kg body weight. Control animals were administered equal volumes of 0.9% normal saline. In all cases the volume of injected suspension or solution was 0.5 mL. 0.2 mL blood was withdrawn from the tail vein at 0, 1, 2, 4, 6, 8, 24, 28 and 32 h post-administration for estimation of blood glucose and serum insulin content. All results expressed are a mean of at least 6 animals.

Blood glucose was determined using Ascensia kit (Bayer Healthcare, range = 10–550 mg/dL). Serum was separated by centrifugation at 10,000 rpm for 10 min at 25 °C. Serum insulin concentration was determined by ELISA method (Mercodia Insulin ELISA kit, Sweden). The test is based on direct sandwich technique consisting of two monoclonal antibodies directed against separate antigenic determinants on insulin. It is followed by the reaction of the substrate 3,3′,5,5′-tetramethyl benzidine with bound horseradish peroxidase conjugated enzyme. The insulin concentration is determined from the absorbance at 450 nm (Microplate reader, ECIL, India) corresponding to a colorimetric endpoint obtained by stopping the above reaction.



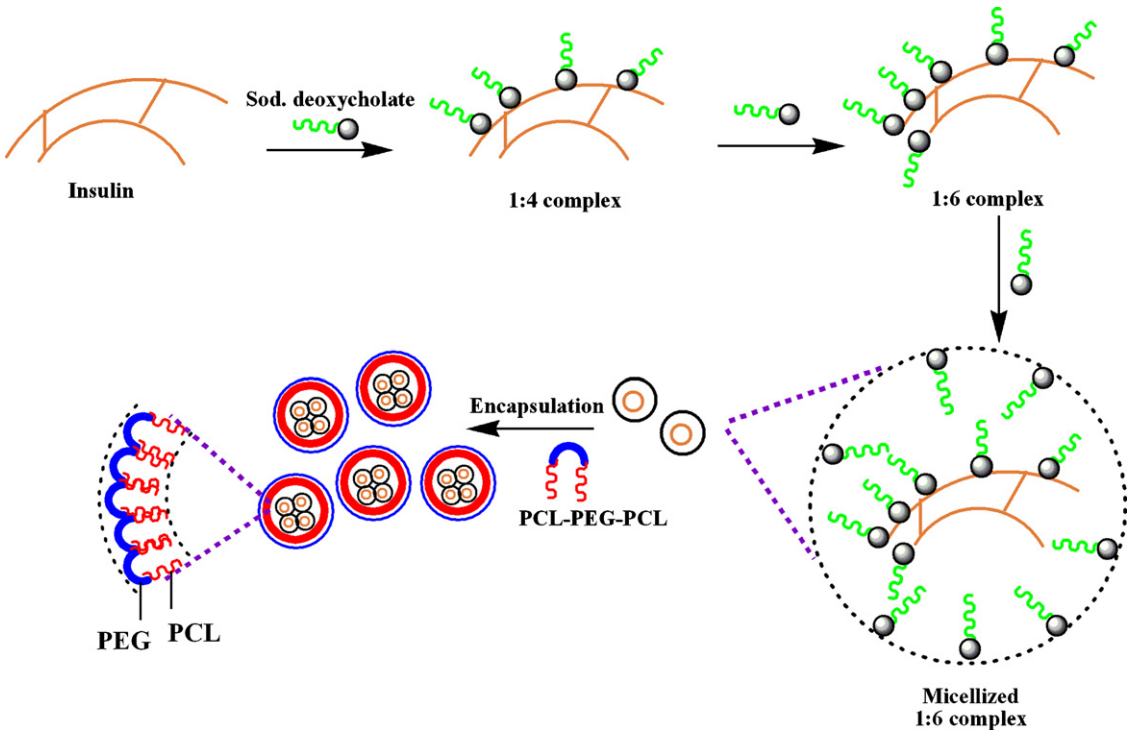
Scheme 1. Representation of the preparation procedure for insulin–'surfoplex'-loaded polymerosomes. Transmission electron micrograph of loaded polymerosomes with average particle sizes of 150–200 nm.

2.12. Statistical analysis

The means and standard errors for all values were calculated. For group comparisons a one-way analysis of variance (ANOVA) followed by Dunnett or a Bonferroni multi-comparison test were applied, using Prism 5 (Graph pad, San Diego, USA). The difference was considered to be significant when $P < 0.05$.

