Pharmaceutical Research, Vol. 24, No. 12, December 2007 (© 2007)
DOI: 10.1007/s11095-007-9378-1

Research Paper

One-Step Surface Modification of Poly(lactide-*co*-glycolide) Microparticles with Heparin

Chengji Cui[1,2] and Steven P. Schwendeman[1,3]

Received April 17, 2007; accepted June 13, 2007; published online August 21, 2007

Purpose. The aim of this study was to modify the surface of poly(lactide-*co*-glycolide) (PLGA) microparticles with heparin. The heparin-coated PLGA may enhance blood and tissue compatibility of PLGA devices and provide a novel approach to deliver growth factors.
Materials and Methods. A one-step method using heparin to replace traditional emulsifiers (e.g., PVA) during emulsion-solvent evaporation process was employed to surface-entrap heparin in PLGA microspheres. The emulsifying activity of heparin was modified *via* varying counter ion form, including univalent (Na^+, K^+, Li^+, and NH_4^+) and divalent (Ca^{2+}, Mg^{2+}, Ba^{2+}, and Zn^{2+}) cations, and complexation with amino acids (Arg, Lys, Leu, Val, Gly and Glu). Surface accessible and total heparin loading were determined by a modified toluidine blue assay and elemental analysis, respectively.
Results. Heparin bound with univalent counter ions and amino acids exhibited emulsifying activity to varying degrees, whereas divalent heparin salts tended to cause complete aggregation of the PLGA o/w emulsion. Increasing pH (≥7.4) of hardening medium enhanced heparin adsorption and significantly stabilized the PLGA o/w emulsion. The initial surface density of heparin on the PLGA microspheres prepared using univalent heparin salts was around 8–33 mg/m^2. Surface associated heparin desorbed quickly; potassium heparin showed the best retention, with ~0.2 and 0.1 mg/m^2 detected on PLGA microsphere surface following 1- and 14-day incubation in PBST at 37°C, respectively.
Conclusions. PLGA microparticles were successfully surface-modified with heparin. Univalent salts and amino acid complexes of heparin, as effective emulsifiers, can become surface-immobilized in PLGA microspheres.

KEY WORDS: heparin; poly(lactide-*co*-glycolide); surface modification.

INTRODUCTION

In numerous biomedical and pharmaceutical applications it becomes necessary to functionalize the surface of the biomaterials, such as commonly used poly(lactic-*co*-glycolic acid; PLGA). A number of methods to accomplish this include modifying the bulk polymer such as PEG-containing PLGA (PEG-PLGA, PEG-PLA; 1–3) or blending an additional compound of interest (e.g., 1,2-dipalmitoylphosphatidylcholine DPPC) or polymer [e.g., poly(ε-CBZ-L-lysine)] with PLGA during microsphere preparation (4,5), and copolymerization of a monomer bearing a functional side chain, e.g., polylysine (6). As complex polymer synthesis can be time consuming and expensive, the resulting polymer quantities for use on the laboratory scale is often limited. Moreover, difficulties with other surface modification methods include safety and regulatory hurdles related to the new polymer (more toxicological and clinical studies may be required for human use) and significant change of the bulk PLGA properties such as degradation and release profile due to the high content of non-PLGA materials.

Therefore, in the late 1990s, we were motivated to explore alternative methods to simplify surface modification of PLGA and other biomaterials. We devised a method based on the well-known surface physical entrapment of the commonly used emulsifier, polyvinylalcohol (PVA), during solvent evaporation processes (7). In this patented method (8), PVA is replaced with a polymeric emulsifier bearing functional (FUN) groups and surface-entrapped in the PLGA particle surface (8,9). We were able to modulate the hydrophile–lipophile balance (HLB) and improve emulsifying activity of a model polypeptide functional emulsifier, polylysine, initially by partially acetylating the charged α-amino groups (8) and later by modulating the dissociation degree (10) to provide conjugatable (11) or DNA condensable amino groups at the surface of PLGA microspheres. The resulting process, which was also applicable to other polymer configurations such as tissue engineering scaffolds, could surface modify the PLGA surface by simple mixing of the FUN emulsifier and PLGA, providing grams of stable

[1] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, Michigan 48109-1065, USA.
[2] *Present Address:* Formulation Development, Johnson & Johnson, Consumer & Personal Products Worldwide, 199 Grandview Rd, Skillman, New Jersey 08558, USA.
[3] To whom correspondence should be addressed. (e-mail: schwende@umich.edu)

surface-modified material on the laboratory scale after a 1-day batch process (8,10). Similar processes are also in use by several other laboratories (12–15).

To expand on our surface modification approach, in this report we have selected as our second FUN emulsifier the proteoaminoglycan, heparin, because of its use to impart blood and tissue compatibility (16–21), stealth (22,23), and growth factor binding (24,25) characteristics to biomaterials. Similarly, a two-step method has been developed recently to covalently surface-immobilize heparin in PLGA microspheres (25). Heparin is an unlikely FUN emulsifier candidate because it is not well known to possess emulsifying activity. We describe here our formulation approach to modulate the hydrophile–lipophile balance of heparin by adjusting the counter ion form and external pH of the polyanion to surface entrap heparin in PLGA microspheres by a one-step method. Although with more difficulty than polylysine (10), we found that heparin can be converted to an effective emulsifier to form PLGA microspheres, and then in fact retained on the polymer surface at significant levels for weeks.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-*co*-glycolide) 50/50, end-group capped by a lauryl ester, with an inherent viscosity of 0.24 dl/g in HFIP at 30°C and a $\overline{MW}_w$ of 26,000 Da (provided by manufacturer) was obtained from Alkermes, Inc. (Cambridge, MA). Heparin sodium and zinc salts from porcine intestinal mucosa (Grade I-A, ~170 USP units/mg) were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise specified. Glycine, L-lysine, L-glutamic acid, NH_4Cl, KCl, $CaCl_2$, $MgCl_2$, and $BaCl_2$ were also from Sigma Chemical Co. L-Valine, L-arginine, and L-leucine were purchased from Fluka Chemical Corp. (Milwaukee, WI). Toluidine blue was obtained from Aldrich Chem. Co. (Milwaukee, WI). All other reagents were of analytical grade or higher and used as received.

Preparation of Uni- and Di-valent Heparin Salts

Roughly 10 mg/ml sodium heparin solution in d.d. H_2O was dialyzed against excess 0.2 M $CaCl_2$, $MgCl_2$, or $BaCl_2$ or 1 M NH_4Cl, KCl solutions, followed by exhaustive dialysis against d.d. H_2O using Spectrum/Por® dialysis membrane (MWCO 1,000 Da; Spectrum Laboratories, Inc., Rancho Dominguez, CA) at 4°C. The purified heparin salt solution was freeze-dried thereafter.

Preparation of Amino Acid and Protamine Complexes of Heparin

Sodium heparin solution in d.d. H_2O (0.5–1.2 g heparin, 25 ml) was passed through an ion-exchange column (1.9×40 cm) containing a 50 ml bed volume of Dowex 50WX8 (H^+) resin (100–200 mesh). Heparinic acid was collected in an ice bath by washing the resin with 125 ml of d.d. H_2O. L-Amino acids, including lysine, arginine, glutamic acid, leucine, valine and glycine in their *free* form, were immediately added at a 10% mole excess of counter-ion binding sites (derived from Na content in sodium heparin) and mixed well. The mixture was filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA) and freeze-dried immediately thereafter, except for the glutamic acid complex. Unlike other amino acids, glutamic acid did not form a soluble complex with heparinic acid initially. Therefore, before freeze-drying, the heparin–glutamic acid complex was slowly and fully dissolved at 4°C for overnight. All lyophilized heparin–amino acid complexes appeared as white solids. To prepare heparin-protamine complexes, varying amounts of protamine with Arg residues equivalent to 15–110% (mol/mol) of the total counter-ion binding sites were added to heparinic acid and freeze-dried.

Potentiometric Titration of Heparin Salts

One milliliter of 25 mg/ml or 2.5 ml of 5 mg/ml heparin salts solution in d.d. H_2O was titrated with 0.4 N HCl at room temperature (24±0.5°C). Five microliters of HCl solution were added each time and a total of ~20 additions were made. Back titration of 2 ml Na heparin (25 mg/ml) was made with 0.5 N NaOH (4 μl NaOH was added each time) following addition of excess (100 μl) 1 N HCl. The pH was measured with a Corning combination electrode (Corning, NY), which was calibrated with pH 4, 7, and 10 buffer solutions before each use. The equivalence points for the carboxyl groups of heparin were determined from the peaks of the first derivative curve of the pH titration curve.

