

Design and Evaluation of Hydrophobic Ion-Pairing Complexation of Lysozyme with Sodium Dodecyl Sulfate for Improved Encapsulation of Hydrophilic Peptides/Proteins by Lipid-Polymer Hybrid Nanoparticles

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Abstract

Hydrophobic ion-pairing (HIP) complexation-based approach has been employed to reduce aqueous solubilities of peptide and protein drugs. The solubility of a protein molecule is due to presence of charged amino acids present on the surface. HIP complexation is a technique to complex ionizable functional groups of protein and peptide molecules with oppositely charged functional groups of a complex forming agent. The main objective of this study was to formulate and evaluate HIP complexes of lysozyme with sodium dodecyl sulfate (SDS) as an ion pairing agent. Results of % binding efficiency shown that the formation of HIP complexes were dependent on pH of lysozyme solution and molar ratio of lysozyme to SDS. Aqueous solubilities of HIP complexes were very low compared to lysozyme alone. The effect of HIP complexation on enzymatic activity of lysozyme was also studied. Further, lipid-polymer hybrid nanoparticles (LPNs) loaded with lysozyme and lysozyme:SDS HIP complex were prepared by using a solid-in-oil-in-water (s/o/w) emulsion solvent evaporation method and characterised with respect to morphology, size and encapsulation efficiency. We observed significant improvement in encapsulation efficiency for lysozyme:SDS HIP complex-loaded LPNs. This study demonstrates a novel approach of formulating protein-loaded nanoparticles which can also be employed for delivery of proteins.

Keywords: Hydrophobic ion-pairing complex; Protein; Sodium dodecyl sulfate; Lipid-polymer hybrid nanoparticles

Introduction

The double-emulsion solvent evaporation techniques are widely used for encapsulation of most water soluble peptides/proteins to micro- and nanoparticulate drug delivery systems. However, the main problems were reported that, by this technique, the instability of water-in-oil pre-emulsion induced the lower drug encapsulation efficiency and the peptide or protein drug was inactivated during the preparation process [1,2]. To solve these problems, basically three approaches were considered to be adoptable: (I) Increasing the lipophilicity of hydrophilic drugs, such as by means of hydrophobic ion pairing (HIP), namely pairs of hydrophilic drugs and the amphipathic molecules with opposite charges [3], or lipophilic prodrug formed by chemical conjugation with hydrophobic component [4]; (II) Non-aqueous techniques during encapsulation so as to reduce drug leakage, for example, compressed gas anti-solvent precipitation techniques [5], oil-in-oil (O/O) emulsion-evaporation method [6], etc.; (III) Improving the hydrophilicity of nanoparticles by using hydrophilic materials or introducing functional groups that can be easily substituted or conjugated to the particle matrix. For example, substituting acyl groups on poly(glycerol adipate) backbone (PGA) with various amounts of pendant C18 chain by which the dexamethasone phosphate loaded nanoparticles were yielded [7].

The hydrophobic ion-pairing (HIP) complexation has emerged as an alternative approach to formulate dosage form for hydrophilic molecules especially for peptide and protein drugs [8-12]. HIP complex is formed by electrostatic interactions between ionizable groups of protein and peptide molecules with oppositely charged functional groups of complex forming agents such as surfactants or other amphiphilic molecules at suitable pH. Basic amino acids have been employed (mainly lysines and arginines) to form a HIP complex with anionic surfactant molecules. The complex is reversible in nature and can easily dissociate in the presence of excess of oppositely charged ions [13]. The formed HIP complex is highly lipophilic in nature and

is able to partition largely in to hydrophobic polymer matrix during encapsulation process rather than the external aqueous medium [14,15]. As a result, HIP complexation significantly enhances encapsulation efficiency. This approach offers several advantages including (i) alteration the aqueous solubility of a hydrophilic protein molecule in a reversible manner, (ii) higher solubility of complex in the lipid phase which may result in higher permeation across cell membrane, (iii) enhanced encapsulation of protein molecule in a polymeric matrix, (iv) and (v) providing conformation stability to the protein molecule in the presence of organic solvents. Due to above mentioned reasons, HIP complexation-based technique has started to get acceptance in the preparation of colloidal dosage form such as nanoparticles for protein-based therapeutics [14,16,17].

