Surface Entrapment of Polylysine in Biodegradable Poly(DL-lactide-*co*-glycolide) Microparticles

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ABSTRACT: The purpose of this study was to entrap poly(L-lysine) (pLys) as a surface active functional (SAF) polymer in the surface of biodegradable poly(DL-lactide-*co*-glycolide) (PLGA) microparticles. This was performed by stabilizing a methylene chloride-in-water emulsion with PLGA dissolved in the dispersed phase and using modified pLys as an emulsifier. The microparticles form as the organic solvent evaporates and PLGA precipitates entrapping the pLys. Modification of surface activity of pLys was accomplished by adjustment of ionization and secondary structure. PLys with ≥68% degree of dissociation and ≥50% α-helix content stabilized the PLGA oil-in-water emulsion and promoted surface entrapment of the polypeptide. The elevated α-helix content of ionization-modified pLys was essential for the stabilizing effect probably by formation of a strong interfacial film. The ζ-potential measurement of PLGA microparticles prepared with 85% dissociated pLys showed a strongly positive charge, compared with negative values on control microparticles prepared with an un-ionized emulsifier, indicating the presence of the pLys on the microparticle surface. PLys was retained to >60% on the microparticles following exposure to physiological conditions for 3 weeks.

Introduction

The surface modification of biodegradable poly(DL-lactide-*co*-glycolide) (PLGA) microparticle/nanoparticle systems currently has attracted much attention because of the possibility of modulating interaction of these particles with components in body fluid and the potential delivery of these colloidal carriers of bioactive substances to specific organs or tissues.[1−4] For example, surface modification of PLGA microparticles has been performed in order to adsorb DNA vaccines on the positively charged particle surface, which are then capable of inducing a desirable immune response.[3] Similarly, efforts to reduce the particle−protein interaction and to extend the circulation time of nanoparticles have been accomplished by incorporating a hydrophilic layer, e.g., poly(ethylene oxide) (PEO), on the polymer surface.[5,6]

Three major approaches have been reported to achieve surface modification of PLGA particles. First, hydrophilic block segments such as PEG[7] or functional branches such as poly(L-lysine) and poly(L-aspartic acid)[8] can be incorporated into the PLGA backbone to produce a new functionalized block or graft copolymer. Microparticles can be produced with these new polymers either with (e.g., for copolymers of lactic and amino acids) or without (e.g., for amphiphilic PEG−PLGA copolymer) emulsion stabilizers. Partially hydrolyzed poly(vinyl alcohol), PVA, is the most commonly used emulsifier. Second, PLGA and an additional polymer or surfactant of interest such as poly(ϵ-CBZ-L-lysine)[1] and 1,2-dipalmitoylphosphatidylcholine (DPPC), a major lung surfactant,[9] can be blended or codissolved in the dispersed phase, and micro/nanoparticles are produced with the aid of PVA. In both these methods, the functional groups along with the identities of base polymer and other stabilizers generally appear on the surface of these particles. Third, water-soluble surface-active molecules with one or more functional groups, which we *term* surface-active functional (SAF) polymers (e.g., poly(ethylene glycol)−dextran conjugate[6]) or functional (FUN) emulsifiers (e.g., cetyltrimethylammonium bromide, CTAB[3]), can be used as the *principal* emulsifier in place of PVA and added in the continuous phase to stabilize the emulsion formed during production of micro/nanoparticles by emulsion−solvent evaporation methods. As the polymer is dried in liquid, the FUN emulsifier becomes entrapped into the surface of the polymer.

The third approach of entrapping SAF polymers into microparticles, which is the focus of this paper, may have several advantages relative to the other surface-modifying techniques. It is non-labor-intensive, and gram quantities of surface-modified particles can be prepared very rapidly. Unlike the first technique, the SAF polymer approach does not necessarily require synthesis and characterization of new polymers not currently FDA-approved in biomedical products. In addition, only a very small fraction of the SAF polymer on a mass basis (e.g., <0.1% loaded) normally would become embedded in the base polymer (e.g., PLGA), which allows the bulk properties of well-characterized base polymers to be unaltered during surface modification. This is in contrast to both the first and second techniques, which typically use >10% (w/w) of the surface-modifying non-base-polymer materials and, in addition to changing the surface of the polymer, will alter the bulk properties as well.

An important obstacle to the SAF polymer/FUN emulsifier method is the difficulty in making the functional macromolecules of interest sufficiently surface active so that both surface entrapment can occur and microparticles are produced with good dispersive stability. According to emulsion theory, emulsion stability is determined by an interplay of hydrodynamic forces related to the Gibbs elasticity, bulk viscosity, surface diffusion, and the disjoining pressure due to the forces described by Deryagin, Landau, Verwey, and Overbeek

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(DLVO forces) and oscillatory forces applied on the thin plane-parallel film between particles.[10] In the case of an emulsion system stabilized by macromolecules including polymers and proteins, a combination of steric and electrostatic repulsion as well as viscoelasticity of the surface film is believed to be responsible for emulsion stability.[11] Most work on adjustment of the surface activity of SAF polymers has achieved this by conjugating hydrophilic segments such as PEG and dextran, with predominantly hydrophobic polymers such as PLA and poly(propylene oxide) (PPO). During emulsification, SAF polymers will distribute between both water and oil phases, with mainly hydrophobic segments anchored in the PLGA phase and hydrophilic tails extending into the outer phase as tails and loops to impose steric stabilization.

In the present study, the potential use of the homopolypeptide, poly(L-lysine) (pLys), as a model SAF polymer, and its surface entrapment in PLGA microparticles was investigated. To adjust the surface activity of pLys, the dissociation degree of its ϵ-amino groups and its secondary structure were adjusted over a wide range. Herein, the novel surface entrapment of pLys in PLGA microparticles and the specific physicochemical properties of pLys necessary in attaining this result are described.