3. Results and discussion

Numerous approaches put forth to improve the encapsulation efficiency of protein-based drugs in colloidal carriers while maintaining their structural integrity include co-encapsulation of substances such as PEG, PVP, sugars, albumin and many other additives and alteration in formulation processing parameters (refer to



Scheme 2. Graphical representation of the formation of insulin–surfoplex on addition of sodium deoxycholate solution and its encapsulation in PEG and PCL co-polymer vesicles.

review by Bilati et al., 2005). The ion-pairing approach has also been experimented by various researchers with inconsistent results (Powers et al., 1993; Dai and Dong, 2007; Meyer and Manning, 1998; Kendrick et al., 1997; Choi and Park, 2000). The present study has therefore been aimed at preparation and characterization of insulin–deoxycholate complex, followed by its encapsulation in polymer vesicles. The ultimate goal is to determine whether ion-pair formation can assist in not only increasing the loading efficiency but also improve the properties of the formulations in biological systems.

Insulin is a 51 amino acid protein comprising of A and B chains each consisting of 21 and 30 amino acids, respectively, with a disulfide bridges on the A chain and 2 connecting the chains. At low pH in the range of 2–3, much below the isoelectric point, it is expected that the amino acids, histidine (pK_a ~6.0), arginine (pK_a ~13.0) and lysine (pK_a ~10.5) are protonated and are readily available for interaction with the $-COO^-$ group of deoxycholate. A report by Powers et al. (1993) has suggested that even the carboxylate side residues are reactive at this pH and can interact with SDS, the same has not been observed in the present study (Powers et al., 1993). Thus, from the pK_a values of the amino acids, it can be judged that the probability of complexation of deoxycholate with the $-NH_2$ group of Lys, the guanidine residue of Arg and the imidazole nitrogens of the two His is higher, thereby resulting in the formation of 4:1 adducts of deoxycholate to insulin. Further there is a possibility of interaction with the –N terminals of the A and B chains viz. the $-NH_2$ groups of Gly and Phe resulting in the formation of 6:1 complex. This phenomenon has been proven to be credible by the observations of the various studies conducted. The prepared surfoplexes have been encapsulated in polymer vesicles and their efficacy evaluated in vitro as well as in vivo. Scheme 2 is a representation depicting the formation of insulin–surfoplex with deoxycholate and its encapsulation thereafter in PCL and PEG-based vesicles. It should be noted henceforth, that the surfoplexes in discussion have been prepared by 1:4 insulin–deoxycholate molar ratio unless specified.

3.1. Formation of insulin–deoxycholate complexes

Addition of graded molar aliquots of deoxycholate to insulin solution resulted in alterations in the turbidity of the system. It has been postulated that amorphous aggregation of proteins can be detected by turbidity changes in solutions at 350 nm (Panyukov et al., 2008). In the present case, a uniform rise in the absorbance was observed till insulin to deoxycholate molar ratio of 1:4 was attained; thereafter a non-linear increase was noted with visible cloudiness in the solution (Fig. 1). Further addition of deoxycholate (above 8 moles) showed a steep rise in the concentration of adducts visualized as turbulence followed by a plateau phase suggesting a stage of equilibrium with nearly complete complexation and formation of dense white flocculating precipitates. In comparison to a report by Dai and Dong, 2007, the solution did not trend towards clarity as in insulin–SDS complex; however the system showed a visual increase in viscosity after addition of 20 moles of deoxycholate.

Zeta-potential measurements showed an instant neutralization of charge with a molar ratio of 1:1 (inset, Fig. 1). Further addition depicted a rearrangement in the insulin–deoxycholate complex till a molar ratio of 1:3. Turbidity was observed with higher concentration of deoxycholate in the ratio 1:4 and measurements could not be carried out beyond this value.

3.2. Chromatographic analysis

RP-HPLC was used to determine the amount of free insulin present after complexation. As observed from Fig. 2 a maximum of 6.97% of free insulin was detected after addition of 0.048 moles of deoxycholate to the medium (equivalent to 1:4 insulin–deoxycholate molar ratio). Further addition of deoxycholate till a molar ratio of 1:20 (insulin–deoxycholate) showed a gradual decrease in the uncomplexed insulin with a maximum of 97.2% complexation. No degradation products such as A-21 desamido insulin were detected during the time course of the

