International Journal of Pharmaceutics 358 (2008) 50–59

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Role of a novel multifunctional excipient poly(ethylene glycol)-*block*-oligo(vinyl sulfadimethoxine) in controlled release of lysozyme from PLGA microspheres

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ARTICLE INFO

Article history:
Received 20 December 2007
Received in revised form 4 February 2008
Accepted 11 February 2008
Available online 16 February 2008

Keywords:
PEG-*b*-oligo(vinyl sulfadimethoxine)
Ion-pairing
Lysozyme stabilization
Aqueous/organic interface
PLGA microspheres
Release modulation

ABSTRACT

This study investigated the effect of ion-pairing of anionic polyelectrolytes: our novel poly(ethylene glycol)-*block*-oligo(vinyl sulfadimethoxine) (PEG-OVSDM) and poly(ethylene glycol)-*block*-poly(L-aspartic acid) (PEG-PAA) with cationic lysozyme on retention of protein stability during emulsification. Soluble lysozyme recovery after exposure to the deleterious interface was 42–88% (when ion-paired with PEG-OVSDM, PEG-OVSDM concentration dependent) compared to only 30% for free lysozyme. PEG-OVSDM provided a higher stabilization of lysozyme than PEG-PAA (36–60%). Lysozyme when recovered in the aqueous phase and analyzed by chromatography, enzymatic assay, fluorescence, and mass spectrometry showed no significant physicochemical change when compared with a lysozyme standard. Lysozyme was incorporated into poly(lactide-co-glycolide) (PLGA) microspheres via the typical double emulsion method. Incorporation of lysozyme complexes led to a higher encapsulation efficiency and loading amount, and a lower incidence of insoluble lysozyme aggregates compared to the control microspheres containing lysozyme only. More significantly, ion-pairing was able to dramatically reduce the initial lysozyme release to 18% compared with 50% from control microspheres and provided an overall better control of protein release. PEG-PAA was less effective than PEG-OVSDM in controlling the release probably due to weaker interactions between this polyelectrolyte and lysozyme. Manipulation of such polyelectrolyte–protein complexation may play a role in protein-controlled delivery.

1. Introduction

Proteins are typically administered by injections because of their poor oral bioavailability (Mahato et al., 2003; Shen, 2003). Formulating proteins as controlled-release depot systems has the potential to reduce the frequency of injections, provide sustained delivery of the protein, and thereby reduce patient discomfort and possibly improve therapy (Varde and Pack, 2004). Development of such a controlled-release system of a protein may involve encapsulation in matrices prepared from biocompatible and biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) (Cohen et al., 1991). Fabrication methods used for encapsulating protein in matrices often adversely affect protein stability due to exposure to aqueous/organic interfaces, hydrophobic surfaces and vigorous agitation (Wise et al., 1987; Weert et al., 2000a). In the most commonly employed double emulsion microencapsulation process, an aqueous protein solution is emulsified into an organic solvent (usually methylene chloride containing PLGA) to form the primary water/oil (w/o) emulsion. Formation of the primary emulsion is considered the main cause of protein inactivation and aggregation (Sah, 1999a,b; Weert et al., 2000b). A commonly employed approach to tackle protein instability during microencapsulation is to add stabilizing excipients such as surfactants, serum albumins, and poly(ethylene glycol) (PEGs) to the inner aqueous phase of the primary emulsion. These excipients compete with the proteins for adsorption at the aqueous/organic interface and thereby prevent emulsification-induced protein aggregation (Pean et al., 1999; Weert et al., 2000b; Kang et al., 2002; Perez et al., 2002; Perez-Rodriguez et al., 2003). No single excipient has shown promise as a general stabilizer for proteins prompting efforts to stabilize the proteins by a different approach.

Protein instability can be overcome by ion-pairing proteins with oppositely charged polyelectrolytes prior to microencapsulation. This approach is based on a hypothesis that a reversible 'molecular shield' for a given protein (by induction of ionic and/or hydrophobic interactions between polyelectrolytes and proteins) may prevent destabilization of protein during formulation. Numerous reports about enzyme stabilization by complexation with polyelectrolytes have appeared in the literature (Ganhorn et al., 1987; Teramoto et al., 1992, 1996).

Induction of ionic and/or hydrophobic interactions between a PEGylated polyelectrolyte (diblock copolymers) and proteins may

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0378-5173/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2008.02.010

prevent protein destabilization during microencapsulation. PEG and the polyelectrolyte components having steric hindrance and 'protein conformational rigidification' properties, respectively may prevent protein aggregation, interfacial destabilization of proteins during microencapsulation and also protein's interactions with the hydrophobic PLGA matrix. The strong binding between the polyelectrolytes and the proteins is expected to minimize the initial burst release commonly observed during protein release studies of microspheres by enlarging the complex size. In studies carried out in our laboratory, this hypothesis has been proved by demonstration of improvement in the interfacial stability and modulation of release of anionic proteins such as bovine serum albumin (BSA) and insulin by ionically complexing them with a cationic polyelectrolyte PEG-poly(L-histidine) (Kim et al., 2005; Taluja and Bae, 2007). The poly(L-histidine) component of PEG-polyhistidine may be physically attached to the anionic surfaces of BSA and insulin through ionic interactions with minimal disturbance of protein's tertiary structure. Hydrophilic PEG is proposed to cover the surface of the protein acting as the corona. This feature may have prevented protein interfacial adsorption and reduced the extent of denaturation of both proteins.

Along the same principle, an anionic polyelectrolyte is proposed to be tested for the stabilization of a cationic polyelectrolyte. In the present study, the potential benefit of an anionic polyelectrolyte; poly(ethylene glycol)-*block*-oligovinyl-sulfadimethoxine (PEG-OVSDM, Fig. 1A, a novel smart copolymer synthesized in our laboratory) in reduction of lysozyme aggregation at the aqueous/methylene chloride interface was investigated. Another polyelectrolyte, poly(ethylene glycol)-*block*-poly(L-aspartic acid) (PEG-PAA) was employed as a positive control (Fig. 1B, it should be noted that intramolecular isomerization as well as racemization of the aspartic acid units in PEG-PAA take place to form the β-aspartic units during the deprotection process by alkaline hydrolysis). Lysozyme was subsequently encapsulated into microspheres. A systematic attempt for understanding the factors affecting complexation of lysozyme with the polyelectrolytes, the stabilization effects on lysozyme during the microencapsulation process by employing ionic and nonionic polymeric excipients is reported here. The release profile of lysozyme from microspheres and the stability of the released lysozyme were also investigated.