Experimental Section

Materials. Poly(L-lysine) hydrobromide (pLys: 150−300 kDa), sodium dodecyl sulfate (SDS), and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Poly(DL-lactide-*co*-glycolide) 50/50, end group capped, with an inherent viscosity of 0.20 dL/g in HFIP at 30 °C and a $\overline{MW}_w$ of 9440 Da (provided by manufacturer) was obtained from Birmingham Polymers, Inc. (Birmingham, AL). *o*-Phthalaldehyde was purchased from Alltech Associates Inc. (State College, PA). L-Leucine was from Fluka Chemical Corp. (Milwaukee, WI). All other reagents were analytical grade or higher and used as received.

Potentiometric Titration of pLys Solution. 20 mL of 0.63 mg/mL or 5 mL of 5.0 mg/mL pLys solution, in CO_2-free dimineralized double distilled water or 0.19 N KCl were titrated under stirring at 25 °C. CO_2-free and water-vapor-saturated N_2 was continuously passed through the solution during titration. The pH was measured with a Corning (Corning, NY) combination electrode, which was calibrated with pH 4, 7, and 10 buffer solutions before each use. After each addition, pH was recorded until equilibrium was established.

Circular Dichroism (CD) Spectroscopy. All CD measurements were carried out on an AVIV circular dichroism spectrometer, model 202, at 25 °C using 0.1 mm (for 5.0 mg/mL or 1 mm (for 0.1 mg/mL) quartz cells. All of the pLys solutions were made in either CO_2-free dimineralized double-distilled water or 0.19 N KCl. The degree of dissociation (α) of pLys solution was modified by the addition of 0.1 or 1.0 N NaOH. For 68% dissociated pLys solution (5.0 mg/mL), secondary structure was disturbed and monitored by CD after heating the solution at 50 °C for 1 h and subsequent cooling at 25 °C for 1.5 h.

Preparation of PLGA/pLys Microparticles. PLGA microparticles were prepared by oil-in-water emulsion−solvent evaporation methods.[12] To prepare PLGA/pLys or PLGA/PVA microparticles, PLGA was dissolved in methylene chloride at 500 mg/mL. PLys (MW 150−300 kDa) solution (0.5%, w/v) at various degree of ionization was prepared by titrating the polypeptide solution with 0.1 or 1.0 N NaOH. A 0.25 mL PLGA solution was emulsified with 0.25 mL 0.5% pLys solution at 13, 45, 68, 85, and 97% degree of dissociation or 0.5% PVA in distilled water solution by vortex-mixing (Fisher Genie 2, Scientific Industries, Inc., Bohemia, NY). The resultant PLGA/pLys emulsion was hardened in 60 mL of dimineralized double-distilled water for 3 h under stirring. All microparticles were collected by centrifugation and washed three times with 0.15 N NaCl, suspended in a solution containing 7% (w/v) sucrose and 0.04 N NaCl, and freeze-dried. For freeze-drying, samples were flash frozen in liquid nitrogen and placed on Labcono freeze-dry system (Kansas City, MO) at 133×10^{-3} mbar or less at a condenser temperature of −46 °C for at least 24 h.

Evaluation of Emulsion Stability. The o/w emulsion stability was monitored by the kinetics of particle size distribution during in-liquid drying of the microparticles in order to evaluate the emulsifying activity of pLys. Photographs of the particles were taken through a microscope (Axiolab, Zeiss, Thornwood, NY) at 0, 1, and 3 h during solvent evaporation. The size of the particles on the microphotographs was calibrated with a stage micrometer. For convenience, the measured particles were divided into five classes according to their size: 0−9, 9−18, 18−26, 26−35, and >35 μm, and the populations of each group were counted ($n = 100-700$). Two or more preparations were analyzed for all formulation conditions. This method has been reported to be reliable and widely used for measuring course particles (>2 μm).[11,13] Other indicators of emulsion stability were also monitored, namely, (1) polydispersity, (2) appearance of collected microparticles (see below), (3) observed aggregation on the stirrer bar, and (4) microparticle yield.

Determination of pLys Content Entrapped in PLGA/pLys Microparticles by a Precolumn OPA Derivatization RP-HPLC Method. The amount of entrapped pLys was determined by a precolumn *o*-phthaldialdehyde (OPA) derivatization lysine assay where the PLGA/pLys particles were completely hydrolyzed in 6 N HCl. The pH of acid-hydrolyzed sample was kept within a narrow range of 9.40−9.42 to avoid changes in reactivity with OPA.[14] To promote lysine-OPA fluorescent product stability, derivatization time was very carefully controlled (exactly 1 min) and SDS was added in the derivatization mixture.[15,16] The analytical method is summarized as follows:

(i) Preparation of the o-Phthaldialdehyde/2-Mercaptoethanol (OPA/2-ME) Derivatizing Solution. 250 mg of OPA was dissolved in 6 mL of absolute methanol. 250 μL of 2-ME was added, and the solution was adjusted to 60 mL with 0.2 M sodium carbonate buffer (pH 9.5). The mixture was flushed with nitrogen and filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA). The solution was stored in the dark at 4 °C before use. 2-ME was added every 2 days to maintain the reagent strength.