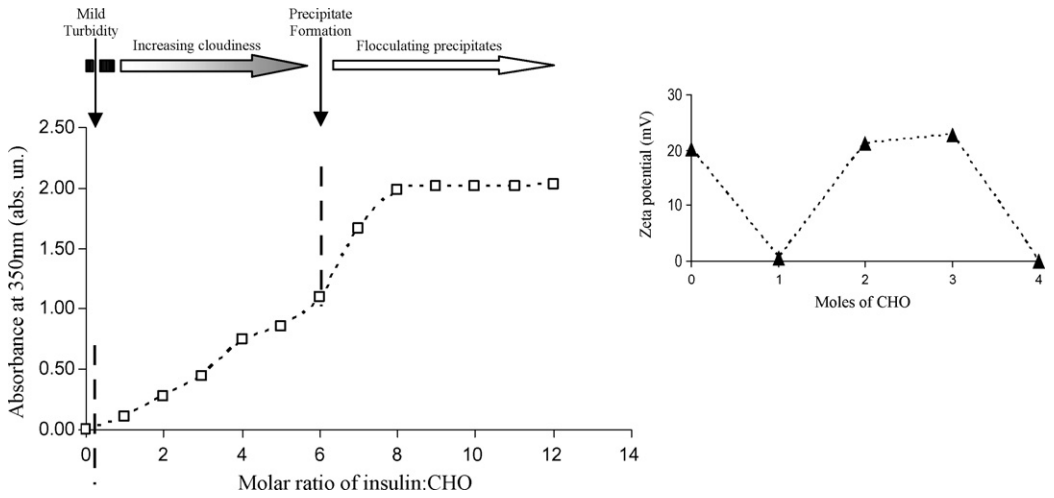


Fig. 1. Determination of turbidity changes with increasing molar ratio of deoxycholate measured as absorbance at 350 nm at 25 °C. Inset shows the changes in potential observed on addition of deoxycholate to insulin solution.

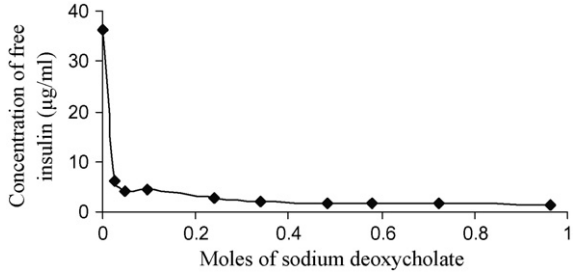


Fig. 2. Amount of uncomplexed insulin remaining on addition of graded concentrations of sodium deoxycholate as determined from RP-HPLC at 213 nm.

experiment suggesting that the formation of a complex assisted in stabilizing insulin in aqueous media.

To further understand the effect of complexation on protein stability, the formed adducts were separated by high speed centrifugation followed by freeze drying. The lyophilized samples were re-suspended in PBS pH 7.4 at 25 °C to assess whether the complex was stable and not easily dissociable. Fig. 3 shows the cumulative insulin released from the complex over a 72 h period. It was

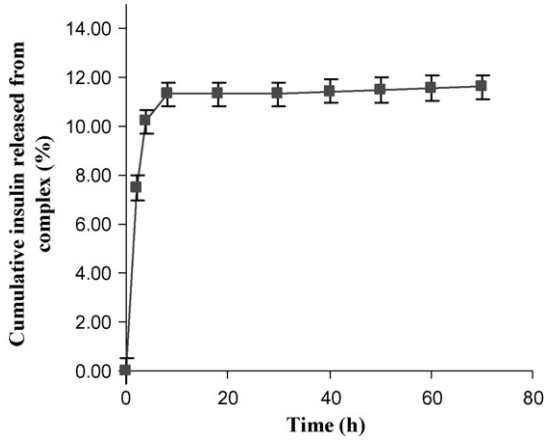


Fig. 3. Extent of insulin released from the deoxycholate complex (1:4; insulin:deoxycholate) in phosphate buffered saline pH 7.4 at 37 °C. The concentration of free insulin in solution was determined from RP-HPLC at 213 nm.

observed that after an initial release of approximately 7–10% of insulin within 2 h, the dissociation profile reached a constant with a maximum of 12% free insulin in the medium up to 72 h.

3.3. Mass determination of the formed complexes

Fig. 4 shows size exclusion chromatographic separation of insulin and the various complexes generated during the course of complexation. The molecular weight of the products formed was verified by mass analysis of the eluted sample (data not shown). Insulin was seen to elute around 12.5 min (Fig. 4A) with some minor peaks between 15 and 16 min. Analysis of the solution after complexation (1:4) showed the formation of a product observed as a peak at 15.3 min (Fig. 4B). Above 1:6 molar ratio, a further peak was observed at 16.4 min with further increased molecular weight. Beyond 10 moles deoxycholate concentration a stage of dynamic equilibrium between the two formed products was noticed signifying that insulin–deoxycholate complexation was an interactive process. Furthermore, the delayed elution times imply that the hydrophobicity of insulin has increased after complexation.

Fig. 5 shows the results of mass analysis carried out after graded additions of deoxycholate to insulin solutions. The products were separated by centrifugation and re-suspended in acidified water–acetonitrile mixture. The approximate mass of insulin was calculated to be 5807 amu. Addition of 1 mole of deoxycholate to a molar solution of insulin resulted in the generation of a minor peak corresponding to the attachment of 4 units of deoxycholate to a molecule of insulin with an average calculated mass of 7260 amu. Continued addition of deoxycholate to the system (4 moles), depicted a rearrangement with the formation of a species with average calculated mass = 9100 amu equivalent to association of approximately 8 deoxycholate molecules with that of insulin along with traces of 1:4 complexes. Successive increase in deoxycholate in excess resulted in stabilization of the 1:8 aggregates as well as the formation of a new species with an approximate mass of 11,200 amu.