Circular Dichroism (CD) Spectroscopy

CD measurements were carried out at 25°C on an AVIV 202 or Jasco J-810 circular dichroism spectrometer, using 1 mm quartz cells. Na, K, NH_4, and Zn forms of heparin were dissolved in d.d. H_2O at a concentration of 2.5 mg/ml for CD measurements. The effect of pH was also determined following adjustment of the pH of Na heparin solution to 4.3 or 9.2 using 0.1 N HCl or NaOH with the ionic strength maintained constant with 0.01 N NaCl. The baseline of CD spectroscopy was calibrated with blank solutions. The molar elipticity (ME or [θ]), in deg cm^2 $decimole^{-1}$, was calculated according to:

$$\mathrm{ME} = \frac{\theta(\text{in milidegree}) \times \overline{\mathrm{MW}}_{\text{disaccharide}}}{\text{cell length(cm)} \times 10 \times \mathit{conc}(\text{mg/ml})} \qquad (1)$$

The concentration of heparin was normalized to heparinic acid content assuming counter ions completely replaced sodium ion in the heparin salts. $\overline{MW}_{disaccharide}$ is the molecular weight of the repeating disaccharide of heparin and was taken as 537 as described elsewhere (26).

Preparation of PLGA Microspheres Using Heparin Salts or Complexes as the Emulsifier

PLGA microparticles were prepared by an oil-in-water emulsion-solvent evaporation method. First, PLGA 50/50 was dissolved in 1 ml methylene chloride (CH_2Cl_2) at a concentration of 500 mg/ml. Two milliliters of heparin salt or

heparin–amino acid complex solution or suspension in d.d. H_2O with a concentration of 25 mg/ml were added. The mixture was homogenized at 10,000 rpm for 1 min on a Model IQ^2 Homogenizer (VirTis Co., Gardiner, NY). The resultant emulsions were in-liquid hardened in 120 ml hardening medium (d.d. H_2O or buffers) under mild stirring at room temperature for about 3 h. The microparticles were sieved through US standard 500 μm steel sieve, washed with d.d. H_2O for three times and lyophilized on a Labcono freeze dry system (Kansas City, MO). The yield of resultant microspheres, which was calculated by dividing the weight of collected microparticles by the weight of polymer initially added, 500 mg, was recorded.

Determination of Total and Surface Accessible Heparin

The total heparin loading in heparin-entrapped microspheres was determined by elemental analysis of sulfur by Galbraith Laboratories, Inc. (Knoxville, TN). The surface accessible heparin was determined by a modified toluidine blue assay. Briefly, roughly 20 mg microspheres were suspended in 3.85 ml 0.2% (w/v) NaCl solution containing 0.1% (w/v) Tween 80. To this suspension 3.75 ml of toluidine blue solution (0.01%, w/v) containing 0.2% NaCl, 0.1% Tween 80 and 0.02 N HCl were added. Heparin binds with toluidine blue and precipitates out from the aqueous solution. In previous studies, heparin–toluidine complexes were removed by extraction with hexane and the decrease in the absorbance of toluidine solution was used to correlate with heparin concentration (27,28). Here, we found that the hexane/water interface is not sufficient to completely remove the complexes. Filtration or centrifugation provides a better means to separate the complexes. For heparin associated with the microparticles, heparin-dye precipitates remain on the particle surface and can be removed by centrifugation/filtration as well. Therefore, the mixture was vigorously vortexed for 30 s, followed by filtering through a 0.22 μm syringe filter (Millipore, Bedford, MA). The absorbance of 120 μl filtrate was determined at 630 nm on a MRX II microplate reader (Dynex Technologies, Inc., Chantilly, VA). The amount of heparin was derived from a series of standards containing 31–250 μg heparin (100 μl), in the same form or salt as to be determined in PLGA microspheres, following the same procedure. For microsphere samples of limited amount, 1/5–1/20 amount of samples and reagents were used; the mixture were centrifuged at 12,000 rpm for 5.5 min and the supernatant dye solution was removed to determine absorbance.

For microsphere samples with limited heparin content, e.g., samples as in retention study, a more sensitive toluidine blue assay—toluidine/DMSO assay—was developed, in which the amount of exact dye precipitated with heparin was determined. Briefly, after centrifugation of heparin-bound dye suspension, the supernatant dye solution was discarded and the precipitate of heparin–toluidine complex, or the microspheres with surface heparin complexed with the dye, were washed twice with 0.1% Tween 80 solution to remove nonspecifically adsorbed toluidine and dissolved in DMSO following incubation at 37–60°C for 2–4 days. The absorbance of toluidine in DMSO solution of both standards and samples were read at 630 nm. The toluidine solution was filtered to remove any possible precipitate before use.

Morphology and Particles Size of Heparin-Immobilized PLGA Microparticles

The morphology of PLGA/heparin microparticles was analyzed with a scanning electron microscope (Hitachi S-3200 N). The dry particles were coated with a thin gold layer using a Denton Vacuum Desk II Cold Sputter-etch Unit (Denton Vacuum, Inc.). For the purpose of particle size determination, more than 100 particles were counted and measured. Number averaged mean (length-number mean, $d_{\ln}$) and volume averaged mean (weight moment mean, d_{wm}) particle sizes were calculated according to Eqs. 2 and 3, respectively (29). Specific surface area (SSA) was calculated by Eq. 4, as previously described (10).

$$d_{\ln} = \frac{\sum nd}{\sum n} \tag{2}$$

$$d_{\mathrm{wm}} = \frac{\sum nd^4}{\sum nd^3} \tag{3}$$

$$SSA = \frac{\sum 4\pi r_i^2 \cdot N_i}{\rho \cdot \sum \frac{4}{3}\pi r_i^3 \cdot N_i} = \frac{6\sum d_i^2 N_i}{\rho \cdot \sum d_i^3 N_i} \tag{4}$$

where N_i is the number, 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/heparin microparticles.

Retention of Heparin on PLGA Microparticles

Around 10–20 mg dry microspheres were incubated in phosphate buffered saline solution containing 0.02% (w/v) Tween 80 (PBST) under mild agitation at 37°C. The microsphere samples were taken at pre-determined time intervals. After removal of the release medium, the microspheres were firstly dispersed by manually breaking up the loose aggregates, followed by centrifugation and washing with PBST three times. The microsphere pellet was then suspended in 0.1 M citrate buffer (pH 2.0) containing 0.1% Tween 80 to counteract possible pH change caused by residual PBST and polymer hydrolysis products in the microsphere samples. Surface heparin retained on PLGA microparticles was determined by toluidine blue assay or toluidine/DMSO assay, as previously described. The release medium was changed frequently (every 3–4 days) and the pH was controlled to between 6.4 and 7.4 for the period of retention study to prevent the possible change of heparin retention and polymer degradation by the acidic pH caused by polymer degradation.

RESULTS

Characterization of Uni- and Di-valent Salts of Heparin

The emulsifying activity of heparin was first adjusted by exchanging the counter ions, as certain ions may significantly modify the aqueous solubility of heparin, and thus modify the

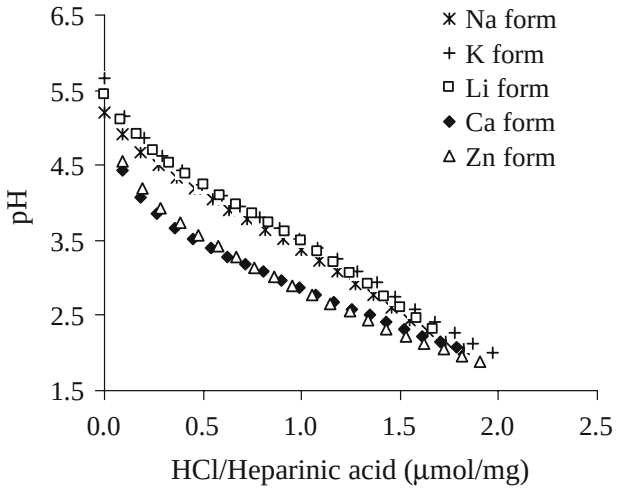

Fig. 1. pH titration curve of heparin salt solutions in d.d. H_2O at room temperature (24±0.5°C). Unless otherwise specified, all heparin salts are of a concentration of 25 mg/ml. Zn heparin is obtained from Sigma; other forms were prepared as described in "MATERIALS AND METHODS."

hydrophile–lipophile balance (HLB) of the polyelectrolyte. To avoid heterogeneity or inconsistency in the composition of commercial heparin, heparin salts were prepared by exhaustively dialyzing Na heparin (from Sigma Chemical Co.) against either d.d. H_2O or the chloride forms of various uni- (Na^+, Li^+, K^+, NH_4^+) and divalent (Zn^{2+}, Ca^{2+}, Mg^{2+}, and Ba^{2+}) cations, unless otherwise specified.