(ii) Sample Preparation, Derivatization, and HPLC Analysis About 3 mg of PLGA/pLys microparticles and 2 nmol of L-leucine as internal standard were hydrolyzed in 6 N HCl at 110 °C for 22 h after sealing under light vacuum. The hydrochloric acid was removed by centrifugation under vacuum (Eppendorf Vacufuge, Brinkmann Instruments, Inc., Westbury, NY) at 43−45 °C. 0.5 mL of 1 M sodium carbonate buffer (pH 9.5) was added to dissolve and neutralize the residual hydrolysis products. After centrifuging at 10 000 rpm for 5 min, 50 μL of supernatant was removed and added to 50 μL of 2% (w/v) SDS in 0.2 M carbonate buffer (pH 9.5) solution and 50 μL of OPA/2-ME derivatizing solution. The solution was mixed thoroughly by vortex. Exactly after 1 min, 120 μL of mixture solution was injected to an ODS column (Nova-Pak C_{18}, 3.9 × 150 mm, 4 μm, Waters, Milford, MA) fitted with a guard column (Nova-Pak C_{18} Guard-Pak, Waters) by a Waters 717plus autosampler, eluted with degassed methanol/0.05 M sodium acetate buffer, pH 6.8, 58/42, at a rate of 1.5 mL/min. The signals were detected by a Waters 474 scanning fluorescence detector ($\lambda_{Ex}/\lambda_{Em} = 340/455$ nm). The method was validated before sample determination.

Surface Characterization of PLGA/pLys Microparticles by Scanning Electron Microscopy (SEM) and ζ-Potential Measurement. The morphology of the microparticles was analyzed with a scanning electron microscope (Philips XL-30). Microparticles were freeze-dried after removal of salts and sucrose in the sample before coating with a thin gold layer in an argon atmosphere using a Pelco model 3 sputter coater for SEM use. The measurement of the ζ-potential of PLGA/pLys microparticles was carried out by a Zetasizer

(Malvern, 3000HS, Southborough, MA) in distilled water from pH 4 to 8 with an autotitration module.

Retention Kinetics of pLys on PLGA/pLys Microparticles. Sucrose was removed from the lyophilized microparticles formulation by washing twice the PLGA/pLys microparticles with the release medium, PBS (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.02% Tween-80, followed by centrifugation. The washed microparticles were incubated in 0.5 mL of the release media and incubated at 37 °C under gentle agitation on an IKA KS 125 shaker at 120 rpm. The microparticles were collected at predetermined time intervals (1, 3, 7, 14, 21 days) and frozen after removal of the supernatant solution. The content of pLys remaining in the microparticles was determined as described above after acid hydrolysis.

Data Analysis. *Determination of Apparent pK_a (pK_{app}) of pLys from pH Titration Curves.* Apparent dissociation constant pK_{app} was calculated by

$$\mathrm{p}K_{\mathrm{app}} = \mathrm{pH} - \log \frac{\alpha}{1-\alpha}$$

where α is the average degree of dissociation, defined by

$$\alpha = \frac{[\mathrm{RNH_2}]}{[\mathrm{RNH_2}] + [\mathrm{RNH_3}^+]}$$

where $[\mathrm{RNH_2}]$ is obtained from the amount of NaOH titrated. Degree of ionization is equivalent to 1 − α.

Determination of Conformational Composition from CD Measurement. The data obtained from CD measurement were analyzed with the G&F program according to the method of Greenfield and Fasman.[16] This specific program was used mainly because pLys was used in the method development as one of the standards.[16] Ellipticities at 13 data points in the range 208−240 nm were used to calculate the conformational composition: random coil, α-helix, and β-structure.

Determination of Surface Density of pLys on PLGA/pLys Microspheres. Surface pLys density (mg/m^2) was obtained by dividing the total pLys content per weight of microspheres by the total specific surface area (SSA). The SSA (total surface area of particles divided by the total weight) was calculated by

$$\mathrm{SSA} = \frac{\sum 4\pi r_i^{\,2} N_i}{\rho \sum \frac{4}{3}\pi r_i^{\,3} N_i} = \frac{6\sum V_i/d_i}{\rho \sum V_i} \quad \left(V_i = \frac{4}{3}\pi r_i^{\,3} N_i\right)$$

where N_i is the number and V_i the volume of particles with mean class radius r_i and mean class diameter d_i. ρ is the particle density, which was assumed to be 1 g/cm^3 for all PLGA/pLys particles. For this calculation, it is assumed that no pLys was encapsulated in the bulk PLGA, since pLys is not soluble in the carrier solvent, methylene chloride.

Results and Discussion

Overall Approach for the Formation of Surface-Modified PLGA Microparticles by Adjustment of Surface Activity of pLys. In order for pLys to be useful as a SAF polymer and to coat the entire microparticle surface, the polypeptide must be able to effectively replace common emulsifiers such as PVA used in the solvent evaporation method. However, because of its high charge density, pLys at neutral pH is unable to stabilize the o/w emulsion used to form PLGA microparticles. To reduce this charge and to promote its surface activity, attempts to introduce a hydrophile−lipophile balance in the polypeptide were performed. In control studies, this was accomplished by varying the degree of acetylation of the ϵ-amino groups of pLys. With acetylated pLys (MW = 4−15 kDa) synthesized at ≥5:1 molar ratios of acetic anhydride/lysine residues, microparticles were well formed and of a useful size (data not shown). Excess acetic anhydride was required in the reaction mixture due to the competition of hydrolysis with acetylation.