As discussed earlier, an in-depth analysis of the structure of insulin yields a maximum of 6 probable sites of interaction on the basis of their pK_a value. Prior studies report the aggregation number of deoxycholate in the range of 5.82–6 by freezing point depression and static light scattering experiments (Jover et al., 1997; Reis et al., 2004). Jover et al. (1997) have also speculated that a higher value could arise as bile salt aggregates can be modified by the introduc-

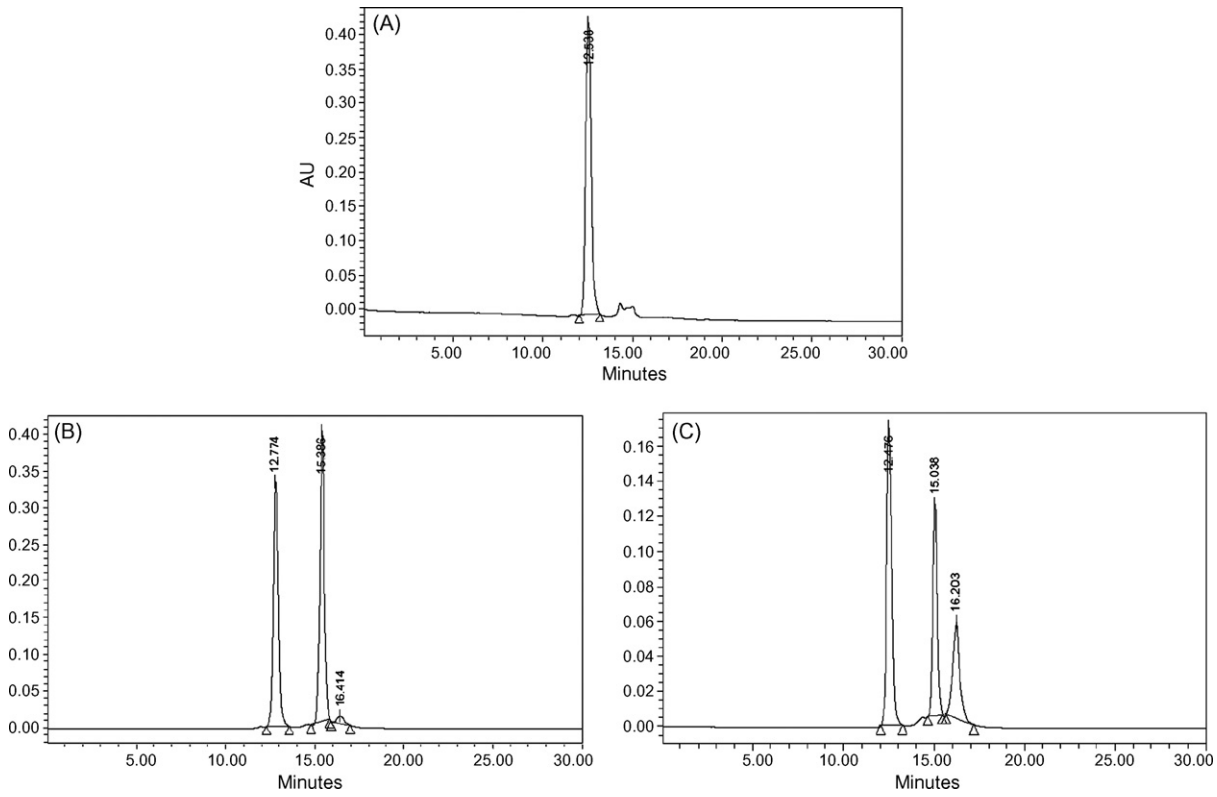


Fig. 4. SEC-HPLC separation of insulin (A), 1:4 (B) and 1:6 (C) complexes.

tion of guest molecules. Thus on the basis of the findings of the mass spectra we propose that the peak corresponding to 11,200 amu, might be a 1:6 insulin–deoxycholate complex enclosed within a deoxycholate micelle comprising of approximately 7 units.