The decrease in the pH of heparin solution was observed following exchange of counter ions, as shown in Fig. 1 and Table I. The pH drop of heparin with different counter ions followed the order of Li^+, Na^+, K^+ < Ca^{2+}, Zn^{2+} < NH_4^+. Besides weakly acidic cations (e.g., NH_4^+) contributing to pH reduction, the pH drop has been correlated with enhanced cation binding, which increases acidity of heparin carboxylic acid groups (30). When heparin is either passed through a Ca^{2+} ion-exchange column or placed in the presence of divalent ions, similar pH decreases have been observed (30). The results here indicated that binding of divalent cations to the heparin chain is stronger than that of univalent ions, which is consistent with the results derived from the ion-exchange reaction and methylene blue replacement (31,32). 0.15 M NH_4Cl, with NH_4^+ at the same concentration as in ammonium heparin, resulted in a pH of 5.2, suggesting the additional pH drop to 4.0 in ammonium heparin was due to NH_4^+ binding to heparin chain. The reduced solubility of ammonium heparin (Table I) further confirmed the interaction of ammonium ions with heparin.

The highly ordered structure of macromolecules such as occurring in α helices has been found to be important for the remarkable emulsifying activity of polylysine (10). Heparin, about 16 nm in length, has been proposed to adopt a single helix structure with sulfate and carboxylate groups lying on the outside of the helical chain (33). In addition, heparin conformation has been reported to be cation-dependant. For example, binding of Ca ions induced major changes in both optical rotation and circular dichroism (34). Therefore, we monitored the circular dichroism (CD) spectrum of heparin to determine if heparin conformation played a role in emulsifying activity and microsphere formation. As shown in Fig. 2a, the CD spectra of Na and K heparin were almost identical, indicating similar chain configuration. The downward shift of ammonium heparin was similar to that of Na heparin at acidic pH. Although both acidic, the CD spectra of Zn heparin and ammonium heparin shifted to opposite directions as compared to Na/K heparin, suggesting a different conformation of Zn heparin (similar to the reported conformational shift of heparin upon Ca binding (34)).

Table I. Characteristics of PLGA Microspheres Prepared by Emulsion-Solvent Evaporation Method, Using Uni- and Divalent Heparin Salts as the Emulsifier

Salt Form	Characteristics of Emulsifier Solution (2.5%, w/v)		Microsphere Yield (%)	Particle Size (μm)		Polydispersity Index[a]
	PH	Appearance		Number Averaged Mean	Volume Averaged Mean	
Univalent						
Li^{+b}	6.8	Soluble	19	ND	ND	ND
Na^{+b}	6.3	Soluble	34	16±15	42	2.6
K^+	5.8	Soluble	7	5±6	25	5.1
NH_4^+	4.0	Slightly turbid	58	17±13	42	2.4
Divalent						
Mg^{2+}, Zn^{2+}, Ca^{2+}	5.4	Soluble	0	N/A	N/A	N/A
Ba^{2+}	5.4	Very turbid	0	N/A	N/A	N/A
Controls						
PVA	ND	Soluble	86	10±8	24	2.4
Emulsifier-free[c]	N/A	N/A	0	N/A	N/A	N/A

ND Not determined; *N/A* not applicable
[a] Polydispersity Index was indicated by the ratio of weight or volume averaged mean particles size to number averaged mean particle size
[b] From Sigma
[c] d.d. H_2O w/o heparin was used in place of heparin solution

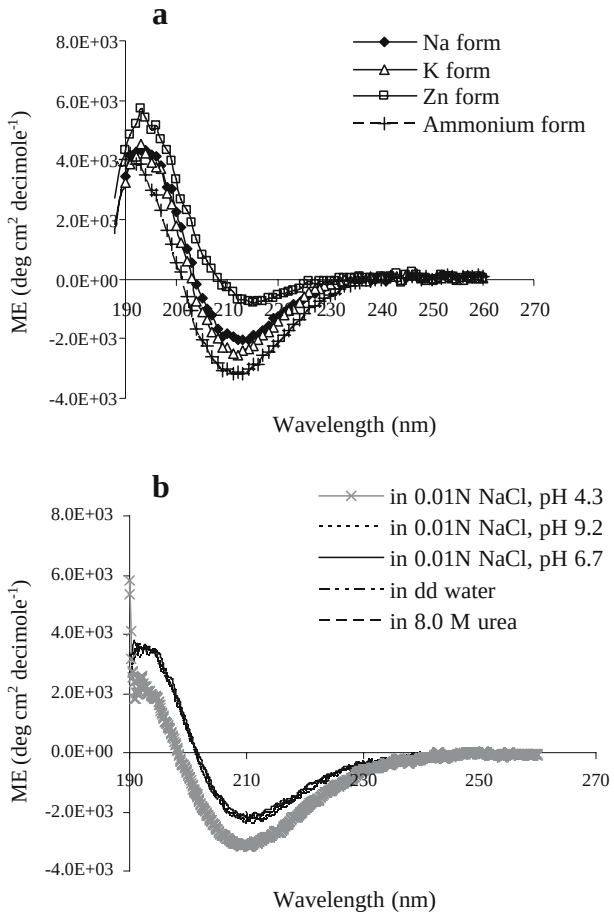

Fig. 2. **a** Circular dichroism spectra of heparin salts, 2.5 mg/ml, in d.d. H_2O at 25°C. Zn form of heparin is from Sigma. **b** CD spectra of Na heparin, 2.5 mg/ml, in d.d. H_2O at 25°C at pH 6.7 (unadjusted), 4.3, and 9.2, as compared with Na heparin in d.d. H_2O and in 8 M urea solution. The pH was adjusted with 0.1 N HCl or NaOH; 0.01 N NaCl was used to maintain constant ionic strength.

PLGA Microparticles Prepared Using Heparin Salts as the Emulsifier

PLGA microspheres were prepared by the oil-in-water emulsion-solvent evaporation method using heparin salts as the sole emulsifier. The emulsifying activity of heparin salts was evaluated by the yield and particle size of the resultant PLGA microspheres. In general, the higher the yield and the smaller the particles, the better the emulsifying activity. The most commonly used emulsifier in the preparation of PLGA microspheres, PVA, was used as reference. PVA, an amphiphilic copolymer of vinyl alcohol and vinyl acetate, is well-established as an effective emulsifier for the PLGA oil-in-water emulsion. In addition, as a negative control, microspheres were also prepared with emulsifier-free solution (i.e., d.d. H_2O) to illustrate the necessity of the use of an emulsifier during PLGA microsphere preparation.

The characteristics of the PLGA microparticles prepared using heparin salts as the emulsifier are shown in Table I. Among the heparin salts used, ammonium heparin resulted in the highest microspheres yield (i.e., 58%). Other counter ions followed the order of $Na^+ > Li^+ > K^+ >$ divalent ions. PLGA emulsions prepared with divalent heparin salts aggregated quickly and completely during the solvent evaporation process probably due to the bridging of the droplets in the presence of heparin and divalent ions, which also led to the aggregation of calcium-bound fat emulsions with the addition of heparin (35). The microspheres prepared using univalent heparin salts as the emulsifier were spherical and well-formed, as shown in Fig. 3. As shown in Table I, the particle size of the microspheres prepared using Na and ammonium heparin as the emulsifier was larger than PLGA/PVA control microspheres. Potassium heparin produced smaller PLGA microspheres, yet poorly produced and highly polydispersed, with a yield of only 7% and polydispersity index of 5.1 in contrast with ~35–85% yield and ~2.4 polydispersity index for microsphere prepared using Na and ammonium heparin or PVA. The overall lower yield and/or larger particle size suggested poorer emulsifying activities of heparin salts as compared with PVA, which is not surprising for a highly charged and hydrophilic polysaccharide such as heparin.