2. Materials and methods

2.1. Materials

Sulfadimethoxine [4-amino-*N*-(2,6-dimethoxy-4-pyrimidynyl) benzenesulfonamide] (SDM), *N*-hydroxysuccinimide (HOSu), dicyclohexyl carbodiimide (DCC), triphosgene, polyethylene glycol

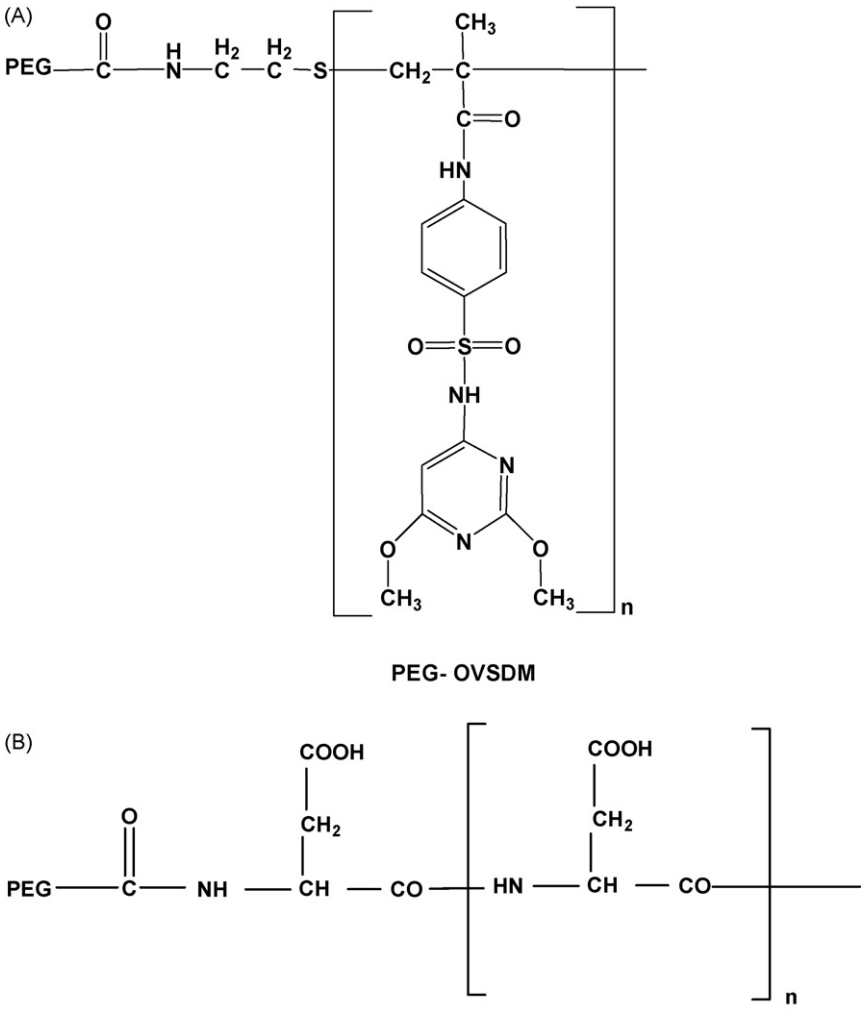

Fig. 1. Chemical structure of: (A) sulfonamide-based oligomer, PEG 2 K-OVSDM 3 K; (B) PEG-block-poly(L-aspartic acid), PEG 2 K–PAA 4 K.

(MW 2000 and 6000) were purchased from Aldrich (Milwaukee, WI) and used without further purification. α-Hydroxy-ω-carboxymethyl poly(ethylene oxide) (PEG monoacid, MW ~2 K) was synthesized and purified (Zalipsky et al., 1992). Methacryloyl chloride (Aldrich) was distilled under reduced pressure (10 mm Hg) at 30 °C, and dimethyl sulfoxide (DMSO, Aldrich) was purified by vacuum distillation at 75 °C (12 mm Hg). 2,2′-Azobisisobutyronitrile (AIBN, Aldrich) was recrystallized twice in methanol.

Lysozyme from chicken egg white (E.C. 3.2.1.17), polyvinyl alcohol (cold, water-soluble, MW 8000), β-benzyl-L-aspartate, and *n*-hexylamine were sourced from Sigma (St. Louis, MO). Poly(lactide-co-glycolide) (PLGA, Resomer™ 502H, MW 8600) was purchased from Boehringer Ingelheim (Germany). BCA protein assay kit was sourced from Pierce (Rockford, Illinois). SDS-PAGE kit was obtained from Invitrogen Corporation. Pluronic F-127 and Pluronic F-68 were purchased from BASF Group (New Jersey). Spectrapor™ dialysis membranes for the purification of polymers were obtained from Spectrum Labs (VWR) and are designated as molecular weight cut off (MWCO). All other chemicals and reagents used in the study were reagent grade or higher.

2.2. Synthesis and characterization of poly(ethylene glycol)-block-oligo(vinyl sulfadimethoxine) (PEG-OVSDM)

The synthetic scheme involved converting sulfadimethoxine to vinyl-sulfadimethoxine for free radical polymerization into sulfonamide-based polymers (Han et al., 2003). Sulfadimethoxine (SDM, 0.01M) was dissolved in aqueous NaOH (0.5N)/acetone mixture (1:1, v/v). Methacryloyl chloride (0.01M) was added dropwise into the SDM solution at 10 °C with vigorous stirring. The monomer: methacrloylated SDM (VSDM) precipitate was filtered, washed with deionized water, collected and dried, and recrystallized in methanol for purification. The sulfonamide-based polymer was synthesized by free radical solution polymerization of the monomer (VSDM) in DMSO at 65 °C for 20 h in the presence of AIBN (initiator; 0.2 mol% of the monomer) and AET (2-aminoethanethiol, 0.15 mol%, a chain transfer agent). The reaction mixture was degassed by three freeze–thaw cycles. After polymerization, the contents were poured into deioinized water to precipitate the initial product. Unreacted monomer was removed by dialysis of the initial product for 3 days against deionized water (molecular weight cut off, MWCO 2 K) adjusted to pH 10. The yield of the polymer: OVSDM after dialysis was 77%. The peak originating from monomer double bond disappeared during polymerization and the benzene peak was down-shifted to ~7.9 ppm (δ 7.8 ppm in VSDM). ^{1}H NMR: δ 1.95 (–CH_2=CH–CH_3), δ 5.5–5.7 (–CH_2=CH–CH_3), δ 10.2 (–CONH–), δ 7.9 (phenylene-*H*), δ 11.2 (–SONH–), δ 6.7 (pyrimidinyl-*H*), δ 3.6 (pyrimidinyl –N=C–OCH_3), δ 2.3 (H_2N–CH_2–CH_2–S–), δ 2.7 (H_2N–CH_2–CH_2–S–). The average molecular weight of OVSDM was estimated by ^{1}H NMR to be 3 K (degree of polymerization ~9). Hence the product was named an oligomer rather than a polymer.

PEG monoacid (MW ~2 K) was activated to yield PEG – *N*-hydroxysuccinimide ester. The activated PEG was conjugated to the amine terminal of OVSDM (introduced by the chain transfer agent: AET) to produce the diblock copolymer PEG-OVSDM. The final product was characterized by ^{1}H NMR to support the coupling reaction between PEG and OVSDM blocks. The diblock copolymer (dissolved in DMSO) was dialyzed against deionized water adjusted to pH 10 for 3 days (MWCO 3.4 K) and lyophilized to yield the final purified diblock copolymer. The yield of the final diblock polymer: PEG-OVSDM after dialysis was 64%. ^{1}H NMR: δ 1.95 (–CH_2=CH–CH_3), δ 5.5–5.7 (–CH_2=CH–CH_3), δ 10.2 (–CONH–), δ 7.9 (phenylene-*H*), δ 11.2 (–SONH–), δ 6.7 (pyrimidinyl-*H*), δ 3.6 (pyrimidinyl –N=C–OCH_3), δ 2.3 (H_2N–CH_2–CH_2–S–), δ 2.7 (H_2N–CH_2–CH_2–S–), and δ 3.6–3.8 (protons on repeating units, PEG).

MALDI-TOF spectrum showed the distribution of molecular weight of PEG-OVSDM to be between 4 and 6 K with maximal intensity around 5 K (spectrum not shown here). This oligomer was used for complexation studies with a model protein ; lysozyme.