3.4. Free amino groups are the site of interaction

To further prove that the accessible $-NH_2$ groups of the amino acid side chains are the only probable sites of interaction with deoxycholate, pure insulin and various complexes were analyzed by TNBS assay. 2,4,6-trinitrobenzene sulphonic acid is reported to interact with the free $-NH_2$ groups of proteins to form a colored complex (Habeeb, 1966). To ensure the absence of any interference, 1 mg/mL deoxycholate solution was also taken as sample. It was observed that increasing the molar ratio of deoxycholate in the protein solution resulted in decrease in absorbance thereby suggesting the utilization of the $-NH_2$ groups in the complexation. Further addition of deoxycholate to the system showed a rearrangement of the protein molecule which can be observed in Fig. 6 as a sudden increase in the absorbance at an insulin:deoxycholate ratio of 1:6. However, continued addition of deoxycholate suggested no changes in nature of the formed complex as the absorbance values were maintained. Thus the findings of the assay support the observations of the size exclusion chromatography and mass analysis suggesting that the mechanism of complexation is highly dynamic in nature wherein the reaction begins at the most accessible $-NH_2$ group (depending on the pK_a value) and continues till a stabilized micellar structure is obtained.

3.5. Assessment of conformational changes by FTIR

Fig. 7 shows a comparison of the FTIR spectra of insulin, deoxycholate and complex. Distinct amide I and II peaks of insulin are observed at 1643.33 and 1516.66 cm^{-1}. The hydrocarbons of deoxycholate are observed as asymmetric and symmetric stretching vibrations at 2933.33 and 2866.53 cm^{-1}. A hypsochromic shift in the hydrocarbon symmetric vibration to 2873.48 cm^{-1} is observed in case of the complex. Further an increase in the intensity of the Amide I vibration in the complex shows the incorporation of insulin. The amide II peak has a contribution of the C=O bending vibration and is observed in all the three samples. However in the spectra corresponding to deoxycholate and complex it is observed at 1556.24 cm^{-1}.

3.6. Native PAGE

It is known that movement under native conditions is an outcome of the charge to mass ratio of the biomolecule of concern (Zheng et al., 2007). On the other hand, presence of SDS in denaturing PAGE imparts a uniform charge on the polypeptide chain. Since formation of an ion-pair is dependent on the inherent charge on the protein molecule, it also depicts a possibility of competitive displacement of the ion-pairing agent viz. deoxycholate by SDS depending on the affinity for the protein. This phenomenon is observable in our findings comparing the two techniques. Adequate separation was obtained in 18% gels with 4% cross-linker concentration as seen in Fig. 8. Native electrophoretic separation showed formation of two bands, a dark band matching with insulin and a weaker one corresponding to the ion-pairs. In comparison, SDS-PAGE depicted a solitary band for the complex parallel to insulin. This clearly suggests that electrophoretic separation under native conditions will be an important tool to identify charge-based protein complexes.

3.7. Partition coefficient and entrapment efficiency of the complex in Nps

Since the main goal of complexation was to increase the lipophilicity of the peptide to ensure maximum loading in nanoparticulate carriers, the partition coefficient of the complex was