Characteristics of Heparin–Amino Acid Complexes and PLGA/heparin–Amino Acid Microspheres

The positively charged amino groups of amino acids can electrostatically interact with sulfate and carboxylate groups on heparin chain. Herein, the emulsifying activity of heparin was modified by replacing its counter ion with basic, neutral or acidic amino acids with varying hydrophobicity. Heparinic acid is very poorly stable and decomposes so quickly that it is hardly isolatable by lyophilization (36). The interaction between the amino acid and heparinic acid was confirmed by the enhanced stability of the complexes as compared with the heparinic acid itself (*personal observation: heparin–amino acid complexes showed no color change following lyophilization, while heparinic acid turned to brownish*). Complexes formed between heparin and neutral or acidic amino acids (Gly, Val, Glu and Leu) showed very acidic pH (~2.5); most carboxyl groups on heparin chain [pK_a ~3.9–5.1 depending on concentration, (37)] may be protonated and sulfate groups remain ionized and bound with amino acids. By contrast, Arg and Lys resulted in higher pH when complexed with heparin (pH 8.5 and 5.5, respectively) because of the basicity of side chains; heparin chains are expected to be fully ionized and interact electrostatically with the basic groups of amino acids.

The characteristics of PLGA microspheres prepared using heparin–amino acid complexes as the emulsifier are shown in Table II. The morphology of the representative microparticles prepared using Arg– and Gly–heparin complexes was shown in Fig. 3. The use of amino acid complexes of heparin resulted in reasonable microspheres yields (35–55%), comparable to ammonium heparin, likely due to the higher hydrophobicity of amino acids bound with heparin. The particle size of the microspheres produced followed the order of Arg < Lys < Leu < Glu < Gly, Val. Arg–heparin produced microspheres with the smallest particle size (~11 μm), which is comparable to PLGA/PVA control microspheres, presumably due to stronger interaction between heparin and the positively charged side groups of Arg (see "DISCUSSION"). The PLGA/heparin–amino acid microspheres were well-formed as shown

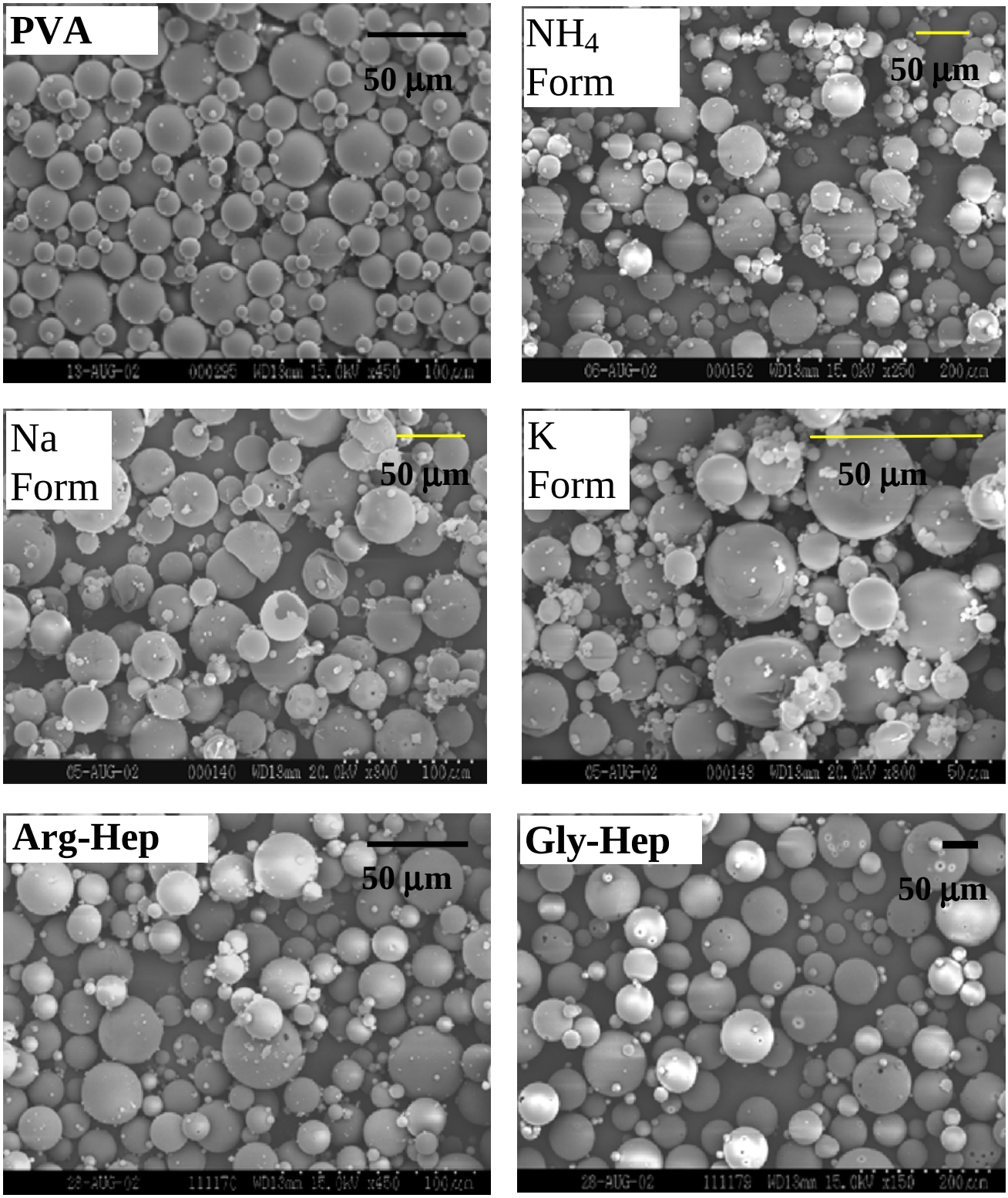

Fig. 3. Scanning electron micrographs (SEM) of PLGA microspheres prepared by emulsion-solvent evaporation method using various salt forms and complexes of heparin or PVA (control) as the emulsifier.

by the SEM micrograph and the polydispersity indices were comparable to PLGA/PVA microparticles (*data not shown*).

The strong interaction between arginine and heparinic acid was further explored by using polypeptide containing multiple arginine residues—protamine to complex with heparin and prepare PLGA microparticles. Multiple site interaction of Arg residues on protamine with heparin was expected to provide a better blockage of negative charges on heparin and thus provide better emulsifying activity. Varying amounts of protamine with Arg residues equivalent to 15–

Table II. Characteristics of PLGA Microspheres Prepared by Emulsion-Solvent Evaporation Method, Using Heparin-amino Acid Complexes as the Emulsifier

Amino Acid Form	Microsphere Yield (%)	Particle Size (μm)	
		Number Averaged Mean	Volume Averaged Mean
L-Arginine	54	11±9	28
L-Lysine	34	21±14	44
Glycine	39	38±26	73
L-Valine	46	37±27	77
L-Leucine	53	28±22	61
L-Glutamic acid	40	31±20	63
Control (PVA)	86	10±8	24

110% (mol/mol) of the total counter-ion binding sites on heparinic acid were added. Precipitation was observed with the addition of a higher percentage of protamine (>30%). The heparin–protamine complexes unexpectedly resulted in complete aggregation of the PLGA emulsions and no microparticles were produced. This could also be due to the bridging of the droplets by the polypeptide able to interact with more than one heparin chain simultaneously.