In this study, a more liposoluble lysozyme-sodium dodecyl sulfate complex was prepared using the hydrophobic ion pairing method. Sodium dodecyl sulfate (SDS) was used as an ion pairing agent. Then, effect of lysozyme solution pH and molar ratio of lysozyme to SDS on properties of HIP complex were investigated. Furthermore, lipid-polymer hybrid nanoparticles (LPNs) loaded with lysozyme and lysozyme:SDS HIP complex were prepared by using a solid-in-oil-in-water (s/o/w) emulsion solvent evaporation method and characterized with respect to morphology, size and encapsulation efficiency.

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A wide variety of compounds with significantly diverse physicochemical properties have been developed in the last decade due to adaptation of combinatorial chemistry and high-throughput screening based approaches. Formulation development of such a diverse set of compounds poses a very significant challenge. Colloidal dosage forms have been introduced and widely investigated as drug delivery systems for both hydrophilic and hydrophobic molecules. Nanoparticulate based formulation approach has been very successful for the delivery of small and large molecules.

Hydrogen bond: These interactions arise from partial sharing of hydrogen atom between H bond donor and acceptor. OH and NH serve as hydrogen bond donors while nitrogen and oxygen serve as hydrogen bond acceptors. In the protein structure, the peptide backbone and other polar amino acid side chains also serve as hydrogen bond donors and acceptors. A hydrogen bond is considered as a type of dipole-dipole interaction. Secondary structure of the protein (α helix and β sheet) originates from the hydrogen bonds in protein molecule.

Materials and Methods

Chemicals

Lysozyme, egg white was obtained from Vivantis (USA). Sodium dodecyl sulfate (SDS) was obtained from Sigma (USA). Poly(ϵ -caprolactone) (PCL) (Mw=14 kDa) was purchased from Aldrich (USA). *Micrococcus lysodeikticus* (ATCC No. 4698) were obtained from Sigma-Aldrich (Germany). Microbicinchoninic acid (microBCA) protein assay reagent kit was purchased from Pierce Biotechnology (Rockford, IL). Ultrapure water (Millipore Model Milli-Q Water Purification System, Canada) was used for all the experiments. All other chemicals used were analytical grade.

Preparation of HIP complexes of lysozyme

Briefly, 10 mg of lysozyme was dissolved in pH 6 phosphate buffer solution (PBS) and pH of the lysozyme solution was adjusted with 0.1 N HCl to four different pH values (4, 4.5, 5 or 5.5). Following pH adjustment, the solution of SDS as ion pairing agent was added slowly to lysozyme solution. HIP complex was formed spontaneously as both aqueous solutions were mixed in optimum ratios. The four different molar ratios (0.042, 0.056, 0.083 and 0.167) were evaluated in this study. Once formed, the HIP complex was vigorously vortexed for 5 min followed by centrifugation at 14 000 rpm for 12 min to separate supernatant. The resulting HIP complex was redispersed in MilliQ water and lyophilised into powder. Uncomplexed lysozyme was measured in the supernatant using microBCA assay.

Characterisation of the HIP complexes

Determination of lysozyme binding efficiency: The amount of lysozyme within the complexes was determined by indirect method. Briefly, uncomplexed lysozyme remaining in the supernatant was measured by microBCA assay kit. The percentage of binding efficiency was expressed as the percentage of lysozyme difference between the initial amount of lysozyme and the free amount in the supernatant is relevant with the initial amount of lysozyme added for the complex preparation. The percentage of binding efficiency of lysozyme was calculated by the following equation:

$$\% \text{ Binding efficiency} = \frac{(M_i - M_f)}{(M_i)} \times 100 \quad \% \text{ Binding efficiency} = \frac{(M_i - M_f)}{(M_i)} \times 100$$

where M_i denotes initial amount of lysozyme added and M_f represents amount of free lysozyme measured in supernatant.

Dissociation of lysozyme from HIP complexes: Dissociation of lysozyme from HIP complex was studied to characterize the nature of interaction between lysozyme and SDS. Initially, HIP complex of lysozyme and SDS were prepared at the molar ratio of 0.083 in pH 4.5 PBS followed by measurement of uncomplexed lysozyme in the supernatant. Then, 3 mg of lyophilised HIP complex was incubated in the PBS having different concentrations of NaCl (0.1, 0.5 and 1 M). Moreover, HIP complex was also redispersed in the MilliQ water for comparison. The solution was mixed and kept for equilibrium for 24 h at room temperature. Then, the solution was subjected to centrifugation at 14 000 rpm and supernatant was collected. Concentration of dissociated protein in the supernatant was then measured with microBCA assay.