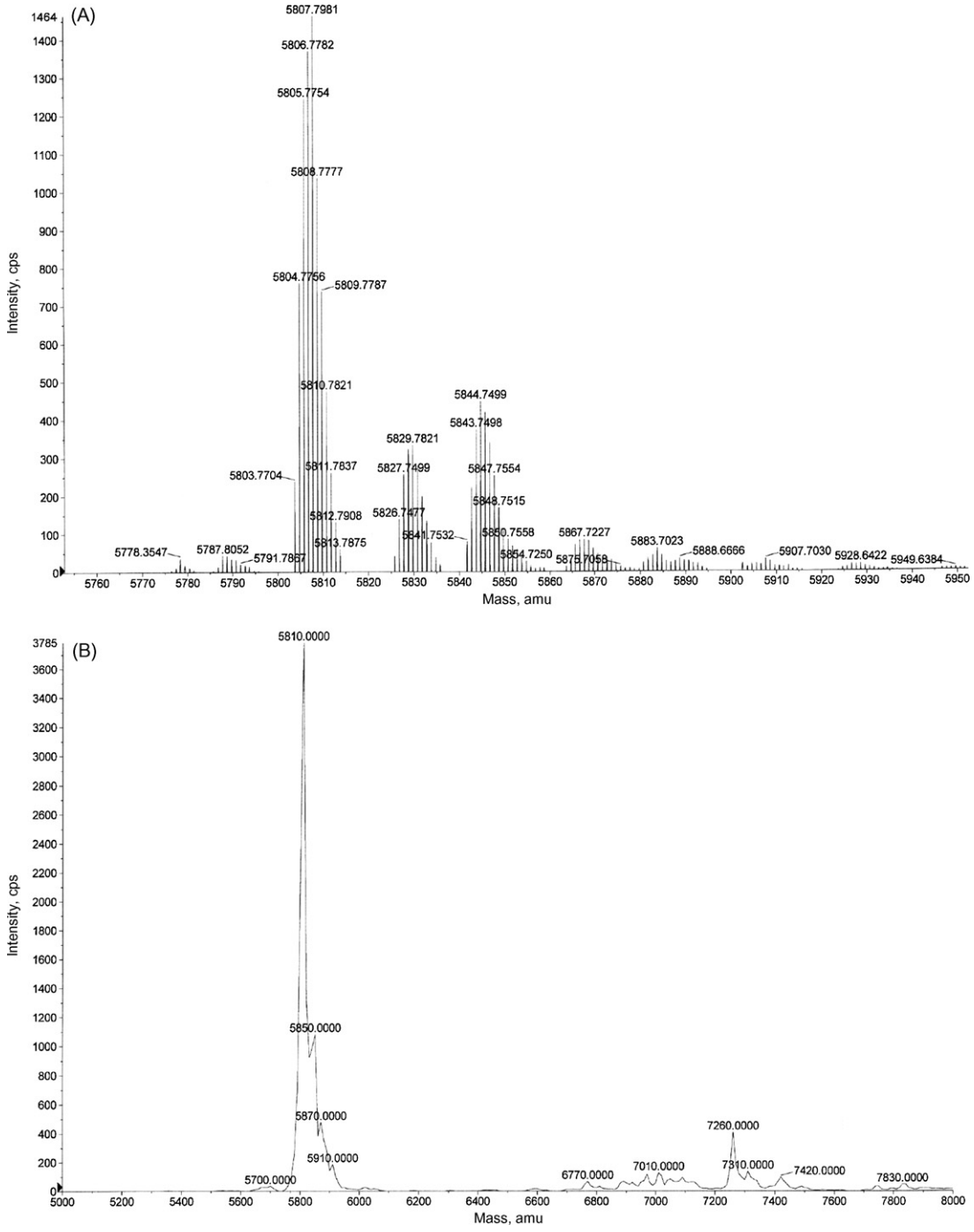


Fig. 5. ESI-MS spectra of insulin (A) and 1:4 insulin complex (B). Free insulin is seen as a main peak corresponding to 5807 amu in (A) and as 5810 amu in (B). The formation of a complex has been shown as a group of peaks with maximum at 7260 amu for insulin–deoxycholate molar ratio of 1:4.

determined. The octanol/water distribution coefficient of the complex was found to be 1.926 at room temperature as compared to 0.32 of pure insulin. This depicts that the deoxycholate interaction imparts a high degree of lipophilicity to the peptide.

The enhancement in the lipophilicity of the complex resulted in increase in the entrapment efficiency of insulin in various Np formulations as shown in Table 1. The data displays the augmentation in the drug loading upon complexation. The enhancement was found to be approximately 10% and 50% for CEC1 and CEC2 Nps. CEC1 Nps have a PCL content of 46.96% only while caprolactonic part is 68.43% in CEC2 Nps (Rastogi et al., submitted for publication). Thus, the variation observed in surfoplex loading is due to the difference in the hydrophobicity of the particle cores. CEC2 Nps being more hydrophobic show an increased affinity for the hydrophobic complex in comparison to CEC1 Nps.

A striking feature observed was the increase in the cumulative amount of insulin released from CEC1 Nps. This suggests that the formation of the complex reduced the extent of electrostatic interaction between the amino acids and the polymer chains as noticed earlier (Rastogi et al., submitted for publication). However, the hydrophobic interactions between the complexed protein and the PCL regions of the micellar nanoparticles still seem to persist thereby accounting for the controlled release of insulin from the Nps. On the other hand, in case of CEC2 Nps the total insulin

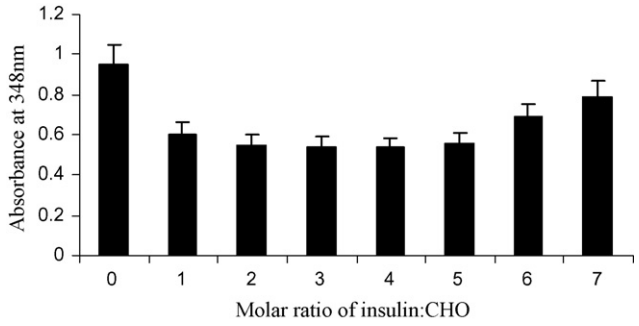


Fig. 6. TNBS assay of the insulin:deoxycholate complexes with increasing molar ratios of deoxycholate. The absorbance measured at 348 nm depicts the extent of free $-NH_2$ groups in the peptide.

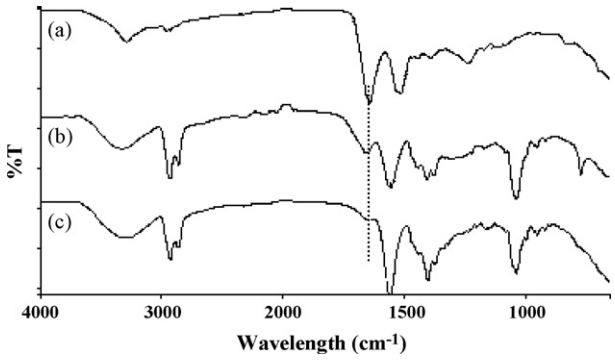


Fig. 7. ATR-FTIR spectra of insulin (a), 1:4 insulin–deoxycholate complex (b) and sodium deoxycholate (c). Dotted line highlights the Amide I band of insulin and its presence in the complex.

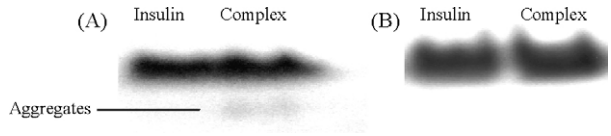


Fig. 8. Separation of complexes by native (A) and SDS- (B) PAGE in 18% acrylamide–bisacrylamide content with 4% cross-linker concentration in both the cases. Gels were run for 5 h at pH 8.3 at 100 V per gel.

detected in the medium was less in comparison to that observed in our previous study (Rastogi et al., submitted for publication). This is agreeable as the nearly 30% of burst release observed with insulin-loaded CEC2 Nps was not seen in the present case (Fig. 9). Thus, formation of surfoplex resulted in increased encapsulation efficiency as well as reduction in the burst release due to enhanced hydrophobicity of the macromolecule.

3.8. Pharmacological evaluation of surfoplex-loaded nanoparticles

The serum insulin levels were observed to be in the range of 35–40 mIU/mL for both free insulin and the surfoplex at 1 h (Fig. 10). Administration of free insulin (5 IU/kg body weight)

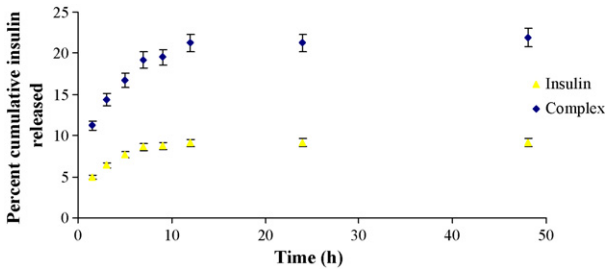


Fig. 9. Complexation of insulin also suppressed the burst release profile observed in the case of CEC2 Nps with reduction in the cumulative amount of drug released (50.9%).

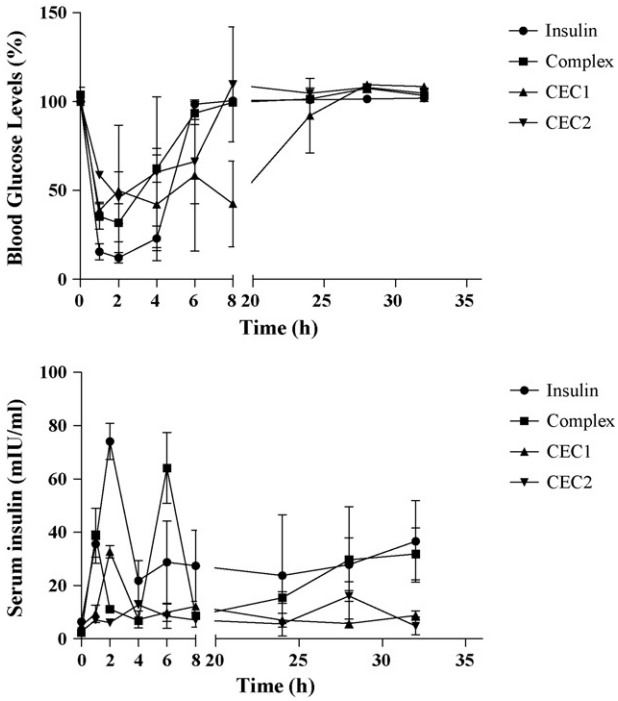


Fig. 10. Reduction in blood glucose (%) and changes in serum insulin concentration (mIU/mL) as observed after i.v. administration of 30 IU/kg body weight insulin–surfoplex-loaded nanoparticles in comparison to s.c. injection of free insulin (30 IU/kg body weight) and the surfoplex (equivalent to 30 IU/kg body weight).

resulted in decrease in %BGL to 15.54 ± 4.45% (serum insulin = 74.48 ± 6.28 mIU/mL) of the original level. The serum insulin concentration was seen to dwindle and tend towards the baseline values thereafter with negligible activity beyond 6 h. On the other hand, s.c. injection of surfoplex (equivalent to 5 IU insulin/kg body weight) showed lowering in BGL values to 26.58 ± 1.58% (serum insulin = 64.15 ± 13.28 mIU/mL; $P < 0.03$). Insulin–surfoplex was found to be active for 8 h in comparison to 6 h activity of free insulin. This proves that the therapeutic potential of insulin is maintained in spite of complexation. Moreover, surfoplexes being active for an increased duration suggests that complexation enhanced the serum stability of insulin also.