Total Loading and Surface Density of Heparin in PLGA Microspheres

The total content of heparin entrapped in PLGA microspheres and the portion of heparin entrapped at the surface were determined by elemental analysis (sulfur) and a modified toluidine blue assay, respectively. For elemental analysis, heparin standards in different salt or complex forms were

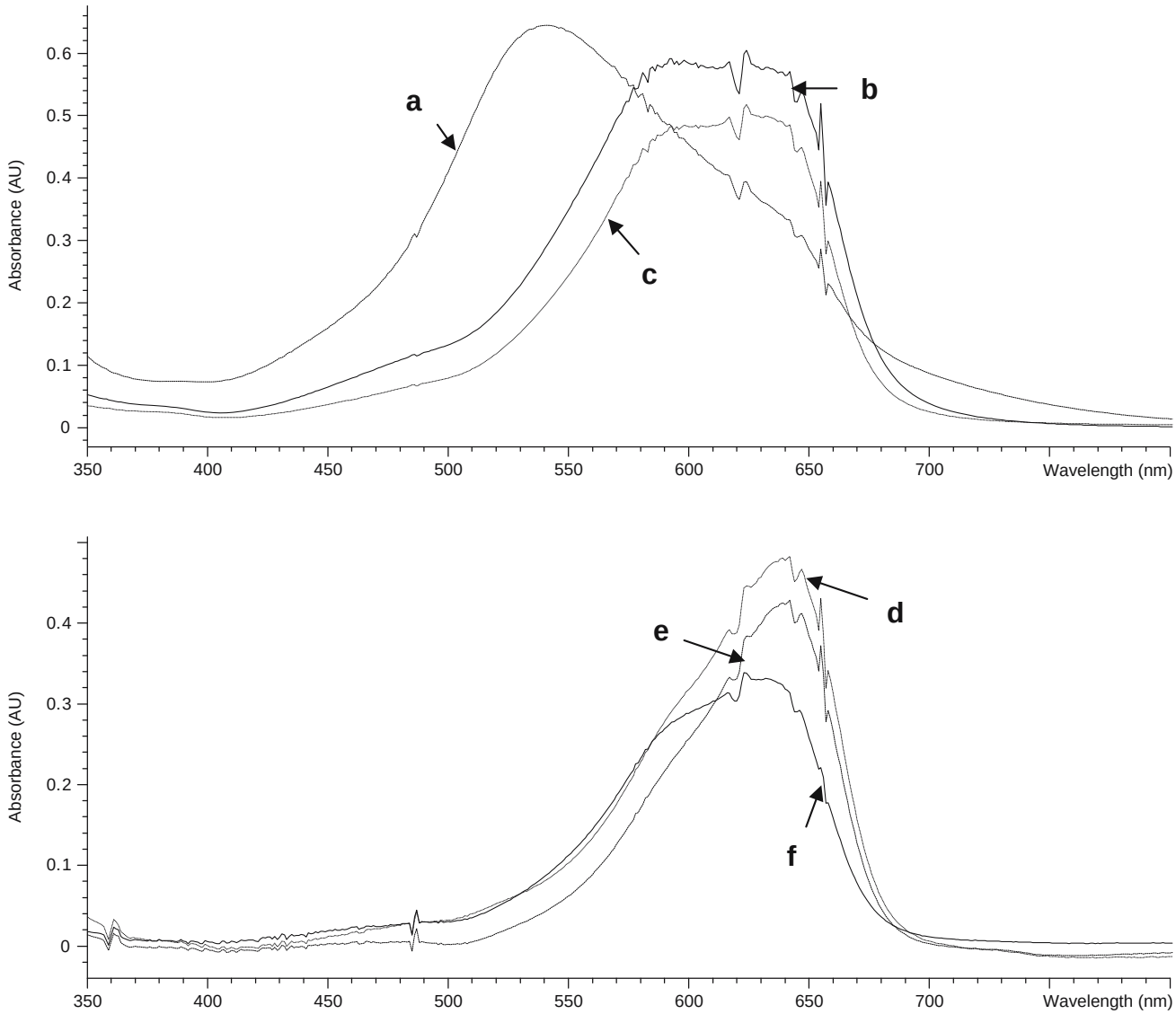

Fig. 4. UV/Vis spectra of toluidine blue and its complexes formed with heparin as in heparin assay (see "MATERIALS AND METHODS"). Curve **a** and **b**: remaining toluidine blue solution after removal of heparin-toluidine complexes formed by addition of 5.0 (**a**) or 2.5 mg/ml (**b**) Na heparin to 0.01% toluidine blue solution; **c**: diluted toluidine blue solution; **d**: complex formed between microsphere-associated heparin and toluidine blue, which was dissolved in DMSO; **e**: toluidine in DMSO solution; **f**: toluidine in water solution at the same concentration as in **e**.

Table III. Total and Surface Immobilized Heparin Content in PLGA Microspheres Prepared Using Univalent Heparin Salt and Arg–heparin Complex as the Emulsifier

Counter Ion or Amino Acid	Hardening Medium	Total Heparin Loading (μg/mg)[a]	Surface Accessible Heparin[b] (μg/mg)	SSA[c] (m^2/g)	Surface Density[d] (mg/m^2)
Na^+	H_2O	2.7	1.5±0.1	0.16	9.4
	PBS	7.2	3.9±0.3	0.20	19.9
K^+	PBS	11.5	4.3±0.1	0.13	33.1
NH_4^+	PBS	10.7	1.4±0.1	0.18	7.8
Arg	H_2O	0.8	0.15±0.01	0.25	0.7
	PBS	2.3	0.13±0.02	0.24	0.6

[a] Determined by elemental analysis of sulfur
[b] Determined by toluidine blue assay as described in "MATERIALS AND METHODS"
[c] Calculated as in "MATERIALS AND METHODS"
[d] Surface density (mg/m^2) is calculated by dividing surface accessible heparin (μg/mg) by SSA (m^2/g)

tested as reference. For the toluidine blue assay, within a certain concentration range (≤2.5 mg/ml), heparin bound with toluidine blue and precipitates without affecting the UV/Vis spectrum of the remaining dye solution at λ_{max} 630 nm, as shown in Figs. 4a–c (A spectral shift or color change was observed at higher heparin concentration, 5.0 mg/ml, or lower toluidine blue level, 0.001%, representative of a soluble toluidine–heparin complex (38). The altered toluidine blue spectrum also caused a deviation from the linear standard curve). The absorbance of the remaining solution at 630 nm showed an inversely linear correlation (r^2>0.99) with the concentration of heparin standards. The results obtained from PLGA or PLGA/PVA control microspheres w/o heparin showed an absorbance corresponding to a heparin concentration of <0.3 mg/ml, regardless of microsphere amount used. The minimal non-specific adsorption of toluidine blue onto PLGA falls below the range of standard curve, therefore did not interfere with the assay.

The results of total heparin loading and surface accessible heparin determination are shown in Table III. The total heparin loading was 2.7 and 0.8 μg/mg for PLGA microspheres prepared using heparin sodium salt and Arg–heparin complex as the emulsifier and in-liquid hardened in d.d. H_2O, respectively. The surface accessible heparin (1.5 vs 0.15 μg/mg) and surface density (9.4 vs 0.7 mg/m^2) were much higher in PLGA/Na heparin microspheres than the PLGA/Arg–heparin microspheres, suggesting less adsorption of Arg complex than Na form of heparin onto PLGA o/w emulsions. However, the PLGA o/w emulsion was stabilized with such a lower surface adsorption, suggesting that Arg heparin may possess better emulsifying activity than other heparin forms.

Effect of Heparin Solution pH and Hardening Medium on PLGA Microsphere Formation

Interestingly, when we replaced d.d. H_2O with PBS as the hardening medium, aggregation of PLGA/methylene chloride-in-water emulsion with sodium heparin salt as the emulsifier was completely inhibited (Table IV). Therefore, we further investigated the effect of the pH of Na heparin solution and hardening medium on the emulsifying activity of heparin.

As shown in Fig. 5a, acidification of sodium heparin solution has a deleterious effect on the emulsifying activities of heparin. The microspheres yield dropped to only ~10% when the pH of Na heparin was adjusted to ~4 and surface accessible heparin was barely detectable. On the other hand, increasing the pH of heparin solution to 8.4 appeared to enhance microsphere production. Most significantly, as shown in Fig. 5b, the use of hardening buffer only with neutral to basic pH (pH 7.4 and 8.5) completely stabilized the o/w emulsion (yield ~80%; normally a ~20% decrease in polymer yield during microsphere preparation is caused by

Table IV. Effect of Hardening Medium on the Characteristics of PLGA Microspheres Prepared Using Heparin Salts and Amino Acid Complexes

Counter Ion or Amino Acid	Hardening Medium	Microsphere Yield (%)	Particle Size (μm)	
			Number Averaged Mean	Volume Averaged Mean
Na^+	H_2O	34	16±15	42
	PBS	80	16±11	35
K^+	H_2O	7	5±6	25
	PBS	83	20±17	54
NH_4^+	H_2O	58	17±13	42
	PBS	75	17±13	38
Arg	H_2O	54	11±9	28
	PBS	86	13±10	29
Control (emulsifier-free)[a]	H_2O	0	N/A	N/A
	PBS	15	23±23	85

[a] d.d. H_2O was used in place of emulsifier solution to prepare PLGA microparticles.