Evaluation of enzymatic activity of lysozyme after dissociation from HIP complex

Initially, HIP complex was incubated with 1 ml of PBS containing 0.1, 0.5 or 1 M NaCl for 24 hrs. This incubation resulted in dissociation of the HIP complex. This solution was further subjected to centrifugation. The enzymatic activity of lysozyme in supernatant was measured and compared with freshly prepared lysozyme solution. Briefly, 0.2 mg/ml dispersion of *M. lysodeikticus* (Sigma-Aldrich, USA) was prepared in a 66 mM phosphate buffer pH 6.6. To 2.9 ml of this suspension, 0.1 ml of the lysozyme-containing solution was added. The activity of lysozyme was determined by measuring the reduction in optical density of a *M. lysodeikticus* suspension at 450 nm. Enzyme activity was deduced from the slope of the curve.

Preparation of lipid-polymer hybrid nanoparticle (LPNs)

The LPNs were prepared by a solid-in-oil-in-water (*s/o/w*) emulsification-solvent-evaporation method. Briefly, 200 mg PCL and 45 mg PC:glyceryl tripalmitate mixture were dissolved in 8 ml DCM:acetone mixture as organic solvents to form the oil phase (o). The lyophilised HIP complex of lysozyme with SDS as solid phase (s) was dispersed in the oil phase by sonication for 2 min in an ice bath. The resultant dispersion (*s/o*) was poured into 10 ml %1 (w/v) Pluronic F-127 solution (w) and was sonicated again for 5 min in an ice bath. Afterwards, the nano-emulsion (*s/o/w*) was stirred overnight at room temperature to evaporate off organic solvents, and the resultant LPNs were collected by centrifugation using Vivaspin 20 centrifugal concentrator (Sigma-Aldrich, USA), washed with ultrapure water (MilliQ water) and lyophilized to obtain free flowing powder. As a control, LPNs were prepared by using powder lysozyme alone.

Physicochemical characterizations of HIP complex-loaded LPNs

Morphology of nanoparticles was analysed by atomic force microscopy (AFM). Firstly, the nanoparticles were diluted in deionized water to an appropriate concentration and ultrasonicated for 30 minutes. 50 μ l of the dilution were put on a mica freshly cleaved before use, incubated 30 minutes, and the residual solution was blown by a stream of nitrogen. Then, AFM images were obtained by scanning the mica surface in air under ambient conditions using AFM (eZAFM, Nanomagetics, Turkey; eZAFM analysis software Version 3.23).

The size, polydispersity index and zeta potential of the LPNs in an aqueous suspension were measured by dynamic light scattering (DLS) using the Malvern Zetasizer (Malvern, UK); all measurements were taken at 25°C.

The drug encapsulation efficiency was determined from the ratio of

the encapsulated lysozyme to the lysozyme initially added. The amount of lysozyme was determined by using microBCA protein assay reagent kit according to the instructions of the manufacturer [18,19]. All experiments were performed in triplicate. Background readings were corrected for using the supernatant of blank LPNs of a corresponding batch.

Results and Discussion

Preparation of HIP complexes of lysozyme

HIP complexation has been investigated due to its potential to form a hydrophobic reversible complex with small molecules, peptides, and macromolecule using oppositely charged molecules. Basically, HIP complexation lowers the aqueous solubility of a charged molecule and, hence, encapsulation of the molecule can be significantly enhanced in a polymeric matrix. Other major advantages of HIP complexation include restriction of conformational flexibility of macromolecules thereby imparting physical stability. In this study, application of HIP complexation was demonstrated lysozyme as a model protein. It is a basic protein (129 amino acids) and contains numerous basic amino acids which can be complexed. SDS was used as an ion pairing agent. Effect of lysozyme solution pH and molar ratio of lysozyme to SDS on properties of complex were investigated.

Characterization of HIP complexes

Determination of lysozyme binding efficiency: HIP complexes of lysozyme were prepared and characterized with respect to % lysozyme binding efficiency. HIP complexation was optimized with respect to pH of lysozyme solution and molar ratio of lysozyme to SDS. Effects of four different pH conditions on binding efficiency of lysozyme have been evaluated. When molar ratio of lysozyme to SDS was kept constant, a higher binding efficiency (more than 90%) has been observed when pH was less than 5 which might be attributed to ionization of basic amino acids in greater extent under these conditions (Figures 1a-1d). Protonation of these amino acids are dependent on pH of surrounding medium which promote complexation by ionic interactions amongst oppositely charged species. Subsequently, effect of different molar ratios of SDS to lysozyme on binding efficiency was also evaluated (Figures 1a-1d). Four different molar ratio of lysozyme to SDS were investigated. Results suggest that molar ratio of lysozyme to SDS affected % binding efficiency of lysozyme dependent on pH of lysozyme solution. At pH 4, maximum % binding efficiency of lysozyme was observed with the