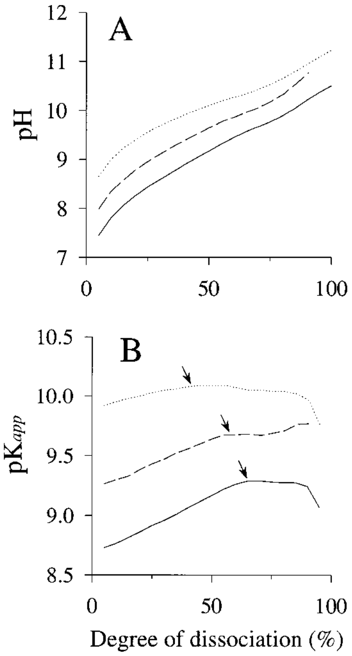

Figure 1. Potentiometric titration of pLys: (A) titration curve of pLys and (B) effect of concentration and ionic strength on apparent dissociation constant of pLys, pK_{app}; the starting points of conformational transition were indicated by arrows on the curve. Concentration of pLys was 5.0 mg/mL in distilled water (− −), 5.0 mg/mL in 0.19 N KCl solution (···), and 0.63 mg/mL in distilled water (—).

The particles formed from acetylated pLys exhibited very low amount of available ϵ-amino groups and a poor dispersive stability and were, therefore, not pursued further. To improve the number of amino groups available at the surface and particle dispersibility, another strategy to surface-entrap pLys in PLGA microparticles was adopted: namely, by adjustment of the ionization degree of the polypeptide. It should be noted that although the performance of the control microparticles entrapping acetylated pLys was poor, the foregoing experiment confirmed a simple, but important, principle: poorly surface-active polymers with functional units (e.g., ϵ-amino groups) can be modified into SAF polymers and incorporated in PLGA microparticles by the emulsion−solvent evaporation technique.

Characterization of Native pLys by Potentiometric Titration and CD Spectroscopy. The ionization degree and secondary structure of pLys as a function of pH were determined in order to later correlate these properties for the specific pLys selected (MW 150−300 kDa) with the surface entrapment of the polypeptide (see below). The titration curves of pLys are displayed in Figure 1A. The concentration and ionic strength of the pLys solution affected the solution pH throughout the whole span of ionization. The pH titration curve was shifted upward when either pLys concentration was increased (from 0.63 to 5.0 mg/mL) or salt (0.19 N KCl) was included. Similarly, the increase in concentration caused a shift in pK_{app} to a higher value at a given α (Figure 1B). The addition of salt had a similar effect on pK_{app} by increasing the net charge on the molecule at a given pH and an increased shielding

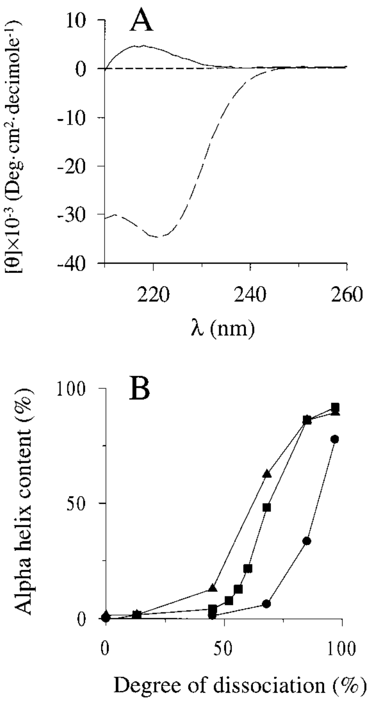

Figure 2. Secondary structure of pLys determined by circular dichroism spectroscopy. (A) Typical CD spectra of pLys in water (5.0 mg/mL) possessing either mostly random coil (—) at $\alpha = 0$ or mostly α-helix (– –) conformation at $\alpha = 1.0$. (B) Effect of ionization, concentration, and ionic strength on the α-helix content of pLys obtained by fitting the CD data with Program G&F (by Greenfield and Fasman).[17] Concentration of pLys was 0.1 mg/mL in distilled water (●), 5.0 mg/mL in distilled water (■), and 5.0 mg/mL in 0.19 N KCl solution (▲). The increase in α-helix content was accompanied mainly by a decrease in random coil content. Content of *β*-structure was less than 20% within the entire range of α and increased steadily with increasing α (data not shown).

of electrostatic repulsion between charged side chains at the increased ionic strength.[18] The well-known ionization-induced conformational transition from random-coil to α-helix of pLys can be located at the critical point where the curve of pK_{app} vs α levels off.

To more accurately determine the point of transition and the range of ionization degree over which the transition occurs, the secondary structure was analyzed by CD spectroscopy. As shown in Figure 2, the conformational transition from random coil to α-helix in pLys in distilled water solution (5.0 mg/mL) was initiated at $\alpha \cong 0.5$ and completed by $\alpha \cong 0.8$. A higher dissociation degree was needed to initiate this transition at lower pLys concentration (0.1 mg/mL). In contrast, the presence of salt facilitated this process at lower α ($\Delta\alpha \cong 0.1$ or less), which confirmed the stabilizing effect of salt on the α-helix structure due to reduced electrostatic repulsion of the side chains and elevated pLys concentration.[19]

Evaluation of Emulsifying Activity of Ionization-Modified pLys by Surface Entrapment Density and Emulsion Stability. The emulsifying activity[20] of pLys at different α was evaluated by the amount of polypeptide incorporated into the interphase and by the ability of pLys to inhibit emulsion coalescence. As shown in Table 1, the amount of pLys entrapped in the microparticles greatly increased with increasing α and approached a plateau of about 0.95 mg/g at 85% dissociation. The surface pLys density calculated from specific surface area and total surface entrapment (for details see Experimental Section) also increased with increasing degree of dissociation and appeared to approach a saturated value (~4 mg/m²) at $\alpha = 0.85$ (Table 1). This value is comparable to the surface density of PVA (1–2 mg/m²),[21] the commonly used emulsifier to prepare PLGA microparticles, and a monolayer surface coverage of proteins adsorbed at oil/water interfaces (1–3 mg/m²).[22]

Table 1. pLys Content Entrapped in PLGA/pLys Microparticles Prepared with pLys with Various Degree of Dissociation, Determined by Precolumn OPA Derivatization HPLC Methods

dissocn deg of pLys	amt of lysine residue incorporated in the microparticles (mg/g) (mean ± SD, $n = 3$)	specific surf. area (m²/g)	surf. pLys density (mg/m²) (mean ± SD, $n = 3$)
13% w/o salt	0.35 ± 0.03	0.27	1.3 ± 0.1
w/ salt	0.30 ± 0.00	0.18	1.7 ± 0.0
45% w/o salt	0.53 ± 0.04	0.24	2.2 ± 0.2
w/ salt	0.38 ± 0.04	0.18	2.1 ± 0.2
68% w/o salt	0.80 ± 0.03	0.23	3.6 ± 0.1
w/ salt	0.74 ± 0.07	0.17	4.4 ± 0.4
85% w/o salt	0.95 ± 0.04	0.22	4.3 ± 0.2
w/ salt	0.80 ± 0.04	0.17	4.8 ± 0.2
97% w/o salt	0.95 ± 0.03	0.23	4.2 ± 0.1
w/salt	0.96 ± 0.02	0.19	5.1 ± 0.1

It has been reported that the amount of adsorbed pLys on solid latex increases with increasing pH, with a sigmoidal-shaped adsorption profile on the surface of positively charged polystyrene latex.[23,24] It was suggested that decreasing charge density of the polyelectrolyte diminishes the strong lateral repulsion between ammonium groups more strongly than the interaction with the surface, which appears to be supported by polyelectrolyte adsorption theory.[25] The same explanation may apply to the data described in Table 1. The increase in the degree of dissociation increased pLys adsorption by the effect of increased hydrophobicity (and thus increasing partitioning in the dispersed phase) in cooperation with decreasing the lateral electrostatic interaction among pLys chains. However, the potential reactivity between amino groups of pLys and the polyester, PLGA, at mildly basic pH cannot be ruled out.

The most relevant types of instability observed in o/w emulsions are Ostwald ripening, coalescence, partial coalescence, aggregation, and creaming/sedimentation.[26] For PLGA o/w emulsions under stirring, coalescence was found to be the dominant instability route when ionization-modified pLys was used. Aggregation and creaming were also observed in the system in some instances (e.g., $\alpha = 0.68$, after heat treatment; see below). As a primary indicator of coalescence, the kinetics of particle size distribution of the PLGA/pLys microparticles are shown in Figure 3A. Each bar represents the average of volume fractions of each particle size class (five classes total) in different preparations ($n = 2$). Reproducible particle size distribution profiles were observed at various pLys ionization degree, which further confirmed the validity of methods used and the significance of pLys ionization on batch emulsion stability. Microparticles prepared with low α (0.13–0.45) showed an increase in size during 3 h with a highly polydispersed size distribution, indicative of emulsion coalescence (and poor emulsifying activity of pLys). In contrast, as α was raised to 0.68–0.97, the particle size distribution was virtually unchanged over the 3 h hardening period with a more monodispersed particle size distribution, indicating a

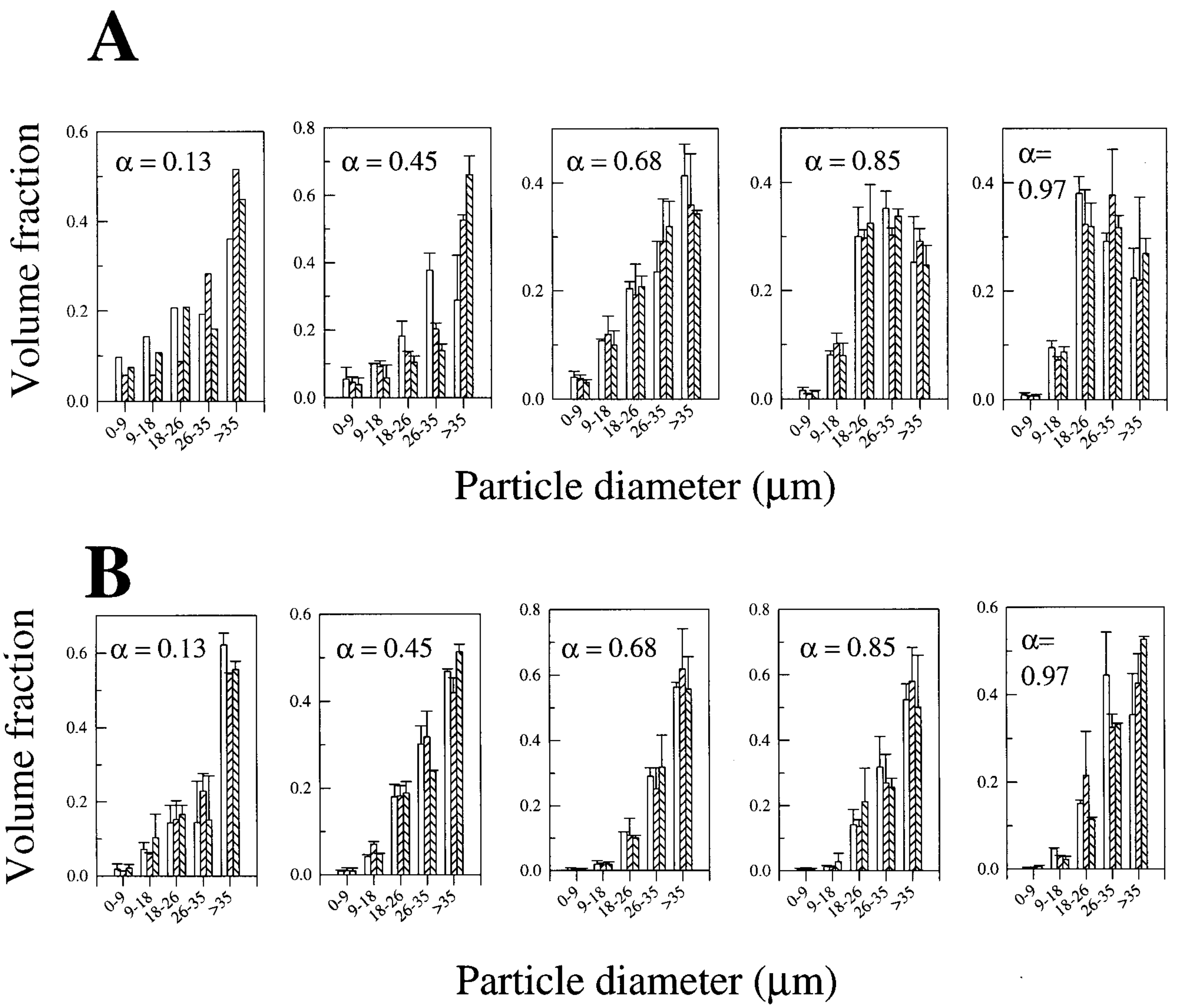

Figure 3. Effect of dissociation degree of pLys and the presence of salt on the stability of the o/w emulsion during microsphere formation. The volume-based particle size distribution of emulsions at 0 (□), 1 (▨), and 3 h (▧), during in-liquid hardening (3 h total) was used to evaluate the bulk emulsion stability in the absence (A) or presence (B) of 0.19 N KCl at various dissociation degree (α). Error bars represent the standard deviation of two preparations, except for the 13% dissociation (without salt batch), in which one of the emulsions failed to form.

stable emulsion. The improved emulsion activity of pLys at α ≥ 0.68 may be related to the higher surface adsorption as well as secondary structure of pLys (see below). It is important to note that since the organic solvent in the oil phase is evaporating from the system that in some cases the particle size actually decreased with hardening time. Furthermore, the dispersive stability of the freeze-dried PLGA/pLys microparticles, similar to emulsion stability, was also dependent on the pLys ionization, with highly dissociated pLys (e.g., α ≥ 0.85), providing a stronger barrier against aggregation during freeze-drying and better dispersibility (data not shown). To further improve the dispersive stability of freeze-dried microparticles, the lyoprotectant sugar, sucrose, was added, and little aggregation was visualized in the microparticle suspension thereafter (data not shown).

As shown in Figure 4, there was a striking similarity between the surface density of pLys and the amount of α-helix in pLys solution. In addition, the range of α (0.5−0.75) of these curves where both these parameters rapidly increased coincided with the critical value of α where the o/w emulsion became stabilized (α ≥ 0.68).

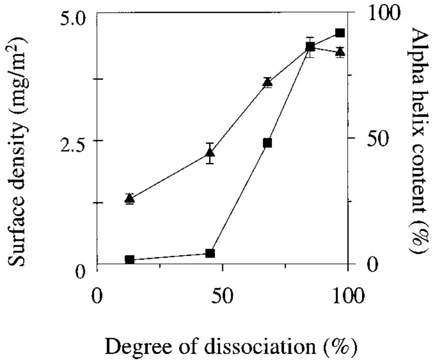

Figure 4. Comparison of the increase in amount of pLys surface-entrapped (▲) (mean ± SD, *n* = 3) in PLGA microspheres and the increase in α-helix content in pLys solution (■) with degree of dissociation.

While the role of increased adsorption of pLys onto the oil−water interface on emulsion stability is relatively unambiguous, the role of pLys secondary structure is less clear. To address this question, we utilized the well-known loss of α-helix with increasing temper-

Table 2. Effect of Heat Treatment on the Conformation and Surface Entrapment of PLys during PLGA Microparticle Preparation

	% secondary structure ($\alpha = 0.68$)			
	α-helix	β-structure	random coil	x
no heat treatment	48.1	7.3	44.6	0.80 ± 0.03
with heat treatment[a]	28.4	12.3	59.4	0.95 ± 0.02

[a] 5.0 mg/mL pLys solution was heated at 50 °C for 1 h and cooled at 25 °C for 1.5 h prior to microparticle preparation. [b] x = amount of pLys entrapped in the microparticles (mg/g) (mean ± SD, n = 3).

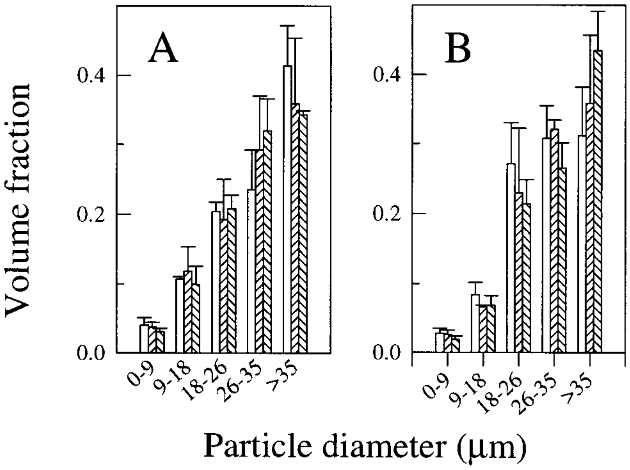

Figure 5. Effect of heat-induced perturbation of α-helix content on the stability of the o/w emulsion during microsphere formation. The volume-based particle size distribution of emulsions, at 0 (□), 1 (⊠), and 3 h (⧅), during in-liquid hardening (3 h total) was used to evaluate the stability of bulk emulsions prepared by pLys, without **(**A**)** or with **(**B**)** heat treatment. Error bars represent the standard deviation of two **(**A**)** and three **(**B**)** preparations. No aggregates were observed in samples prepared with unheated pLys, whereas blocks of aggregates were observed in the samples prepared with pLys following the heat treatment.