2.3. Synthesis of the diblock copolymer; poly(ethylene glycol)-block-poly(L-aspartic acid) (PEG-PAA)

The synthesis of PEG-PAA was carried out as per the established protocols (Daly and Poche, 1988; Yokoyama et al., 1987). Briefly, a stirred mixture of β-benzyl-L-aspartate and triphosgene in dry tetrahydrofuran (THF) was heated at 50 °C under a nitrogen atmosphere until a clear solution was obtained. The reaction mixture was poured into an excess of *n*-hexane. The precipitated β-benzyl-L-aspartic acid *N*-carboxy anhydride (BLA-NCA; monomer) was collected by filtration and dried under vacuum for 48 h. The ring-opening polymerization of BLA-NCA in dioxane/THF (3:1, v/v) was initiated with a primary amine initiator: *n*-hexylamine at room temperature under a nitrogen atmosphere for 3 days. Precipitation with excess ethanol–ether (7:3, v/v), washing with ethanol–ether and drying *in vacuo* yielded poly(β-benzyl-L-aspartate) (PBLA).

NMR spectroscopy is a commonly employed technique to estimate the degree of polymerization of such benzyl-protected polymers. Composition of PEG-PBLA was determined by ^{1}H NMR from peak intensity ratios of methylene protons of PEG (OCH_2CH_2: δ 3.7 ppm) and phenyl protons of β-benzyl groups of PBLA (–$CH_2C_6H_5$: δ 7.3 ppm). The average degree of polymerization of PBLA was determined to be 31 (intended degree of polymerization based on monomer:initiator molar ratio = 35).

The activated PEG (as mentioned earlier) was conjugated to PBLA (having an amine terminus) to yield benzyl-protected polymer PEG-PBLA. PEG-PBLA was treated with 0.1N NaOH to remove the benzyl groups to yield PEG-poly(L-aspartic acid); PEG-PAA. The solution obtained on effective removal of benzyl groups was dialyzed against deionized water for 3 days (MWCO 5 K) and lyophilized to yield the final diblock copolymer; (PEG-PAA). Complete removal of benzyl groups in PEG-PBLA (δ 7.3 ppm) was confirmed after alkaline hydrolysis as per the established procedures (Yokoyama et al., 1987, the ratio of α to β units in the deprotected PAA segment of the diblock copolymer was 1:3, however for simplicity only the α-structure of PEG-PAA is shown in Fig. 1B). The gel permeation chromatography (GPC) measurement was done to estimate the molecular weight distribution of PEG-PAA. Unimodal distribution with M_w/M_n = 1.26 (called the polydispersity index) was estimated for PEG-PAA. The average molecular weight of PEG-PAA was determined to be 6 K consisting of two blocks; PEG, 2 K and PAA, 4 K.

2.4. Preparation of ion-paired complexes of lysozyme and PEG-OVSDM/PEG-PAA

Lysozyme was dissolved in deionized water (wherever needed, NaCl added to deionized water to make different salt concentration solutions) to make a stock solution. The concentration of the lysozyme solution was estimated by UV spectrophotometry at 281 nm using an extinction coefficient value, $E^{1\%}$ = 26.4 (information sourced from Sigma). PEG-OVSDM and PEG-PAA were dissolved in pH 8.0, 0.15 M phosphate buffer (prepared according to the United States Pharmacopoeia (USP, Edition 24) procedure). PEG-PAA was also dissolved in deionized water as a control. The polyelectrolytes and lysozyme were mixed in various weight ratios and incubated for 2 h at room temperature for ion-pairing.

2.5. Binding characteristics

Solution depletion method was used to estimate the extent of binding of polyelectrolytes and lysozyme. The concentration of polyelectrolytes (PEG-OVSDM/PEG-PAA) was varied to estimate the amount of PEG-OVSDM and PEG-PAA needed to completely complex lysozyme (implies no significant amount left in the supernatant, hence the name solution depletion method). A variable volume of PEG-OVSDM stock solution in pH 8.0, 0.15 M phosphate buffer was mixed with a fixed volume of 10.0 mg/ml lysozyme solution to vary the weight ratio of PEG-OVSDM to lysozyme from 0.025 to 0.25. For lysozyme and PEG-PAA, a variable volume of PEG-PAA solution in deionized water (or pH 8.0, 0.15 M phosphate buffer,) was added to a constant volume of 10.0 mg/ml lysozyme solution (similar to PEG-OVSDM) to vary the weight ratio of PEG-PAA to lysozyme from 0.01 to 0.1. At the end of 2 h, the ion-pair mixtures were centrifuged at 10,000 × *g* for 5 min to separate the complexes and residual lysozyme in the supernatant estimated by using a BCA assay kit. HPLC method (details in the following section) was used for the system involving PEG-PAA and lysozyme to avoid interference in the BCA assay from PEG-PAA.

2.6. Effect of salt concentration on intermolecular interactions

Four different salt concentrations: 0.075, 0.15, 0.30, and 0.45 M NaCl were selected to study the binding of lysozyme to PEG-OVSDM and PEG-PAA. Alternatively, the salt concentration required for dissociation of complexes of lysozyme with PEG-OVSDM/PEG-PAA was determined by monitoring the scattering of a suspension of ionic complexes as a function of NaCl concentration. The optical density of the complexes mixture at 500 nm was monitored upon addition of a fixed volume of varying NaCl concentration solutions to PEG-OVSDM: lysozyme complexes in pH 8.0 phosphate buffer, and PEG-PAA: lysozyme complexes in deionized water.

2.7. Retention of stability of lysozyme on exposure to aqueous/methylene chloride interface

The concentrations of our novel PEG-OVSDM, a control polyelectrolyte (PEG-PAA), and commonly investigated stabilizers (PEG 2 K and 6 K, Pluronics F-68, and F-127) were varied. Keeping the lysozyme concentration fixed, excipients and the protein were incubated for 2 h at room temperature, and subsequently emulsified into methylene chloride at a 1:5 (v/v) water-to-methylene chloride ratio. Ultra Turrax® T25 basis model apparatus (IKA-Werke) was employed at 19,000 rpm for 30 s for emulsification. The resultant emulsions were destabilized by addition of 5 ml of deionized water adjusted to pH 8.0 ([NaCl] = 0.5 M), necessary to dissociate the complexes between the polyelectrolytes and lysozyme) and phase separation allowed to occur for an hour, aided by centrifugation at 10,000 × *g* for 5 min. Supernatants containing nonaggregated lysozyme were subject to protein content estimation by BCA assay method (or an HPLC method). The precipitate (lysozyme aggregated due to the interfacial stresses, insoluble in an aqueous medium) was collected using a Pasteur pipette and washed 2–3 times with deionized water. Following lyophillization, the precipitate was collected, and weighed for mass balance studies in select cases.

2.8. Structural analysis of recovered lysozyme after emulsification

Lysozyme fluorescence emission spectra were obtained in 300–450 nm wavelength range and excitation was carried at 295 nm. All conformational measurements were carried out under identical conditions. Following samples were subject to spectroscopic evaluation: (i) lysozyme solution freshly made (concentration = 0.1 mg/ml), no interfacial exposure to methylene chloride and (ii) soluble lysozyme recovered after emulsification (concentration = 0.1 mg/ml). To minimize the contribution of buffer and PEG-OVSDM in the fluorescence signal, the signal from the buffer and PEG-OVSDM simultaneously determined, was subtracted from the total signal. Decomplexation of lysozyme from PEG-OVSDM was carried out after phase separation to avoid changes in signal from lysozyme structural alteration due to molecular interactions with PEG-OVSDM.

Lysozyme recovered after interfacial exposure was analyzed by high-performance liquid chromatography (HPLC). Chromatography was carried out in order to detect different degradation products (if any) in soluble lysozyme recovered in the aqueous phase after breaking the emulsions. Also, the lysozyme aggregates present at the interface were dissolved in 6 M urea and dialyzed against deionized water for an hour in a microdialyzer (QuixSep™, Membrane Filtration Products Inc., Seguin, TX) and subjected to chromatographic evaluation lysozyme standard (not exposed to the aqueous/organic solvent interface). The chromatograph was equipped with a HPLC LC pump, an injection valve (Rheodyne), a UV–vis detector set at the wavelength of 215 nm and an integrator combined in an Agilent 1100 series HPLC system. An Extend™ 5-μm C_{18} (4.6 mm × 250 mm) column (Agilent, USA) was employed. Lysozyme samples were analyzed at room temperature using a binary gradient consisting of (A) water/TFA (99.9/0.1) and (B) acetonitrile/TFA (99.9/0.1). The gradient consisted of 15–65% B in 10 min, followed by equilibration at 15% B for 5 min. Flow rate was set at 1.0 ml/min.

SDS-PAGE of the nonaggregated, soluble lysozyme was performed under nonreducing conditions to detect any covalent aggregates possibly formed during emulsification. The recovered protein was mixed with the nonreducing sample buffer. Electrophoresis was run in a constant current mode with constant voltage mode of 160 V, buffer was used on a discontinuous gel (NuPAGE® 4–12% Bis–Tris Gel). The gels were stained with 0.1% Coomassie blue fixative solution, and destained with deionized water.

2.9. Evaluation of enzymatic activity of recovered lysozyme

Lysozyme activity was determined by using the decrease in absorbance intensity at 450 nm of *Micrococcus lysodeikticus* (ATCC 4698) suspension essentially as per the protocol (Weert et al., 2000b). 0.2 mg/ml cells dispersion was prepared in 0.067 M phosphate buffer (pH 6.2). To 1.3 ml of the suspension, 20 μl of the lysozyme-containing solution was added, and the decrease in $A_{450\,nm}$ was monitored. The slope of the linear part of the curve was related to the slope of a standard sample, after normalization of the protein concentration, and is expressed as specific (enzymatic) activity as a percentage of the control (freshly made lysozyme solution).

2.10. Preparation of PLGA microspheres encapsulating lysozyme

Five different batches of microspheres encapsulating lysozyme were fabricated. The control batch contained lysozyme only. Other batches were prepared from a fixed amount of lysozyme and a variable amount of either PEG-OVSDM or PEG-PAA. 1.0 ml of lysozyme or ion-paired lysozyme was emulsified with 5.0 ml of methylene chloride containing PLGA (RG 502H, 20 mg/ml) at 19,000 rpm for 30 s. The resulting primary emulsion was injected into 200 ml of 0.35% PVA solution to form the water-oil-water (w/o/w) emulsion using L4RT-A homogenizer at 4000 rpm for 3 min. Microspheres were hardened by methylene chloride removal by raising the temperature in a sequential manner (10 min at 10 °C, 10 min at 20 °C,

10 min at 30 °C, and 5 min at 35 °C). Finally microspheres were hardened at 38 °C for an hour. Resulting microspheres were centrifuged, washed thrice with deionized water and lyophilized.

2.11. Characterization of PLGA microspheres encapsulating lysozyme

The characterization of microspheres included estimation of the encapsulation efficiency and the weight content of lysozyme, the content of insoluble lysozyme aggregates in the microspheres, scanning electron microscopy (SEM) for surface morphology evaluation, *in vitro* protein release in a simulated physiological medium and enzymatic activity determination of the released lysozyme.

The actual lysozyme loading in the microspheres was estimated via the previously described DMSO/NaOH/SDS method with some modifications (Sah, 2000). Microspheres (~25 mg), accurately weighed, were put into a glass vial to which DMSO (2 ml) was added. The microspheres were dissolved with mild agitation for an hour. 3 ml of 0.05N NaOH solution containing 0.5% (w/v) sodium dodecyl sulfate was then added to the DMSO solution and gently mixed. After mild agitation at room temperature for 6 h, aliquots of the mixture were assayed for lysozyme content by a BCA assay. Blank protein-free microspheres were also subject to the same procedure to investigate whether the control microspheres interfered with the BCA assay. The encapsulation efficiency was determined using the following equation:

$$\%\,\text{Encapsulation efficiency} = \left(\frac{P_a}{P_t}\right) \times 100,$$

in which P_a represents the quantity of protein encapsulated in the microspheres and P_t is the theoretical amount of protein to be encapsulated (obtained from initial feeding conditions).

The weight percentage of lysozyme was calculated by using the following equation:

$$\%\,\text{Lys} = \left(\frac{P_a}{M_a}\right) \times 100,$$

in which P_a represents the quantity of protein encapsulated in the microspheres and M_a is the amount of microspheres containing P_a amount of protein.

The formation of lysozyme aggregates during the microencapsulation was determined after the extraction of soluble lysozyme from microspheres (Perez and Griebenow, 2003). In brief, microspheres (~20 mg) were added to acetonitrile (1 ml) and stirred for 1 h. The mixture was centrifuged for 10 min at 10,000 × *g* and the supernatant acetonitrile (containing dissolved PLGA) was completely withdrawn. The procedure was repeated once. 1 ml of 0.01 M NaOH was then added and insoluble precipitates were separated by centrifugation. These precipitates were dissolved in 0.5 ml of 6 M urea solution. The amount of lysozyme aggregates was measured by a BCA assay against a calibration curve of lysozyme made in 6 M urea. The quantity of insoluble lysozyme aggregate was assessed using the following equation:

$$\%\,\text{Insoluble Lys} = \left(\frac{P_u}{P_a}\right) \times 100,$$

in which P_u represents the quantity of insoluble protein in the microspheres and P_a is the amount of protein incorporated per unit weight of microspheres.

Surface morphology of PLGA microspheres was observed by scanning electron microscopy (SEM, Hitachi S-3000N). Dry microspheres were coated with gold particles using a sputter-coater (Cressington Sputter Coater 108 Auto).

Only three batches of microspheres were evaluated for comparison of protein release: (i) microspheres containing lysozyme only, (ii) microspheres containing lysozyme ion-paired with an optimal amount of PEG-OVSDM (weight ratio of PEG-OVSDM to lysozyme = 0.5) and (iii) microspheres containing lysozyme ion-paired with an optimal amount of PEG-PAA (weight ratio of PEG-PAA to lysozyme = 0.1). Lysozyme release in phosphate buffer, PBT (5.0 ml, pH 7.4, 0.15M phosphate 0.02 wt% Tween 80) was determined using a BCA assay. Microspheres (~40 mg) were immersed in PBT and incubated at 37 °C at and agitated at 50 rpm. Entire volume of the release medium was collected at each time interval and assayed for released lysozyme content. Fresh and an equivalent volume of the release medium were added at each interval. The released lysozyme was subject to enzymatic activity determination.

2.12. Statistical analyses

All the experiments were repeated at least three times, unless specified otherwise. The data are reported as mean ± standard deviation. For comparison between three or more groups, one-way ANOVA was employed using the GraphPad Prism software. Comparisons having p values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Complexation of lysozyme and PEG-OVSDM/PEG-PAA

Lysozyme–polyelectrolyte interactions primarily arising from ionic forces led to coacervation. The aim was to find optimal complexing conditions between the polyelectrolytes and lysozyme. The formation of insoluble complexes on mixing lysozyme and PEG-OVSDM may be attributed to the charge neutralization of the sulfonamide moieties by positive charges on the lysozyme molecules. Deionization of the sulfonamide groups lead to increased hydrophobic interactions within the sulfonamide oligomer and a solubility transition takes place (Park and Bae, 1999; Kang and Bae, 2002; Han et al., 2003). Possibly, lysozyme is also entrapped within the nanoaggregates. The fraction of initial lysozyme which complexed with PEG-OVSDM/PEG-PAA was determined by measuring the amount of lysozyme left in the supernatant. Nearly 90% of initial lysozyme amount was complexed with PEG-OVSDM at a weight ratio of 0.25 (final lysozyme concentration, 5.0 mg/mL; PEG-OVSDM, 1.25 mg/ml) in the mixture. Decreasing the weight ratio of PEG-OVSDM to lysozyme led to greater amounts of lysozyme left in the supernatant as expected and results are shown in Fig. 2A. Increasing the weight ratio of PEG-OVSDM to lysozyme to 0.5 and higher led to zero lysozyme in the supernatant.

For the lysozyme/PEG-PAA system in deionized water, keeping the lysozyme concentration fixed and varying PEG-PAA concentration, nearly 100% of lysozyme was complexed at the weight ratio of 0.1 (PEG-PAA to lysozyme) as shown in Fig. 2B. Only 70% of lysozyme was complexed with PEG-PAA in 0.075M pH 8.0, phosphate buffer. Attempts to increase the amount of lysozyme complexed by raising the weight ratio of PEG-PAA/lysozyme to 0.2 or higher were not successful. It was initially expected that PEG-PAA in pH 8.0, 0.075M phosphate buffer would ensure higher complexation as PEG-PAA may become more negatively charged as the pH was raised above its pK_a (~4.0) than in deionized water. Another study involving lysozyme and sodium oleate reported similar higher extent of complexation at a pH higher than expected indicating that ionic contributions are not the only contributing factor (Yoo et al., 2001). For further studies, PEG-PAA was dissolved in deionized water to ensure complete complexation of lysozyme.

The size of insoluble complexes of lysozyme and the polyelectrolytes varied between 400 and 600 nm and were a function of

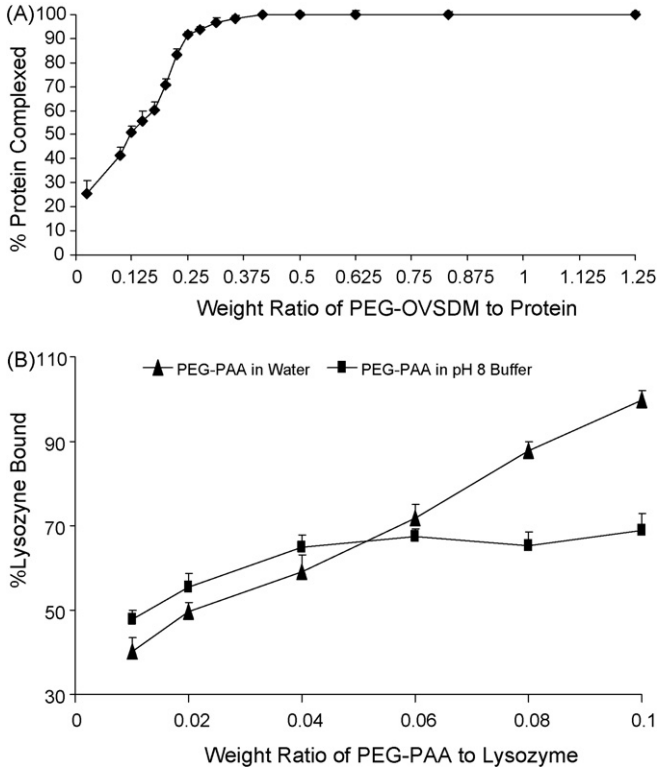

Fig. 2. Binding characteristics of lysozyme and polyelectrolytes, (A) PEG-OVSDM; PEG-OVSDM concentration was varied keeping the lysozyme concentration fixed. (B) PEG-PAA. PEG-PAA concentration was varied keeping the lysozyme concentration fixed.

agitation rate, time of incubation, pH, etc. A clear relationship between the particle size and the protein/polyelectrolyte ratio could not be established. Therefore, this paper is focused more on applications in drug delivery.

3.2. Effect of salt concentration on intermolecular interactions

Protein complexation with polymers in aqueous solution is driven primarily by electrostatic and hydrophobic interactions (Gao and Dubin, 1999; Kaibara et al., 2000). Varying the salt concentration of a solution can provide some insight into the nature of interactions. Table 1 shows that with increasing salt concentration, the percentage of initial lysozyme remaining in the supernatant

Table 1
Effect of salt concentration on the amount of lysozyme bound to PEG-OVSDM

Weight ratio of PEG-OVSDM to lysozyme	A	B	C	D
	% lysozyme remaining in the supernatant			
0.5	0.6	5.9	13.3	18.6
0.25	12.9	23.8	26.4	34.5
Weight ratio of PEG-PAA to lysozyme	**A**	**B**	**C**	**D**
	% lysozyme remaining in the supernatant			
0.1	7.9	16.8	94.5	97.2
0.05	25.8	41.4	98.4	97.6

Four different ionic salt concentrations were employed in the study: 0.075 M (A), 0.15 M (B), 0.30 M (C) and 0.45 M (D) adjusted with NaCl. The pH of the buffer was maintained at 8.0 in all experiments involving PEG-OVSDM. Values are a mean of $n = 2$. Initial lysozyme concentration was fixed at 10 mg/ml. The solutions of PEG-PAA were made in deionized water.

is increased. This is typical behavior for binding that is significantly governed through electrostatic interactions between the proteins and the polyelectrolytes. Interactions between lysozyme and sodium oleate were disrupted by salt concentrations as low as 0.01M (~60% lysozyme dissociated compared to ~5% in water and 80% for 0.045 M NaCl concentration) clearly suggesting that interactions were of a very weak nature and primarily ionic in nature (Yoo et al., 2001). Similarly, complex formation between leuprolide acetate and sodium oleate was carried out in deionized water to minimize the effect of buffer salt ions on ionic interactions (Choi and Park, 2000). To maximize ionic interactions between lysozyme and PEG-OVSDM, the final salt concentration of the pH 8 phosphate buffer was fixed at the minimum possible to achieve complete complexation of lysozyme.

Alternatively, the salt concentration required to "dissolve" the complexes was determined using UV spectrophotometry. About 0.25 M NaCl was required to completely dissociate the complexes between lysozyme and PEG-PAA. On the other hand, no salt concentration tested was sufficient to completely "dissolve" complexes between PEG-OVSDM and lysozyme. Increasing the salt concentration increased the optical clarity of lysozyme/PEG-OVSDM system suggesting lesser amounts of complexes being formed possibly due to reduced ionic interactions. It may be also suggested that stronger hydrophobic interactions between PEG-OVSDM and lysozyme are present compared to lysozyme and PEG-PAA. Significant degree of binding for lysozyme and PEG-OVSDM even at high salt concentrations that may have disrupted purely ionic interactions between PEG-OVSDM and lysozyme is evident.

3.3. Interfacial stabilization of lysozyme upon complexation with PEG-OVSDM

The extent of lysozyme aggregation after emulsification was estimated by measuring the loss of soluble lysozyme. Table 2 summarizes the recovery of lysozyme in the aqueous phase with or without the use of conventional excipients (negative and positive controls, respectively). Without addition of any polymeric excipient, there was about 70% loss of initial lysozyme after emulsification. All four PEG-based polymeric excipients failed to dramatically improve the recovery of soluble lysozyme. No statistically significant differences were obtained on using different molar ratios of PEG-based excipients. These results are in agreement with previous studies involving lysozyme and other proteins (Johnson et al., 1997; Rosa et al., 2000; Weert et al., 2000b).

The use of PEG-OVSDM led to a dramatic reduction in the aggregation of lysozyme during emulsification. The recovery of soluble lysozyme increased to 88% on complexing lysozyme with PEG-OVSDM ($p < 0.001$) in an optimal weight ratio. Evidently, a lesser fraction of lysozyme was adsorbed and/or aggregated at the interface following complexation with PEG-OVSDM than plain lysozyme. Both polyelectrolytes employed in the study have a molecular weight distribution; therefore weight/weight ratio was adopted instead of conventionally employed molar ratios or charge ratios. Suggesting an apparent molar ratio would not imply anything of consequence. PEG-OVSDM decreased aggregation of lysozyme in a concentration-dependent manner as shown in Table 2. Statistically significant differences in lysozyme recovery were obtained even when less than optimal weight ratios were used.

Complexation of lysozyme with PEG-PAA also led to enhanced retention of stability of lysozyme on emulsification. Statistical significance with the negative control (lysozyme only) was reached only when optimal amounts of PEG-PAA were used for complexation. The results with PEG-PAA were however not as dramatic as seen with PEG-OVSDM. Restricting the molecular mobility of

Table 2
Lysozyme recovery after emulsification of protein solutions with methylene chloride with excipients

Excipient used	Molar ratio[a]	Lysozyme recovery (%)	Relative enzymatic activity (%)
None	–	30 ± 5	93 ± 2
PEG 2000	8 to 1[a]	35 ± 4	94 ± 3
	2 to 1[a,b]	40 ± 2	90 ± 2
PEG 6000	8 to 1[a]	37 ± 3	91 ± 2
	2 to 1[a,b]	35 ± 3	87 ± 4
Pluronic F-68	8 to 1[a]	38 ± 3	92 ± 3
	2 to 1[a,b]	42 ± 3	90 ± 2
Pluronic F-127	1 to 1[a]	41 ± 2	90 ± 4
	1 to 2[a,b]	45 ± 2	93 ± 2
Weight fraction of PEG-PE to protein[c]			
PEG-OVSDM	0.5[d]	88 ± 2	87 ± 2
	0.4[d]	73 ± 2	91 ± 2
	0.3[d]	62 ± 3	86 ± 3
	0.2[d]	50 ± 2	92 ± 2
	0.1	45 ± 4	90 ± 2
PEG-PAA	0.1[d]	60 ± 2	86 ± 3
	0.08[d]	48 ± 2	94 ± 3
	0.06	41 ± 2	92 ± 3
	0.04	38 ± 2	90 ± 3
	0.02	36 ± 3	89 ± 3

Lysozyme concentration was fixed at 5 mg/ml. Values given are mean ± S.D.
[a] Molar ratios were used for conventional excipients.
[b] Molar ratios selected for microspheres.
[c] Weight ratios were used for novel polyelectrolytes.
[d] Statistical significance ($p < 0.05$) between the negative control (lysozyme only) and the excipient group.

lysozyme by inducing surface interactions with the polyanion and steric hindrance afforded by PEG may explain for the reduced aggregation of lysozyme at the aqueous/organic solvent interface. It is similar to the results when lysozyme complexed with sodium oleate was more resistant to thermal denaturation compared to free lysozyme (Yoo et al., 2001).

It is important to prevent/minimize aggregation of proteins during microencapsulation. Aggregation has been held responsible for incomplete release of lysozyme from PLGA microspheres (Blanco and Alonso, 1998; Diwan and Park, 2001; Jiang et al., 2002).

3.4. Structural evaluation of lysozyme recovered after emulsification

Negligible effects on tertiary structure of lysozyme were observed for soluble lysozyme recovered after emulsification, destabilization of the primary emulsion and collection of the aqueous phase containing nonaggregated lysozyme (spectra not shown here). Lysozyme stability was assessed by HPLC, which allows the simultaneous detection of lysozyme, fragments and covalent aggregates, if any, formed during the emulsification process. Chromatographic evaluation (not shown here) revealed that the soluble lysozyme recovered from interfacial studies had an identical retention time as that of a lysozyme standard. No other peaks were noticed; lysozyme did not degrade to products of different chemical nature in agreement with literature data (Weert et al., 2000b). White aggregates formed at the interface (soluble in 6M urea) showed an identical spectral pattern to that of a lysozyme standard confirming the noncovalent nature of aggregates formed during emulsification.

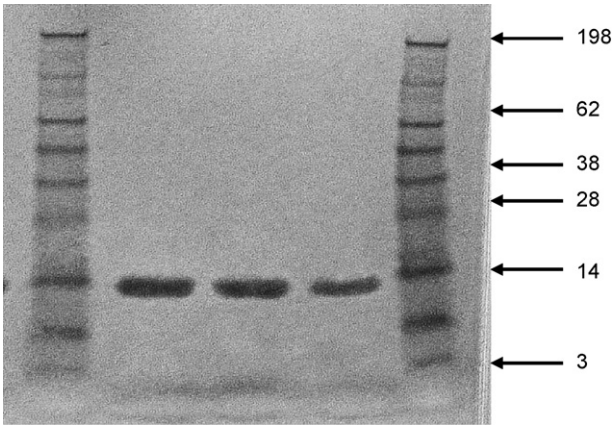

Fig. 3. SDS-PAGE images of (A) lysozyme freshly made, (B) soluble lysozyme recovered after emulsification, and (C) interfacial aggregates in 6 M urea and dialyzed, monomeric species on treatment with 6 M urea reconfirming the noncovalent nature of aggregates.

It is essential that the biological/enzymatic activity of a protein be retained after microencapsulation in PLGA microspheres. Lysozyme is capable of digesting bacterial cell walls; *M. lysodeikticus* was used as a substrate to evaluate the retention of enzymatic activity of the recovered soluble lysozyme. The specific activity of the recovered soluble lysozyme was estimated to be similar to that of native lysozyme at equivalent concentrations (Table 2).

The structural integrity of the recovered lysozyme after emulsification and the aggregates dissolved in 6 M urea (and dialyzed against deionized water) was analyzed by SDS-PAGE under nonreducing conditions. Fig. 3 provides direct evidence that lysozyme after emulsification and recovery maintained its molecular integrity (no fragmentation or covalent modifications) as shown in lane B compared to the native lysozyme in lane A. Lane C shows that the aggregates reverted to the monomeric lysozyme and further confirming the noncovalent nature of the aggregates formed at the interface.

Table 3
Characterization of PLGA microspheres encapsulating lysozyme

Code	Weight ratio of PEG-polyelectrolyte to lysozyme	% encapsulation efficiency[a]	% lysozyme (weight/weight)	% insoluble lysozyme (weight/weight)
LYSO-1	0	61 ± 2	2.9	20 ± 2
LYSO-2	0.05	65 ± 3	3.1	17 ± 2
LYSO-3[b]	0.1	73 ± 2[c]	3.1	22 ± 2
LYSO-4	0.25	78 ± 3[c]	3.9	11 ± 2[d]
LYSO-5[e]	0.50	88 ± 2[c]	4.3	5 ± 1[d]

Values are mean ± S.D. LYSO-2 and 3 contains lysozyme complexed with PEG-PAA. LYSO-4 and 5 contains lysozyme complexed with PEG-OVSDM.
[a] The encapsulation efficiency was calculated using the actual and theoretical loading of the PLGA microspheres.
[b] Optimal conditions for complexation between lysozyme and PEG-OVSDM.
[c] Statistical significance between the control group (LYSO-1) and the groups containing PEG-OVSDM or PEG-PAA (LYSO-3, 4 and 5).
[d] Statistical significance between the control group (LYSO-1) and the groups containing PEG-OVSDM (LYSO-4 and 5).
[e] Optimal conditions for complexation between lysozyme and PEG-PAA.

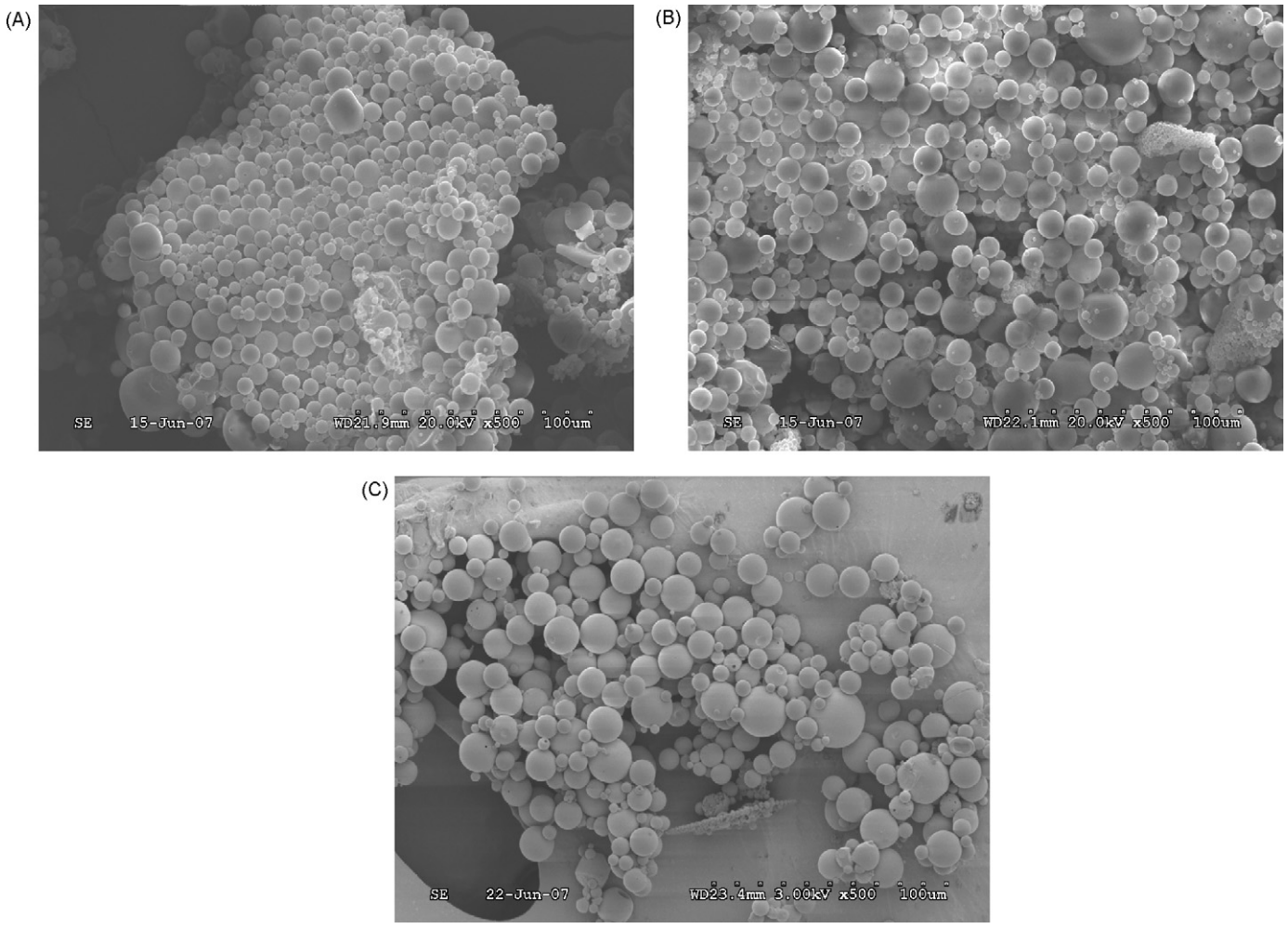

Fig. 4. Surface morphology of the microspheres as seen by SEM; (A) microspheres containing lysozyme only, (B) microspheres containing lysozyme complexed with PEG-OVSDM in an optimal weight ratio, and (C) microspheres containing lysozyme only complexed with PEG-PAA. All micrographs are at 500× magnification to provide a visual comparison of two microspheres batches.

3.5. Characterization of the PLGA microspheres

The PLGA microspheres encapsulated with the ion-paired lysozyme exhibited higher loading amount and greater encapsulation efficiency than microspheres containing lysozyme only. Increasing the amount of PEG-OVSDM enhanced the quantity of lysozyme incorporated in the microspheres. This may be due to the limited availability of water-soluble lysozyme in the presence of polyelectrolytes. Complexing lysozyme with PEG-OVSDM in an optimal weight ratio (0.5) led to the best encapsulation efficiency (Table 3). PEG-PAA also raised the encapsulation efficiency as expected but was less successful than PEG-OVSDM. The increase in encapsulation efficiency coupled with higher recovery of soluble lysozyme after emulsification suggests that complexation of a protein with an appropriate polyelectrolyte can be a useful tool in optimization of microspheres. Similar results have been reported for microencapsulation encapsulating lysozyme (Yoo et al., 2001; Lee et al., 2007), leuprolide (Choi and Park, 2000), and salmon calcitonin (Yoo and Park, 2004) by employing sodium oleate or chondroitin sulfate.

Table 3 shows that the amount of lysozyme aggregates formed during microencapsulation is greater in the absence of polyelectrolytes supporting the interfacial results that shielding of lysozyme by polyelectrolytes may be an effective strategy. Lower aggregation of lysozyme as seen in interfacial studies on addition of PEG-OVSDM suggests that strong binding between lysozyme and PEG-OVSDM may prevent destabilization of lysozyme at the deleterious interfaces/surfaces commonly encountered during the microencapsulation process.

PLGA microspheres showed different particle size distributions. Fig. 4 shows SEM images of three batches of PLGA microspheres encapsulating free lysozyme (A), lysozyme complexed with PEG-OVSDM (B), and lysozyme complexed with PEG-PAA (C). Microspheres containing only lysozyme or lysozyme complexed with PEG-OVSDM/PEG-PAA showed regular, smooth surfaces with very few holes. Most microspheres in the batch A had size in the range of 1–12 μm compared to an increased overall size (1–20 μm) of microspheres encapsulating lysozyme complexed with PEG-OVSDM/PEG-PAA. Differences in size between microspheres batches may be attributed to the viscosity differences between the primary emulsions used for the formation of secondary emulsions. Primary emulsions incorporating the complexes are generally more viscous than that incorporating only protein (Yoo et al., 2001). Nanoparticles aggregation may also be responsible for enhanced particle size of the microspheres in batches B and C. Similar results have been reported while comparing the size of microspheres encapsulating free proteins and complexed proteins (Choi and Park, 2000).

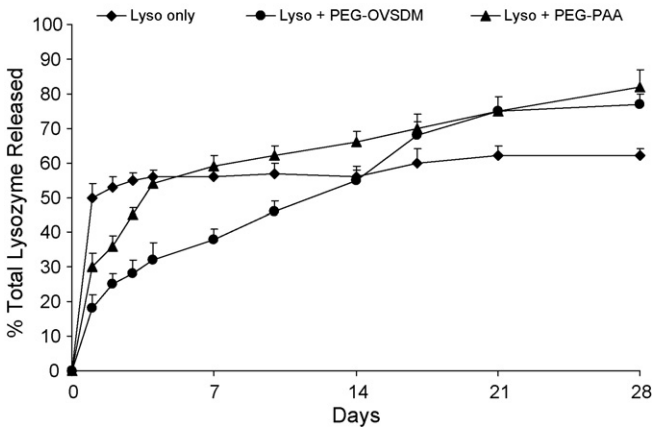

Fig. 5. Cumulative *in vitro* release of lysozyme (wt%) from microspheres containing lysozyme only or lysozyme plus PEG-OVSDM, or lysozyme plus PEG-PAA in an optimal weight ratio, Values are mean ± S.D., $n = 3$.

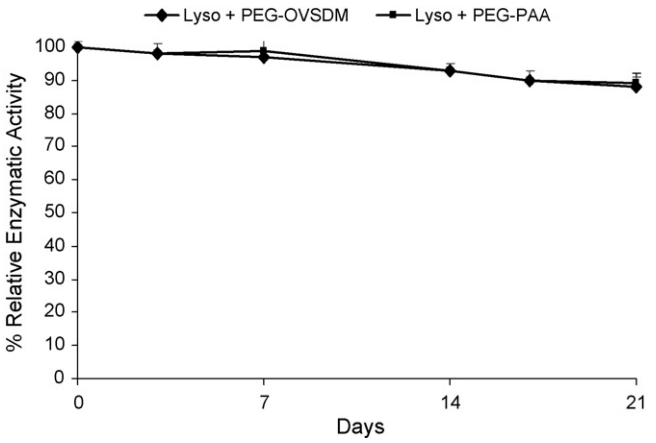

Fig. 6. Relative enzymatic activity of released lysozyme. Released lysozyme was compared with freshly made lysozyme solutions in the release medium (PBT) at equivalent concentrations.

3.6. *In vitro* release of lysozyme

Release profiles of lysozyme from microspheres containing free or ion-paired lysozyme are shown in Fig. 5. Microspheres containing lysozyme/PEG-OVSDM complexes significantly suppressed the initial fast release of lysozyme (~18% after 1 day incubation, 30% from microspheres containing lysozyme/PEG-PAA complexes), ($p < 0.05$ between microspheres containing lysozyme only and microspheres containing lysozyme complexed with the polyelectrolytes) from the microspheres in contrast to microspheres encapsulating lysozyme only. PLGA microspheres containing lysozyme only showed a 50% initial release on the first day, reaching a protein release plateau after 3 days. Complexation reduces the potential for easy diffusion of otherwise freely water-soluble lysozyme by enhancing the nanoparticles size. PEG-OVSDM was more effective than PEG-PAA in reducing the initial release. This may be explained on the basis of difference in strength of intermolecular interactions between lysozyme and the polyelectrolytes. Kamiya and Klibanov (2003) have reported that the release of lysozyme from complexes decreased with increase in hydrophobicity of polyanions. These conclusions are consistent with the view that stronger the lysozyme–polyanion complex (due to enhanced either electrostatic or hydrophobic interactions), the slower the rate of release of lysozyme.

Microspheres encapsulating lysozyme and PEG-OVSDM showed a better control over the release of protein with continuous release up to 21 days and then reaching a plateau. About 77% of the total lysozyme encapsulated was released from the microspheres encapsulating lysozyme/PEG-OVSDM complexes compared to 62% from microspheres containing lysozyme only ($p < 0.05$). Complexation of lysozyme with PEG-PAA also led to an overall higher release of the protein from microspheres than microspheres containing lysozyme only ($p < 0.05$). Possibly, no more nonaggregated lysozyme (monomeric) was left in the microspheres to be released. Unreleased lysozyme was present in microspheres as aggregates; ~30% of initial lysozyme amount compared to about 14% and 16% of initial lysozyme was observed as aggregates in microspheres containing PEG-OVSDM and PEG-PAA, respectively (not insoluble complexes with the polyelectrolytes). It may be speculated that adsorption and denaturation of lysozyme on contact with the hydrophobic PLGA may account for incomplete release of the protein. PEG-OVSDM and PEG-PAA may have been able to prevent these hydrophobic contacts resulting in reduced aggregation in the microspheres to a limited extent. Overall, PEG-OVSDM was more effective than PEG-PAA in modulating the release of lysozyme from PLGA microspheres. Weaker interactions between PEG-PAA and lysozyme might be prone to dissociation by slight changes in the microenvironment. Incomplete release of lysozyme from microspheres encapsulating lysozyme complexed with PEG-OVSDM needs to be altered to achieve a quantitative release for clinical use. Increasing the molecular weight of PEG-block is a viable option that can be investigated for the beneficial effects on modulation of protein release and further preventing contacts with hydrophobic PLGA.

Released lysozyme showed similar retention time as that of a lysozyme standard suggesting that lysozyme is released as a distinct species. There is no evidence yet that PEG-OVSDM altered the degradation of PLGA, which may be attributed to minimal amounts of this proton-buffering polylelectrolyte used in microspheres. Increasing the PEG-OVSDM amount for buffering the pH decline needs to be investigated.

Released lysozyme was subject to enzymatic activity determination and results are shown in Fig. 6. No significant drop in enzymatic activity of the released lysozyme occurred even after 21 days of incubation, which indicates that the tertiary structure of lysozyme was maintained. Complexation with polyelectrolytes may work for labile proteins. Lysozyme serves as a model for several proteins such as interferon and salmon calcitonin.

4. Conclusions

The advantages of PEG-OVSDM over conventional anionic polyelectrolytes such as PEG-poly(L-aspartic acid) is evident in terms of higher retention of lysozyme stability at the deleterious aqueous/organic solvent interface or in contact with the hydrophobic PLGA, higher encapsulation efficiency of lysozyme in the PLGA microspheres, and better modulation of the protein release during incubation in a simulated physiological medium. These differences may be attributed to the nature of interactions between lysozyme and polyelectrolytes. From the results obtained in binding studies between lysozyme and the polyelectrolytes, it may be suggested that interactions between PEG-PAA and lysozyme are more susceptible to be disrupted by change in pH and salt concentration in the medium.

The broad molecular pool of sulfonamides allows the specific selection of both the pH of interest and the extent of aqueous solubility transition at the pH of interest, because sulfonamides demonstrate a range of pK_a (3–11) depending on their side groups

(Foye, 1989). A fine balance between the contributions of ionic and hydrophobic interactions between the proteins and sulfonamide-based polymers would depend on the nature of intended use in therapy.

Acknowledgments

This research was supported in part by an NIH grant GM72612. We thank Dr. Yu Seok Youn for valuable discussion during preparation of the manuscript and help in chromatographic procedures for lysozyme. Dr. Kun Na presently an Assistant Professor at the Division of Biotechnology, The Catholic University of Korea, Gyeonggi-do helped in the synthesis and characterization of the polymers. Appreciation is due to Mike Hanson at the CORE mass spectrometry facility for the mass spectrometry analyses.

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