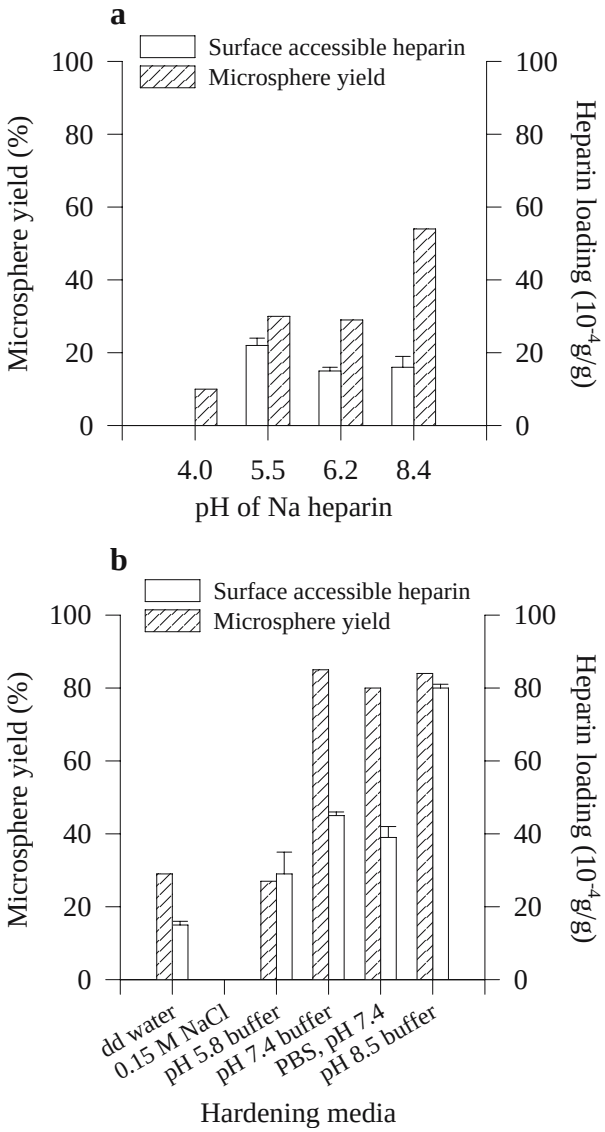

Fig. 5. a Effect of pH of Na heparin solution (2.5%, w/v) on the characteristics of PLGA microspheres in-liquid hardened in d.d. H_2O, including yield and surface heparin content. Unadjusted Na heparin has a pH of 6.2; **b** Effect of hardening medium on the characteristics of microspheres prepared using Na heparin solution (2.5%, w/v) as the emulsifier. The pH of microsphere suspension in d.d. H_2O is ~5.5; pH of hardening media was maintained to 5.8, 7.4, and 8.5 with 10 mM KH_2PO_4/borax buffer (for pH 5.8 and 8.5) or 10 mM Na phosphate buffer (for pH 7.4).

retention of emulsions on the homogenizer and test tube) and enhanced heparin loading as well. The PLGA o/w emulsion aggregated completely when hardened in 0.15 N NaCl, suggesting that ionic strength did not contribute to the enhanced microsphere yield when using PBS as hardening medium. Instead, the control of pH at the neutral range (7.4–8.5) resulted in significant emulsion stabilization.

By using PBS as the hardening buffer, as shown in Table IV, the yield of microspheres was also enhanced for other heparin forms, including K, ammonium and Arg–heparin. Interestingly, for microspheres in-liquid hardened in PBS, particles size was not affected significantly as compared with those hardened in d.d. H_2O, except for K heparin, which showed larger particle size when hardened in PBS. It is noteworthy that inclusion of PBS in emulsifier solutions resulted in microspheres with many broken particles (*data not shown*). This may be due to the high osmotic pressure inside the microspheres, which was caused by PBS and soluble acids in PLGA (see "DISCUSSION"). The polymer membrane became ruptured during hardening, resulting in broken microparticles. In addition, the control microspheres prepared using d.d. H_2O in place of emulsifier solution aggregated completely when in-liquid hardened in d.d. H_2O, and only a small portion of large particles (yield=15%, volume averaged mean particle size=85 μm) were obtained when hardened in PBS buffer probably due to adsorption of ions on the surface providing some charge to stabilize the emulsion.

As shown in Table III, by using PBS as the hardening buffer, both total heparin and surface-associated heparin increased significantly for PLGA microsphere prepared with Na heparin as the emulsifier. The higher amount of heparin adsorbed may have helped to stabilize the emulsions and result in a higher microsphere yield. Among the heparin salts studied here, potassium heparin resulted in highest heparin adsorption (~33 mg/m^2) to the microspheres hardened in PBS buffer.

Retention of Surface-Immobilized Heparin in PLGA Microparticles

The retention of surface heparin is important for the application of the heparin-coated PLGA microspheres. Heparin retained on microspheres following incubation in PBST at 37°C was firstly assessed by toluidine blue assay. As shown in Fig. 6a, surface accessible heparin (i.e., Na, K, ammonium and Arg–heparin) desorbed quickly following incubation, from 1–4 μg/mg to around 0.2 μg/mg, regardless of hardening method (d.d. H_2O or PBS buffer). We initially speculated that this might be due to the leaching of heparin following the rearrangement of polymer during freeze-drying upon rehydration. Thus, heparin retention was also evaluated on the microspheres immediately after in-liquid hardening and collection, without being freeze-dried. The corresponding microsphere content in suspension after collection (suspended in d.d. H_2O) was determined by weighing the freeze-dried aliquots of the microsphere suspension. The results showed that significant desorption occurred similarly (Fig. 7a). Significant heparin desorption during collection following the first washing step and continuous desorption following further washing were also observed (Fig. 7b), regardless of washing medium, d.d. H_2O or PBS. Similarly, quick desorption of heparin or its alkylated derivatives from artificial surface without multiple ionic binding sites was observed when washed with d.d. H_2O or other medium (19,39).

The toluidine blue assay as mentioned above is not sufficiently accurate to determine low heparin content as in retention study (except for initial time point) because only two to three times higher heparin loadings were detected as compared to the control, PLGA/PVA microspheres. In order to improve the sensitivity and accuracy, a toluidine/DMSO assay was developed in which the amount of exact dye precipitated with heparin, instead of the decrease of soluble