ature[19] by comparing the microparticles formed with pLys with and without heat treatment (heating at 50 °C for 1 h, followed by cooling at 25 °C for 1.5 h) of pLys ($\alpha = 0.68$).This dissociation degree was chosen because it falls at the critical value of α when pLys begins to show sufficient emulsifying activity. As shown in Table 2, after brief exposure to 50 °C and subsequent cooling, the transition from α-helix to random coil had occurred as expected[19] with the α-helix content decreasing by ~20%. Emulsification with this heat-treated solution resulted in a less stable emulsion, and aggregation of the forming particle was clearly observed (Figure 5). In contrast, the amount of pLys entrapped in the polymer was actually increased by the heat treatment (Table 2).

The adsorption increase after heat treatment was probably due to the higher mobility of the pLys chains in extended random coil structure. However, ordered α-helical structure imparted a higher stabilizing effect on the newly formed emulsion even with less surface entrapment. This effect can be explained by the increased strength of interfacial films[11] formed with α-helix polylpeptides relative to their less ordered counterparts.[27] For example, native BSA with its highly α-helix content loses surface activity when denatured.[20]

Effect of Salt on Emulsifying Activity of pLys Solution. To determine the role of electrostatic interactions of the pLys chains and the effect of ionic strength on emulsion stability, pLys solution in the presence of salt over the same dissociation degree range as in the previous section was used to prepare PLGA microparticles. As shown in Table 1 and Figure 3B, the presence of 0.19 N KCl in pLys solution at various α not only affected the entrapment of ionization-modified pLys but also influenced the stability of the emulsion. At high degree of dissociation, the surface density of pLys on microparticles increased compared with those microparticles formed in the absence of salt (Table 1), which was likely caused by the shielding of electrostatic repulsion between polyelectrolyte chains. In contrast, at low degree of dissociation, the screening effect of salt was insufficient to compensate for the intermolecular electrostatic repulsion of the pLys chains, and no significant increase in entrapment was observed.

Upon addition of salt, interestingly the emulsions prepared with highly dissociated (97%) pLys were no longer stable regardless of the increased pLys adsorption, as indicated by the increase in the particle size (Figure 3B), while apparently (see below) becoming relatively stabilized at low dissociation degree (13% and

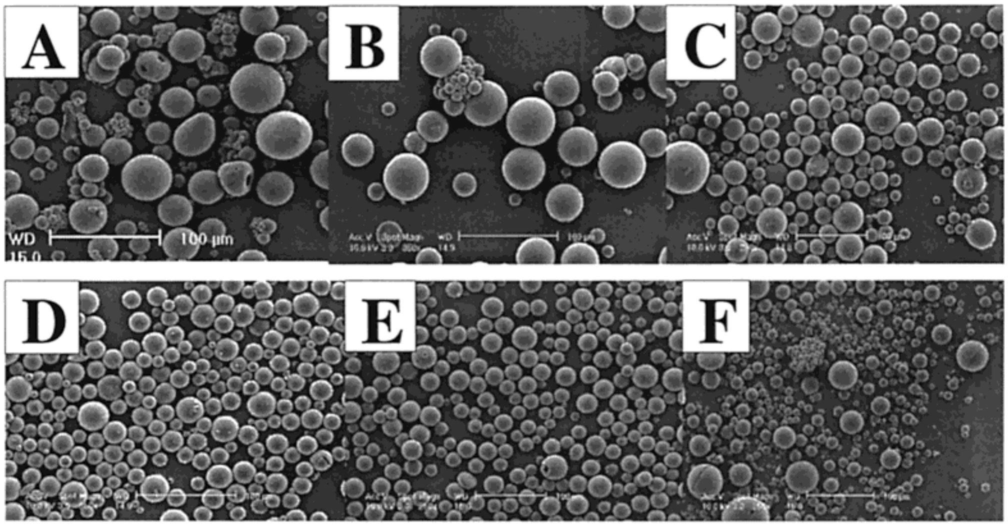

Figure 6. Morphology of PLGA microparticles prepared from ionization adjusted pLys and PVA. Scanning electron micrographs (SEM) of PLGA microparticles prepared from pLys at 13% **(**A**)**, 45% **(**B**)**, 68% **(**C**)**, 85% **(**D**)**, and 97% **(**E**)** degree of dissociation and PVA **(**F**)**.

45%). At the same time, the newly formed emulsion (0 h) had a larger particle size in all formulations relative to the case with no salt included in the pLys solution.

The effect of the pLys adsorption increase accompanying the presence of salt apparently does not correlate with its deleterious effect on emulsion stability. A study from Vliet et al. concluded from the viscosity and dynamic data[28] that in o/w emulsions stabilized by polymethacrylate (PMA), an anionic polyelectrolyte, strong attraction between loops and trains of polyelectrolyte on different emulsion droplets only occurred at low ionization degree. The increase in the viscosity and dynamic moduli following addition of NaCl was also found to be more significant at low ionization degree. In our experiment, the intermolecular forces between highly dissociated pLys on different emulsion droplets in the presence of salt also appeared to bridge the droplets, thereby decreasing emulsion stability. Our observation of the tendency of pLys solution to precipitate in the presence of salt (data not shown) also confirmed the possibility of pLys-induced emulsion coalescence. In addition, the yield of the PLGA/pLys microparticles prepared with 13% and 45% dissociated pLys was found to be extremely low (~20% and 40%, respectively) compared to other formulations (50−65%) prepared with or without salt. (Some losses always occur mainly due to retention of the emulsion on test tube walls used to prepare and transfer the emulsion into the hardening bath.) Therefore, the reason that emulsions prepared with highly ionized pLys appeared to be stabilized in the presence of salt was likely related to the aggregation of particles on the stir bar and thus the diluting of the emulsion system and decreasing particle collision frequency. Hence, the salt effects suggested against the use of a buffer system for preparation of PLGA/pLys microparticles.

Surface Characterization of the PLGA/pLys Microparticles. Scanning electron microscopy was used to examine morphology, particle size distribution, as well as aggregation behavior of PLGA/pLys microparticles. As shown in Figure 6A−E, microparticles produced by highly ionized ($\alpha = 0.13-0.45$) pLys were highly polydispersed and mostly aggregated after freeze-drying, which is consistent with the poorly stabilized emulsions from which they originated (Figure 3A). In contrast, those microparticles prepared with ≥68% dissociated pLys from stabilized emulsions showed satisfactory appearance compared with PLGA microparticles prepared with poly(vinyl alcohol) (PVA). That is, the PLGA/pLys microparticles were monodispersed with narrower size distribution (e.g., for $\alpha = 0.85$, number mean diameter ± SD was 25.3 ± 7.5 μm, $n = 108$) compared with those prepared with highly ionized pLys (e.g., for $\alpha = 0.13$, number mean diameter was 15.3 ± 12.0 μm, $n = 95$), well separated even after removal of sucrose and further freeze-drying, and appeared spherical with smooth surfaces.

Since PLGA/pLys microparticles are expected to possess a thin layer of pLys chains coating the particle surface, a positive ζ-potential is expected. Results from ζ-potential measurement of microparticles prepared with 85% dissociated pLys are shown in Figure 7. Compared with the control PLGA/PVA preparation, the PLGA/pLys microparticles displayed a strongly positive ζ-potential in an acidic to neutral pH environment (pH 4−7.5), indicating the presence of pLys chains on the particle surface. Highly positive ζ-potential at low pH corresponds to a highly ionized fraction of those polypeptide chains on the microparticle surface. The decrease in ζ-potential with approach of media from acidic to neutral pH could be the complicated outcome of increased dissociation of protonated amino groups on pLys chains and dissociation of carboxyl groups on PLGA. This effect became even more pronounced at higher pH and caused the pH of the suspension to drift continuously, prohibiting further ζ-potential titration. The positive surface charge of these particles potentially may be useful to adsorb anionic molecules, especially polyanions, such as DNA. Condensation of DNA onto the surface of PLGA microparticles via electrostatic interaction has shown promise as a unique and efficient way to deliver DNA vaccines and therapeutics.[3]

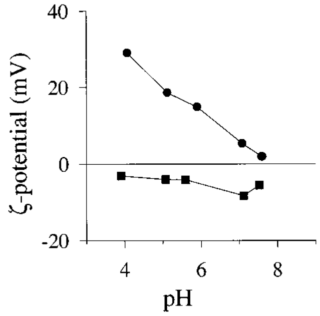

Figure 7. ζ-potential of PLGA/pLys microparticles prepared with 15% ionized pLys and PVA and as a function of pH: (●) PLGA/pLys microparticles; (■) PLGA/PVA microparticles.

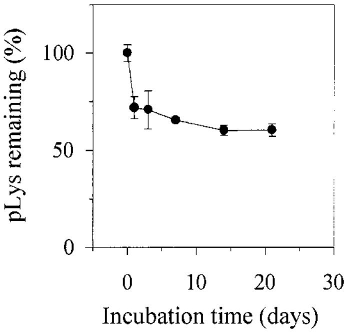

Figure 8. Retention kinetics of pLys on PLGA/pLys microparticles prepared with 85% dissociated pLys. The pLys retained on microparticles (mean ± SD, $n = 3$) after incubation in PBS (with 0.02% Tween-80) at 37 °C was determined by HPLC analysis after acid hydrolysis.

Retention Kinetics of pLys on PLGA/pLys Microparticles. To determine how firmly pLys was entrapped in the surface, the kinetics of pLys retention in the microparticles was examined under simulated physiological conditions. As shown in Figure 8, after a pulse release of ~30% in the first day, most of the pLys was retained on microparticles prepared from $\alpha = 0.85$ for the remaining 3 weeks of the experiment. The long-lasting retention of pLys may be favorable for the function of prolonged association of these either antibody/ligand or vaccine-bearing PLGA particles with cells in vivo and exerting targeting function.

Conclusions

The surface activity of pLys can be adjusted to surface-entrap the polypeptide on the surface of PLGA microparticles. After adjusting the dissociation degree to ≥68% and α-helix content to ≥50%, the surface activity of pLys was suitable for its surface entrapment and proper formation of microparticles. With the addition of salt, pLys displayed higher surface entrapment at higher degree of dissociation, whereas the emulsion stability correspondingly decreased probably due to increased intermolecular interactions of pLys chains on neighboring droplets within the emulsion system. Good dispersive stability of PLGA/pLys microparticles was found only in those preparations with highly dissociated pLys, and PLGA/pLys microparticles prepared with 85% dissociated pLys displayed a strongly positive surface charge at an acidic to neutral pH. These pLys surface-entrapped PLGA microparticles may have a variety of applications in drug targeting and vaccine and gene delivery technologies.

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