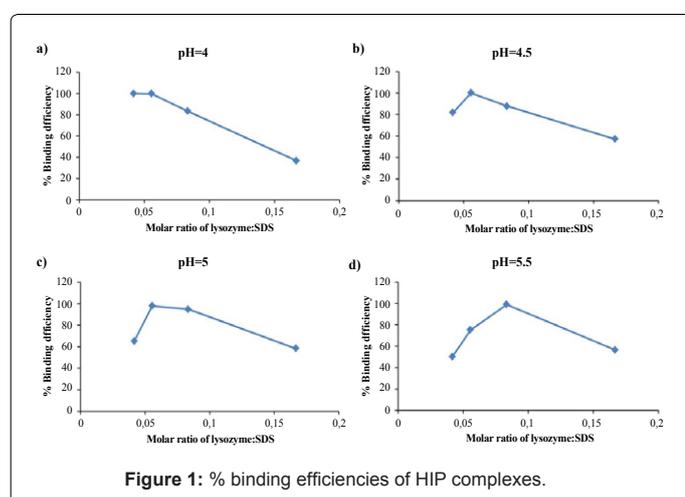


Figure 1: % binding efficiencies of HIP complexes.

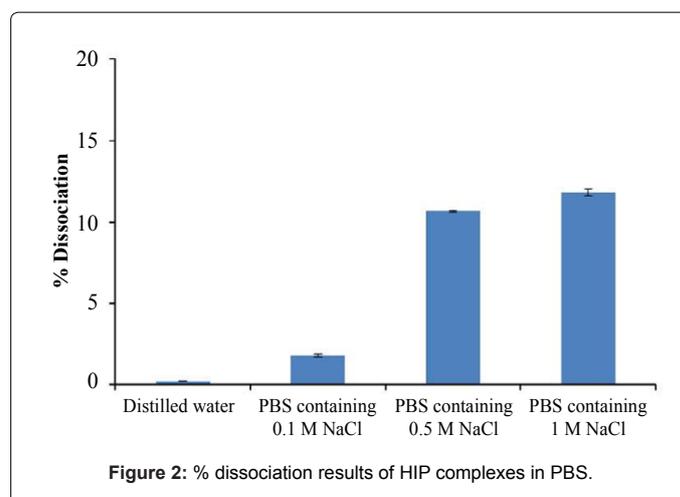


Figure 2: % dissociation results of HIP complexes in PBS.

lysozyme:SDS molar ratios of 0.042 and 0.056. However, % binding efficiency significantly decreased by using the molar ratio of 0.167. As shown in Figure 1b, % binding efficiency of lysozyme significantly increased when molar ratio of lysozyme to SDS was increased from 0.042 to 0.056 at pH 4.5 and 5. Similar to pH 4, further increment in molar ratio showed significant decreased in binding efficiency (Figures 1b,1c). A rise in binding efficiency of lysozyme was noted with increase of molar ratio up to 0.083 at pH 5.5. However, % binding efficiency significantly decreased when molar ratio of lysozyme to SDS to be 0.167 (Figure 1d).

Dissociation of lysozyme from HIP complexes: In this study, hydrophobicity of lysozyme was enriched by complexing with SDS, ion-pairing agent. The octanol:water partition coefficient is more common approach to determine hydrophobicity/hydrophilicity of molecule. However, complexes formed with lysozyme were insoluble in octanol and hence not amenable to this method. Therefore, to assess the augmentation in hydrophobicity, aqueous solubility of resultant HIP complexes was measured. All the complexes have shown very low aqueous solubility compared with lysozyme. As shown in Figure 2, the ability of HIP complexes to reduce the aqueous solubility of lysozyme has been confirmed.

Ionic interactions are the driving forces for the HIP complexation which are delicate and can be dismantle in the presence of higher ionic strength. The dissociation studies indicate how ionic forces may be essential for complexation and can be disrupted in the presence of strong ionic medium such as PBS. To test this hypothesis, dissociation study of complexes was carried out in PBS containing different amount of NaCl (0.1, 0.5 and 1 M) and MilliQ water as well. Results in Figure 2 demonstrate that 11.8% of lysozyme dissociated from lysozyme:SDS HIP complex in PBS presence 1 M NaCl. However, negligible dissociation (0.2%) was observed in water which confirms existence of ionic interactions between lysozyme and SDS.

Liposomes are lipid vesicles containing aqueous core which have been widely exploited in ocular delivery for various drug molecules. Various liposome based formulations were developed and delivered to treat both anterior and posterior segment diseases conditions (Figure 3).

Evaluation of enzymatic activity of lysozyme after dissociation from HIP complexes

Bioactivity assay was carried out on the lysozyme dissociated from

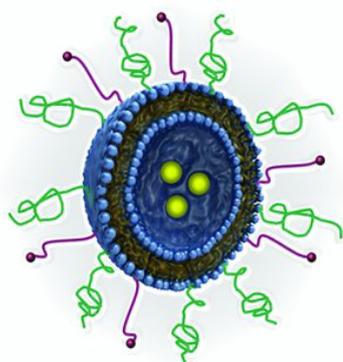


Figure 3: Liposome Hybrid Nanoparticles.

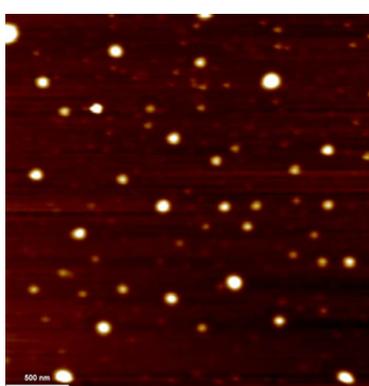


Figure 4: Topography image of lysozyme:SDS complex-loaded LPNs.

Molar Ratio of NaCl in PBS (M)	% Enzymatic Activity
0.1	88.7
0.5	85.4
1.0	81.3

Table 1: % enzymatic activity results of lysozyme dissociated from HIP complex.

lysozyme:SDS complexes by using turbidimetric method. According to the results of bioactivity assay, 81.3-88.7% bioactive lysozyme was recovered from the complexes (Table 1). These results suggested that complexation of lysozyme with SDS didn't cause a significant negative impact on biological activity of lysozyme.

Preparation and characterization of HIP complex-loaded LPNs

LPNs containing HIP complex of lysozyme with SDS were prepared by *s/o/w* emulsification-solvent-evaporation method. PCL was chosen as the polymeric core material due to its great biodegradability and biocompatibility [20]; phosphatidylcholine (PC) and glyceryl tripalmitate were selected as lipids. Furthermore, lysozyme-loaded LPNs were prepared to compare with complex-loaded LPNs.

Atomic force microscopy was performed to study the nanoparticle surface morphology. We have analysed morphology of lysozyme:SDS HIP complex-loaded LPNs. As shown in Figure 4, lysozyme:SDS HIP complex-loaded LPNs have smooth surface and spherical shape.

LPN Formulations	Average Particle Size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
Lysozyme-loaded LPNs	114.43 ± 0.91	0.27 ± 0.003	-5.5 ± 0.05	11.69 ± 0.05
Lysozyme:SDS complex-loaded LPNs	112.53 ± 0.133	0.164 ± 0.012	-2.9 ± 0.04	42.85 ± 0.08

Table 2: Characterization of lipid-polymer hybrid NPs.

As shown in Table 2, a significant change didn't occurred in the size of the LPNs by using lysozyme:SDS complex compared to LPNs prepared with lysozyme alone.

The resultant LPNs were also characterized with respect to zeta potential (Table 2). The zeta potential value of LPNs were tended to slight increase (less negative) with lysozyme:SDS complex.

Higher encapsulation efficiency was achieved with lysozyme:SDS complex with average % encapsulation efficiency of 42.85% (Table 2). However, when lysozyme alone was used % EE was less than 12% (Table 2). The increased encapsulation might be attributed to more partitioning of HIP complex into polymer matrix and lower solubility into aqueous external phase. On the other side, higher water solubility of lysozyme favours its diffusion to aqueous external phase leading to poor encapsulation efficiency.

Conclusions

This study for the first time shows the feasibility of forming HIP complex of lysozyme as a model protein with SDS as an ion pairing agent. % binding efficiency studies showed that pH of lysozyme solution and molar ratio of lysozyme to SDS affected formation of HIP complex. Moreover, there was no any deleterious effect of HIP complexation on biological activity of lysozyme. We have successfully prepared and characterized LPNs of lysozyme in HIP complex form using *s/o/w* emulsification-solvent evaporation method. Significant encapsulation of lysozyme in LPNs has been obtained using the HIP technique. To sum up, HIP complexation can be a promising approach to enhance encapsulation of peptides and proteins in nanocarriers.

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