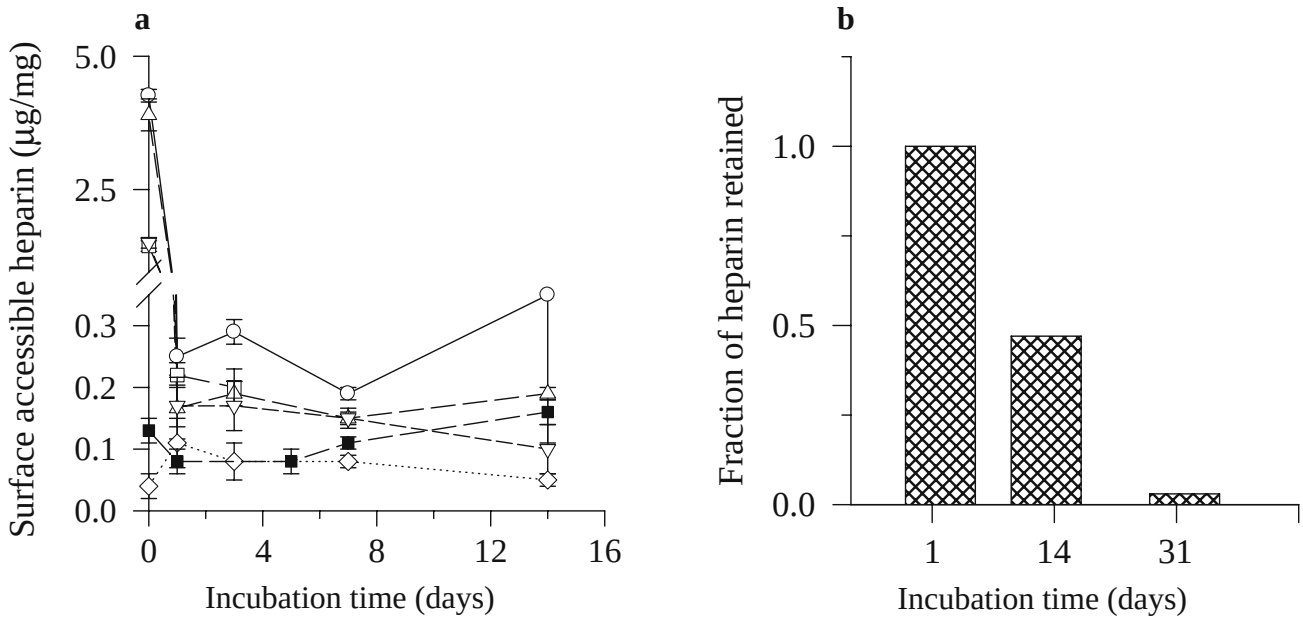

Fig. 6. **a** Retention of heparin on PLGA microspheres prepared using various heparin forms as the emulsifier and in-liquid hardened in d.d. H_2O or PBS. *Open circles*: K heparin and hardened in PBS; *open squares*: NH_4 heparin and hardened in PBS; *open triangles* and *inverted open triangles*: Na heparin and hardened in PBS and H_2O, respectively; *filled squares*: Arg heparin and hardened in PBS; and *diamonds*: PLGA/PVA control microspheres. The surface heparin content was determined by toluidine blue assay; **b** Retention of heparin on PLGA microspheres prepared using K heparin as the emulsifier and hardened in PBS. The surface heparin was determined by toluidine/DMSO assay.

dye concentration upon heparin binding was determined. Toluidine dissociates from the heparin in DMSO as indicated by the essentially identical UV/Vis spectra of toluidine blue and the complex (Figs. 4d–f). To avoid the interference of soluble heparin leached either during incubation or by mechanical breakage of the microsphere aggregates (microspheres flocculate/aggregate during incubation and need to be resuspended because the dye solution cannot penetrate into the aggregates), microsphere samples were washed with PBST three times prior to assay. Addition of 50 mg polymer

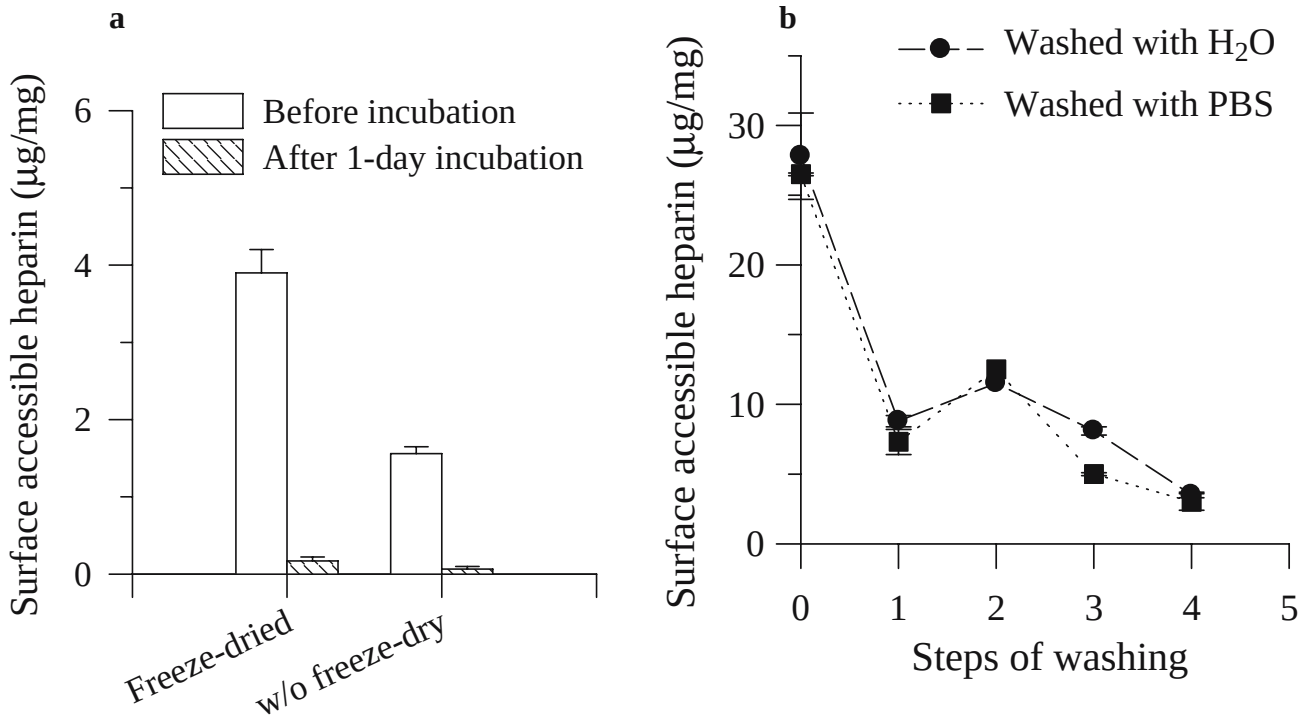

Fig. 7. **a** Desorption of heparin from PLGA microspheres, with or without lyophilization, following 1-day incubation in PBST at 37°C; **b** desorption of heparin upon washing of microspheres during collection by d.d. H_2O or PBS buffer. The surface heparin was determined by toluidine blue assay.

did not affect the assay results of standard heparin solution at either low (0.3 mg/ml) or high (1.25 mg/ml) concentration (*data not shown*).

The content of surface heparin following incubation was further assayed by the toluidine/DMSO method. After 1-day incubation, a surface heparin density of 0.21±0.03 mg/m^2 was retained on the PLGA microspheres prepared using potassium heparin as the emulsifier and hardened in PBS buffer. The retention of heparin following further incubation of these microspheres at 37°C was shown in Fig. 6b. Around 50% of the surface-immobilized K heparin was retained after 2 weeks and 3% was detectable after 1 month. The surface heparin in other formulations, including PLGA microspheres prepared using Arg–heparin and hardened in PBS, or using Na heparin and hardened in d.d. H_2O or buffer (pH 7.4), was very low after 1 day (<0.01 mg/m^2) and became undetectable after 2 weeks. The PLGA/PVA control microspheres were assayed at all time points and were uniformly negative for surface heparin indicating no interference of polymer with the toluidine/DMSO assay.

DISCUSSION

Previously, we demonstrated that the surface modification of PLGA microparticles can be accomplished by one-step entrapment of FUN emulsifiers, e.g., polylysine, by emulsion-solvent evaporation method (10). During emulsification of PLGA in organic solvent solution (oil phase) into aqueous FUN emulsifier solution (water phase), the FUN emulsifier becomes physically entrapped at the o/w interface, thus bearing its desirable functional groups on the surface following solvent evaporation and polymer precipitation. In the present study, heparin, a model polysaccharide, was chosen as another model FUN emulsifier. Remarkably, this important anticoagulant could impart emulsifying activity during microsphere preparation by the o/w solvent evaporation method.

Heparin is a heterogeneous mixture of variably sulfated polysaccharide chains composed of repeating units of D-glucosamine and L-iduronic/D-glucuronic acids. It is not expected to be a good o/w emulsifier because of its high charge density and lack of good hydrophile–lipophile balance to stabilize the o/w interface. The first attempt of using heparin sodium salt as an emulsifier to prepare PLGA microspheres suggested a feasibility to use heparin as an FUN emulsifier, but the yield of PLGA microspheres produced was low (~30%) due to severe emulsion aggregation. We sought to improve the performance of heparin as an emulsifier to be surface-immobilize in PLGA microspheres and to understand the underlying mechanism of heparin emulsifying activity. Three approaches of adjusting the emulsifying activity of heparin were investigated: inorganic counter ion binding, complexation with amino acid/polypeptide, and buffering pH.

Uni- and di-valent counter ions bind to the negatively charged carboxylate and sulfate groups and reduce the charge density of heparin. Potentiometric titration of various heparin salts showed that divalent counter ions bind more strongly to heparin than the univalent counter ions and may shield the negative charges of heparin more efficiently (Fig. 1). However, only univalent heparin salts resulted in reasonable microsphere yield, among which ammonium heparin showed the best emulsifying activity with the highest microsphere yield around 60% (Table I). This may be due to the higher hydrophobicity of ammonium heparin as indicated by its lower solubility. Because of the interference of ammonium ions with pH, the strength of binding to heparin and the chain conformation are uncertain for ammonium heparin, even though this form of heparin exhibited the strongest pH drop upon counter ion binding and a shifted CD spectrum (Table I and Fig. 2a). Other mechanisms, presumably the interfacial viscoelasticity of heparin film and interaction between heparin and polymer may also play an important role for the emulsifying activity of heparin.

By adjusting pH to ≥7.4, the emulsifying activity of heparin was greatly improved. According to counter ion condensation theory of polyelectrolytes (40,41), for a polyelectrolyte exhibiting a high charge density (ξ), counter ions will condense onto the fixed charge unless the net charge density falls below the critical value (ξ_{crit}). For heparin, $\xi \cong 3\ \xi_{crit}$, so that around two-thirds of the anion sites will be neutralized by counter ions; these condensed ions have little mobility with respect to the polyelectrolyte chain. Activity measurement using an ion-selective electrode confirmed the degree of dissociation of Na^+ was ~0.34 in heparin (42). At neutral to higher pH, titration of carboxylic acid groups to carboxylate form increases the axial charge density of the heparin chain, which enhanced counter ion binding; the fraction of anions bound with Na^+ has been reported as 0.43 and 0.59 for unionized and ionized heparin, respectively (43). The present study suggests that neutralization of anion sites of heparin by Na^+ and other counter ions improved its deposition at the o/w interface and thus emulsifying activity as compared to heparin in its free carboxylic form.

In addition, the conformation of heparin may also contribute to its emulsifying activity at neutral to alkaline pH. As shown in Fig. 2b, either increasing the pH of heparin solution from 6.7 (unadjusted) to 9.2 or dissolving heparin in 8 M urea did not affect the CD spectra of heparin, suggesting little conformational change and a relaxed chain configuration of heparin when fully ionized at neutral to high pH. However, at acidic pH, the CD spectrum of heparin significantly shifted with the increase of elipticity (the absolute value), which suggested that a conformational transition of heparin occurred upon the acidification of Na heparin, as observed previously (44). Although it has been argued this could be mainly due to protonation of iduronic acids (45), this explanation was not sufficient to account for the elipticity changes. This transition of chain configuration at acidic pH may have a detrimental effect on heparin adsorption onto the oil/water interface and its emulsifying activity.

It was further illustrated that incomplete neutralization of carboxylic groups in commercial heparin was not responsible for the poor emulsifying activity of Na heparin without pH adjustment. Na heparin (from Sigma) was acidified with excess HCl and back titrated with NaOH (*data not shown*). The results showed that nearly all (~99%) -COOH in the

commercial Na heparin were neutralized. In addition, the CD spectrum of Na heparin without pH adjustment exhibited the same CD properties as heparin at alkaline pH (Fig. 2b). Since an acidic microenvironment has been evidenced in PLGA polymer during polymer degradation, we hypothesize the acidity in this case may also be caused by the polymer. To test this hypothesis, PLGA was dissolved in an ACN/H_2O (80:20, v/v) mixture at a concentration of 2 mg/ml and titrated with 0.01 N NaOH solution. It was found that there is roughly 26 μmol of titratable acids per gram polymer. The presence of acidic products in the polymer raw material could potentially cause a pH drop in the heparin solution, which has a deleterious effect on its emulsifying activity. By adding additional base or using PBS buffer as the hardening medium may have helped to neutralize the acids released from the polymer and thus stabilize PLGA/heparin emulsion.

Heparin–amino acid complexes were previously found to enhance the stability of heparinic acid and the bioavailability of heparin (36). This study showed they also enhanced the emulsifying activity of heparin. Arg–heparin complex showed the best emulsifying activity as indicated by the highest microsphere yield (54%) and smallest particle size. Arginine has been previously shown to bind heparin ~2.5 times more strongly than lysine (K_d=29 and 80 mM, for Arg and Lys, respectively, at 25°C in 10 mM phosphate buffer, pH 7.4) (46). The stronger interaction between Arg and heparin may have shielded the negative charges on heparin chain more intensely to improve the hydrophile–lipophile balance of heparin and thus increase surface activity.

Our previous study on the surface entrapment of polylysine showed around 1 mg/m^2 of emulsifier coverage on PLGA microsphere surface, similar to PVA's surface density in PLGA/PVA microspheres. (47). Formation of a multimolecular interfacial films has been discussed for a few emulsifiers used to prepare PLGA microspheres, including polysaccharides such as sodium alginate (48). From the surface accessible heparin levels (1.5–4.5 μg/mg) and surface density (8–33 mg/m^2) as determined by toluidine blue assay (Table III), a multilayer adsorption of heparin was suggested in the microspheres prepared using univalent heparin salts. On the other hand, the heparin surface density on PLGA microspheres prepared using Arg–heparin complex, ~0.7 mg/m^2, seemed closer to a monolayer of coverage on microparticle surface. The surface accessible heparin accounted for around 6–60% of the total heparin loading, depending on the heparin form and hardening medium used, indicating the existence of surface inaccessible heparin in the polymer.

Surface immobilized heparin desorbed quickly at 37°C, regardless of collection method and lyophilization of the microspheres (Figs. 6 and 7). PLGA microspheres prepared using K heparin and hardened in PBS buffer exhibited the best retention, ~0.1 mg/m^2 surface heparin still remaining on the microsphere surface after incubation for 2 weeks at 37°C. Although potentiometric titration did not differentiate the binding of K^+ and Na^+ to heparin (Fig. 1), it is well known that K^+ has a higher affinity to heparin than Na^+ (49). This higher affinity is explained by the smaller hydrated radius of K^+, giving rise to stronger affinity to the sulfate groups of heparin (sulfate/carboxylate ratio ~2.6–2.7 (50)). The stronger binding of K^+ may have enhanced heparin–PLGA interactions and lead to the longer retention of potassium heparin.

CONCLUSIONS

Heparin was successfully surface-immobilized in PLGA microspheres by a one-step method utilizing univalent heparin salts (Li, Na, K, and ammonium heparin) and heparin–amino acid (Arg, Lys, Leu, Val, Gly, and Glu) complexes as FUN emulsifiers during the solvent evaporation process. Although more strongly attracted, divalent heparin salts (Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}) and heparin–protamine complexes severely destabilized PLGA o/w emulsion. Heparin showed maximum emulsifying activity when fully ionized and bound with univalent counter ions or complexed with amino acids. Increasing pH of heparin solution or using hardening medium with pH ≥7.4 increased both total heparin loading and surface accessible heparin levels (except for Arg–heparin) and completely stabilized PLGA emulsions. Among the heparin-coated microspheres produced in this study, those prepared using K heparin as the emulsifier and hardened in PBS buffer were found to retain surface heparin to the greatest extent. These heparin-modified PLGA microspheres may be useful for adsorption of growth factors and improve blood or tissue compatibility due to the modification of PLGA surface hydrophilicity/hydrophobicity.

ACKNOWLEDGEMENT

This research was supported in part by NIH HL 68345. Helpful discussions with Prof. Victor Yang are also gratefully acknowledged.

REFERENCES

1. M. T. Peracchia, R. Gref, Y. Minamitake, A. Domb, N. Lotan, and R. Langer. PEG-coated nanospheres form amphiphilic diblock and multiblock copolymers: investigation of their drug encapsulation and release characteristics. *J. Control. Release* **46**:223–231 (1997).
2. P. Quellec, R. Gref, L. Perrin, E. Dellacherie, F. Sommer, J. M. Verbavatz, and M. J. Alonso. Protein encapsulation within polyethylene glycol-coated nanospheres. I. Physicochemical characterization. *J. Biomed. Mater. Res.* **42**:45–54 (1998).
3. P. Quellec, R. Gref, E. Dellacherie, F. Sommer, M. D. Tran, and M. J. Alonso. Protein encapsulation within poly(ethylene glycol)-coated nanospheres. II. Controlled release properties. *J. Biomed. Mater. Res.* **47**:388–395 (1999).
4. C. Evora, I. Soriano, R. A. Rogers, K. N. Shakesheff, J. Hanes, and R. Langer. Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effect of 1,2-dipalmitoylphosphatidylcholine. *J. Control. Release* **51**:143–152 (1998).
5. J. Zheng and P. J. