Protein–Fatty Acid Complex for Enhanced Loading and Stability within Biodegradable Nanoparticles

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ABSTRACT: Lysozyme was hydrophobically modified with a fatty acid, sodium oleate, via an ion-pairing mechanism. Ionic binding between an anionic carboxylic group of sodium oleate and basic amino groups in lysozyme was primarily utilized to form lysozyme–oleate complex. The complex formation was pH dependent. The lysozyme–oleate complex dissolved in an organic solvent exhibited much higher conformational stability at elevated temperature compared with free lysozyme in the same solvent. The complex was formulated into biodegradable nanoparticles by a spontaneous emulsion and solvent diffusion method. The resultant formulation showed near 100% encapsulation efficiency of lysozyme within nanoparticles with <100 nm in diameter with a narrow size distribution. Lysozyme could be loaded into the nanoparticles up to 18.6% (w/w) with concomitantly increased particle sizes. This study demonstrates a new formulation method of biodegradable nanoparticles with highly efficient encapsulation of proteins, which are potentially useful for oral protein delivery including mucosal vaccination. © 2001 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 90:194–201, 2001

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INTRODUCTION

Peroral drug administration represents one of the most convenient method among drug delivery routes.1–3 For protein or peptide drugs, however, this route is challenging because of a number of problems to be overcome. Major problems include poor absorption of protein drugs through mucosal membrane and drug instability during drug transition in the gastrointestinal (GI) tract. Many approaches have been made to circumvent these problems, including the employment of microparticulate carriers such as nanoparticles or microemulsions.4,5,7,8 Endocytic uptake of the microparticulates containing protein drugs has been known to readily occur in the region of Peyer's patch located in the specialized mucosal layer of the GI tract. Particularly, oral vaccination based on biodegradable microspheres has utilized such a particulate endocytosis mechanism as a critical route for mucosal immunization. Thus, there have been a growing number of studies on the formulation variables of microparticles (e.g., size, hydrophobicity, surface charge, and the presence of cell-specific ligands on the surface), all of which affect the intracellular uptake of microparticles. It has been shown that microparticles of <5-μm diameter were taken up more efficiently into the cells. The most common method to prepare such small microparticles encapsulated with protein molecules has been based on a water-in-oil-in-water (W1/O/W2 solvent evaporation method.4,6 This method, however, can not guarantee sufficient protein encapsulation efficiency and loading within the microparti-
cles of $<5$-µm diameter. As the size of the microparticles is reduced by increasing the speed of homogenization during the second emulsification step in the W/O/W formulation process, the protein molecules entrapped within the primary emulsion droplets tend to escape into the $W_2$ phase, resulting in low protein encapsulation efficiency and loading. Additionally, a high shear rate generated during the homogenization process makes the entrapped protein molecules susceptible to denaturation and aggregation. Although the formulation of protein-loaded nanoparticles $<1$-µm in size is highly desirable for their efficient endocytosis through cell membrane, it has been very difficult to prepare such protein-loaded nanoparticles. In particular, when the spontaneous emulsion solvent diffusion method is used for the preparation of nanoparticles, protein should be molecularly dissolved in water-miscible solvents, such as acetone and dimethylsulfoxide (DMSO), in which polymer and protein are dissolved together.

Nevertheless, the direct dissolution of proteins into polar organic solvents can be accomplished by covalent and noncovalent modifications of protein molecules. For example, a wide range of therapeutic proteins conjugated with poly(ethylene glycol) (PEG) resulted in enhanced solubility in various organic solvents. In addition, it has been shown that proteins can have an altered solubility in organic solvents by hydrophobic ion pairing (HIP). The ionic interaction of charged groups in proteins by an appositely charged head group of surface active agents results in the formation of hydrophobically modified proteins showing reduced aqueous solubility and dramatically enhanced solubility in organic solvents. Another possible advantage for the use of hydrophobic ion pairing is its capability to enhance the transport of proteins across mucosal membrane.

In this work, lysozyme and sodium oleate were employed as a model protein drug and a hydrophobic ion-pairing agent, respectively. Lysozyme is a positively charged protein at neutral pH, which can be paired with an anionic head group of the fatty acid by ionic interaction. Additionally, as oleic acid has the ability of enhancing permeation through small intestinal membrane, the formation of hydrophobic ion-pairing complex by sodium oleate could be an attractive method for oral delivery of basic protein drugs. Hydrophobic ion-pairing complexes between lysozyme and sodium oleate were generated at various conditions. Ion-pairing complex dissolved in a water-miscible organic solvent was directly formulated into biodegradable nanoparticles based on a spontaneous emulsion solvent diffusion method. It was tested whether the complex showed increased solubility and stability in the DMSO phase. Nanoparticles encapsulated with lysozyme–oleate complex were characterized and compared with those encapsulated with free lysozyme.

**EXPERIMENTAL SECTION**

**Materials**

Chicken egg white lysozyme (3X crystallized, dialyzed, and lyophilized powder), oleic acid (cis-9-octadecanoic acid) sodium salt, and sodium dodecyl sulfate (SDS) were purchased from Sigma. Poly(D,L-lactic-co-glycolic acid) [PLGA] with a lactic/glycolic molar ratio of 50/50 was obtained from Boehringer Ingelheim (Resomer RG502H). Its number average molecular weight as determined by gel permeation chromatography was 8300. Pluronic F-127 was a gift from BASF (Parsippany, NJ). All other chemicals were of analytical grade.

**Preparation of Hydrophobic Ion-Pairing Complex**

Lysozyme completely dissolved in 20 mL of 10 mM Tris or acetate buffer solution at various pH values was adjusted to the final concentration of 2 mg/mL, which was mixed with varying amounts of sodium oleate or SDS dissolved in 1 mL of the same buffer solution. Various molar ratios of sodium oleate or SDS against lysozyme from 1 to 6 were added to the lysozyme solution at room temperature. The solution immediately became cloudy and the precipitate was formed. After equilibrating the solution for 6 h, the white precipitates were recovered by centrifugation for 10 min at 2500 rpm and then freeze dried. The fraction of complexed lysozyme with the two amphipatic molecules was determined by analyzing the amount of remaining lysozyme in the solution after centrifugation.

**Determination of the Dissociable Amount of Lysozyme from Complex by NaCl**

One milligram of dry lysozyme/oleate complex dissolved in 1 mL of 10 mM PBS buffer solution...
adjusted at pH 7.4 was dissociated with an increment of NaCl salt concentration ranging from 0 to 0.45 M. Briefly, the complex incubated in a series of salt solutions was stirred for 6 h at 4°C and subsequently dialyzed against the same concentration of salt solution to remove dissociated olate species with a Spectrapor 7 membrane (molecular weight cut off (MWCO) 10,000). The fraction of dissociated and solubilized lysozyme in the dialysis bag was separated and its amount was determined after centrifugation by a bicinchoninic acid assay (BCA) method.

**Characterization of Lysozyme–Oleate Complex**

The freeze-dried complex dissolved in DMSO was analyzed immediately by gel permeation chromatography (GPC) with a high-performance liquid chromatography (HPLC) pump station (Waters, Milford, MA), and Shodex K803 as a column. Mobile phase was DMSO, which was used at a flow rate of 0.8 mL/min. Lysozyme was detected at 280 nm using a Water 486 ultraviolet/visible (UV) detector (Waters). The GPC was run at room temperature.

**Heat Stability of Lysozyme–Oleate Complex in Organic Solvent**

The freeze-dried complex dissolved in 1 mL of DMSO was incubated at 80°C for indicated time intervals. Fresh lysozyme dissolved in DMSO was used as a control. The sample was diluted with 100 mL of deionized water and dialyzed against 0.045 M NaCl for 4 h (Spectrapor 7 membrane; MWCO 10,000). The dialyzed sample was redialyzed overnight against deionized water to remove any residual salt. The sample was concentrated by an ultrafiltration (MWCO 10,000, Amicon) and then analyzed by circular dichroism (CD). The CD measurement was carried out on a spectropolarimeter (J715, Jasco, Japan) with a 1-mm quartz cell. The concentration of lysozyme was 200 μg/mL for all the CD measurements.

**Formulation and Characterization of Nanoparticles**

Two types of nanoparticles containing lysozyme–oleate complex and free lysozyme were prepared by a spontaneous emulsion solvent diffusion method. For the encapsulation of free lysozyme into nanoparticles, 95 mg of PLGA and 5 mg of lysozyme dissolved in 5 mL of DMSO was slowly added to 100 mL of deionized water containing 1% (w/v) Pluronic F-127 under vigorous stirring conditions. The nanoparticles formed in the aqueous solution were collected by ultracentrifugation (Beckman, Fullerton, CA) at 15,000 rpm for 1 h and resuspended in phosphate buffered saline (PBS) solution. The resuspended nanoparticles were stored frozen (–20°C) until use. The loaded amount of lysozyme within nanoparticles was determined by a spectroscopic method. The known amount of freeze-dried nanoparticles was completely dissolved in DMSO, and then the absorbance was measured at 280 nm. A series of lysozyme concentrations prepared in DMSO solution was used as calibration standards for UV measurement. Encapsulation efficiency was calculated based on the percent ratio of the amount of lysozyme incorporated into nanoparticles to the initial amount used. Size distribution and zeta potential were measured by a laser light scattering technique (ZetaPlus, Brookhaven Instrument Corp.).

**RESULTS AND DISCUSSION**

Hen egg white lysozyme is a relatively small protein composed of 129 amino acid residues, whose structure and function have been intensively investigated. Being positively charged below the isoelectric point of lysozyme (pI = 11.0), lysozyme can be bound with negatively charged amphipatic molecules. The complex formation of lysozyme gradually increases with increasing the relative molar ratio of sodium oleate or SDS to lysozyme, as shown in Figure 1, indicating that lysozyme was equally well ion paired with both of the two surface-active molecules and precipitated out of the solution. The stoichiometric molar binding ratio of four molecules of sodium oleate or SDS to a single lysozyme molecule is sufficient to produce hydrophobically ion-paired lysozyme. This result is consistent with the previous one that lysozyme formed complex precipitates with SDS in a stoichiometric manner. It was reported that six to seven molecules of SDS were bound to each lysozyme. Lysozyme has seven primary amine groups in its structure composed of six ε-amino groups of lysine residues and one N-terminal amino group. In addition, other basic amino acid residues such as arginine, present at the molecular surface, make lysozyme a basic protein at physiological pH. It was postulated that the primary binding site of negatively charged surface-active species to lysozyme is basic amino acid residues.
acid residues of lysozyme, including lysine and arginine. The fraction of complexed lysozyme was determined by measuring the amount of precipitated lysozyme in the solution after centrifugation. With increasing pH of the buffer solution used for complex formation, the greater fraction of lysozyme becomes water insoluble. Because lysozyme gradually becomes more positively charged as the pH value shifts away from its pI value, it was initially expected that more complex would be formed with sodium oleate or SDS at reduced pH value, assuming that pK$_a$ values of carboxylate and sulfate group in oleate and SDS are $< pH$ 4.

However, Figure 1 exhibits contradictory results from the expectation. These results suggest that sodium oleate and SDS might participate in protein precipitation not only by the ion-pairing mechanism, but also by hydrophobic binding to lysozyme molecule. It is conceivable that as the pH approaches the pI value of lysozyme, surface-neutralized lysozyme tends to interact with a hydrophobic hydrocarbon tail of the two surfactant molecules by nonspecific hydrophobic interaction. It should be mentioned that the surfactant-induced protein precipitation is a thermodynamically reversible and cooperative process based on equilibrium between surfactant and protein molecules under given conditions. Thus, complexation and decomplexation rates between the two species are dependent on environmental variables such as concentration, pH, ionic strength, and temperature. Sodium oleate was selected as an ion-pairing agent for lysozyme for further experiments because of its greatest complex formation result. Lysozyme–oleate complex was prepared by maintaining the relative molar ratio value of sodium oleate to lysozyme at 6 and by adjusting the solution pH at 10.

The formation of lysozyme–oleate complex was qualitatively confirmed by gel permeation chromatography (GPC). Freeze-dried complex and free lysozyme were dissolved in DMSO and subjected to GPC analysis operated in DMSO as a mobile phase. The results are shown in Figure 2. The complex is eluted earlier than free lysozyme due to the increase of molecular size caused by binding of oleate to lysozyme. It can be also observed that the complex has a small shoulder peak appearing at the position of free lysozyme, suggesting that the lysozyme–oleate complex contained free lysozyme to some extent. When lysozyme was molecularly dissolved in neat DMSO, a random coil structure was generated.

This fact indicates that the complex dissolved in DMSO phase had a more fully unfolded structure than free lysozyme because of the binding of oleate to the polypeptide backbone. Hence, significantly different elution times between the lysozyme–oleate complex and free lysozyme in the GPC profiles were likely caused by structural conformational difference in DMSO phase. However, the possibility of oleate-induced aggregation of lysozyme molecules in DMSO phase cannot be ruled out. Because ionic interaction between lysozyme and sodium oleate is hypothesized to be a major driving force for the formation of the water insoluble complex, ionic dissociation of the

![Figure 1. Binding curve of amphiphiles to lysozyme.](image1)

![Figure 2. Gel permeation chromatograms of lysozyme and lysozyme–oleate complex. The mobile phase was DMSO.](image2)
complex on exposure to high salt concentration environment was tested to prove the hydrophobic ion-pairing mechanism. Various amounts of NaCl were added to the complex dispersed in aqueous solution (Figure 3). By increasing the NaCl concentration from 0 to 0.01 M, a great extent of oleate was dissociated from the complex and the lysozyme was resolubilized, indicating that the nature of interaction between the two is very weak. As the salt concentration increased up to 0.045 M, more lysozyme was dissociated as the water-soluble fraction from the complex. Interestingly, at salt concentrations > 0.045 M, the lysozyme–oleate complex appeared to be less dissociated. This result can be attributed to the fact that hydrophobic interaction additionally contributed to the complex formation, as already mentioned. Increasing the salt concentration enhances the extent of hydrophobic interaction between lysozyme and sodium oleate while it reduces the ionic interaction between them. Thus, an optimal NaCl concentration for the dissociation of the complex can be seen as a consequence of counteracting effects of the salt addition on ionic interaction and hydrophobic interaction, both of which contributes to the formation of lysozyme–oleate complex.

To evaluate the effect of complex formation on the stability of lysozyme molecularly dissolved in an organic solvent, accelerated heat stability test at 80°C was performed. Circular dichroism (CD) was used for detecting any conformational changes in lysozyme. Intense solvent related absorption in a far-UV region prevented the examination of the secondary structure of lysozyme in DMSO. However, because it has been known that lysozyme regains its original activity and conformation in aqueous solution after exposure to an organic solvent, a near-UV CD spectrum of lysozyme dissolved in water was taken, after the complex was incubated in DMSO for various time intervals and DMSO subsequently removed by a series of dialysis steps. The evaluation of α-helical content in lysozyme was based on ellipticity value at 222 nm according to the established method. As shown in Figure 4, as the exposure time of native lysozyme to DMSO at 80°C increases, the original conformation, as measured by calculating α-helical content of reconstituted lysozyme in the aqueous solution, was gradually lost. On the other hand, lysozyme–oleate complex demonstrates much better structural stability in an organic solvent phase. The improved conformational stabilization of the complex in an organic solvent can be explained by the postulation that sodium oleate bound to lysozyme restricts the polypeptide chain mobility of lysozyme towards unfolding in response to the heat stress. This explanation implies that the problem of using organic solvents during the microparticulate formulation of proteins, which often denatures and unfolds proteins to be encapsulated, could be circumvented by adopting the complex formation approach.

To efficiently formulate oral protein delivery vehicles, such as microemulsions, micropsheres, and nanoparticles, molecular solubilization of

![Figure 3](image_url)  
**Figure 3.** Extraction of lysozyme from the lysozyme–oleate complex. The concentration of sodium chloride was increased from 0 to 0.45 M.

![Figure 4](image_url)  
**Figure 4.** Comparison of relative α-helical contents between lysozyme and lysozyme–oleate complex incubated in DMSO at 80°C (left panel A). Relative α-helical content, which was defined by (α-helical content at indicative time)/(α-helical content at zero incubation time), was calculated by measuring ellipticity at 222 nm. The CD spectra of (A) native lysozyme incubated in water, (B) lysozyme–oleate complex incubated in DMSO for 60 h, and (C) lysozyme incubated in DMSO for 60 h (right panel B) are shown.
proteins in organic solvents should be achieved, which is a crucial step to obtain the high loading of proteins within the various vehicles. The lysozyme–oleate complex showed increased solubility in DMSO, which was about twice as high as that of free lysozyme (free lysozyme = 12.6 ± 4.2 mg/mL; lysozyme–oleate complex = 21.1 ± 3.5 mg/mL). This increased solubility in DMSO enabled nanoparticles encapsulated with lysozyme at high loading to be prepared (listed in Table 1). Nearly all the amount of lysozyme–oleate complex initially added was loaded into nanoparticles, whereas about 80% of free lysozyme was loaded when using free lysozyme. Poor solubility of the lysozyme–oleate complex in water certainly contributed to the high loading amount in nanoparticles as well as the near 100% encapsulation efficiency. Additionally, it is noteworthy that free lysozyme-loaded nanoparticles were much larger in size than lysozyme–oleate complex-loaded nanoparticles under the condition of the same initial loading amount of lysozyme. This difference can be attributed to the viscosity difference of DMSO organic phase between the two formulations having different lysozyme solubilities, which are likely to affect the dissipation tendency of the polymer containing organic phase towards nanoparticle droplets on addition to aqueous medium during the spontaneous emulsion solvent diffusion process. It is also possible that the incorporation of oleate in the nanoparticle formulation produced smaller nanoparticles because of its surfactant action. As listed in Table 2, size distribution of nanoparticles and their zeta potential were measured as a function of initial loading amount of lysozyme–oleate complex in nanoparticles. The amount of lysozyme loaded into nanoparticles was proportional to the amount of lysozyme–oleate complex initially used for loading. The size of nanoparticles was dramatically increased from 99.7 to 2001 nm and the zeta potential was gradually increased from −3.6 to 5.5 mV. The size increment was likely caused by the viscosity effect of organic phase, as already mentioned. Additionally, the size of nanoparticles might increase with increased loaded amount as a consequence of nanoparticle aggregation. During the formulation process of lysozyme nanoparticles, some fraction of excessively loaded lysozyme–oleate complex was inevitably located on the surface of nanoparticles, which might serve as a physical cross-linker between nanoparticles. The size distribution of nanoparticles with lysozyme–oleate complex (loading amount = 10.1%) is shown in Figure 5. It can be seen that lysozyme-loaded nanopar-

### Table 1. Characterization of PLGA Nanoparticles Prepared with Free Lysozyme and Lysozyme–Oleate Complex

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lysozyme and PLGA</th>
<th>Lysozyme–Oleate Complex and PLGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective diameter (nm)</td>
<td>1377.0 ± 2.2</td>
<td>99.7 ± 31.0</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>10.1 ± 1.7</td>
<td>−3.6 ± 0.1</td>
</tr>
<tr>
<td>Loading amount (% (w/w))</td>
<td>8.0 ± 0.9</td>
<td>10.1 ± 1.4</td>
</tr>
<tr>
<td>Loading efficiency (%)</td>
<td>79.6 ± 9.3</td>
<td>101.0 ± 10.4</td>
</tr>
</tbody>
</table>

*Weight ratio of PLGA and lysozyme or lysozyme complex for the formulation nanoparticles was 90/10 (w/w).

### Table 2. Characterization of PLGA Nanoparticles Prepared by Increasing the Loading Amount of Lysozyme–Oleate Complex

<table>
<thead>
<tr>
<th>Initially Added PLGA Amount in the Complex (mg)</th>
<th>Initially Added Lysozyme Amount in the Complex (mg)</th>
<th>Lysozyme Loaded Amount into Nanoparticles (w/w, %)</th>
<th>Encapsulation Efficiency (%)</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
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</table>
particles have a very narrow size distribution centered about 100 nm, with a smaller population appearing at the 50–60-nm range.

CONCLUSIONS

The present study demonstrates that lysozyme–oleate complex enhanced solubility and improved stability in an organic solvent. By hydrophobic ion pairing, the lysozyme complex retained the original secondary structure in water after exposure to DMSO at 80°C, whereas free lysozyme gradually lost the structure as a function of time. The lysozyme–oleate complex was formulated into biodegradable nanoparticles that showed much higher loading amount of lysozyme than those with free lysozyme. Biodegradable nanoparticles as small as 100 nm in diameter could be successfully prepared, which had as much as 10% (w/w) lysozyme loading percent. The particle size of nanoparticles was increased with the increment of initial loading amount of the complex. Hydrophobically ion-paired proteins can be potentially applied to oral microparticulate delivery vehicles, such as microemulsions and nanoparticles, which need the solubilization of proteins in organic solvents to a great extent during the formulation process.

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REFERENCES