Table 1
Variation in insulin entrapment efficiency and cumulative release on loading of insulin–surfoplex in CEC Nps ($n = 3$).

Sample	[a]Entrapment efficiency (%)		Cumulative insulin released in 48 h (%)	
	Insulin	Complex	Insulin	Complex
CEC1	80.1 ± 2.5	88.5 ± 4.7	14.14 ± 6.2	22.52 ± 4.9
CEC2	32.9 ± 7.6	66.1 ± 5.9	90.53 ± 9.7	50.90 ± 8.1

[a] Determined by Bradford assay ($n = 3$ for all batches; $P < 0.05$).

Parenteral administration of surfoplex-loaded Nps in STZ-diabetic rats showed a lowering in the BGL in all formulations (Fig. 10). The maximum reduction in %BGL observed with CEC1 and CEC2 Nps was 38.65 ± 4.68% and 58.70 ± 1.84% of the initial values, respectively, within 1 h of administration (Fig. 10a). Sustained hypoglycemia was maintained for a period of 24 and 8 h, respectively, with the above formulations. CEC2 Np show a sharp decrease in glucose level to 18.92 ± 4.21% of the basal value (serum insulin = 12.77 ± 1.18 mIU/mL) at 4 h followed by complete inactivation with the BGL values returning to the basal levels at 8 h. In comparison, CEC1 Nps showed a persistent decrease in BGL values to 40–60% of the original level for 24 h. Serum insulin levels showed a peak at 2 h corresponding to 32.72 ± 2.34 mIU/mL for CEC1 Nps. This is of importance as loading of free insulin in our previous study was seen to generate intermittent peaks due to erosion of the polymer matrix thereby enhancing the dissolution of the encapsulated protein (Rastogi et al., submitted for publication). Thus, the formation of surfoplex is seen to control the rate of release of insulin from the polymer matrix. However, it failed to prolong the duration of action of the formulations in both the cases (CEC1 and CEC2 Nps).

From the above observations it is summarized that surfoplexes were seen to enhance the duration of therapeutic activity in comparison to free insulin in the present study. It also led to increase in the encapsulation efficiency in all formulations however the insulin release profile from surfoplex-loaded Nps was varied in comparison to that noted in insulin-loaded Nps. In our previous study, we have also shown that loading of insulin in the same carriers have a prolonged action for a period of 22–36 h for various formulations. It was therefore expected that increasing the hydrophobicity of insulin may enhance the duration due to higher loading. However, loading of surfoplex in CEC Nps had a deleterious effect on the efficacy as the formulations were active for a maximum of 24 h only. These findings imply that the efficacy of colloidal carriers cannot be predicted merely on the basis of the in vitro results. Extensive in vivo studies are a prerequisite to decide the therapeutic potential of these systems.

Overall the study highlights that an optimum hydrophilic–hydrophobic balance is necessary to ensure minimal interactions between macromolecules and polymer chains such that the efficacy of the formulation is maintained.

4. Summary

Insulin–surfoplexes with sodium deoxycholate were prepared and characterized for stability and extent of complexation. Mass analysis of the formed complexes showed adducts with approximate molecular weights of 7260, 9100 and 11,200 amu corresponding to 1:4, 1:8 and 1:13 insulin–deoxycholate ratios. TNBS assay confirmed the free amino groups as the site of complexation. The prepared surfoplexes were efficiently encapsulated in PCL and PEG-based polymer vesicles. The increase in entrapment efficiency for the complex was found to be in the range of 10–50% in comparison to that of free insulin. Complexed nature of insulin also altered the release profile from the nanoparticles with reduction in burst release. The therapeutic potential of insulin was maintained after complexation as shown by reduction in BGL to approximately 30% of its original value. Also the duration of activity showed an increase by 2 h with respect to free insulin. However, due to considerable increase in lipophilicity the pharmacological efficacy of complex-loaded nanoparticles was lesser than that of insulin-loaded systems. The study therefore suggests that equilibrium between the hydrophilic and hydrophobic nature of the load and the carrier is a prerequisite for in vivo activity. Also, therapeutic potential of formulations cannot be modeled solely on the basis of their in vitro activity and studies in biological systems are absolutely necessary.

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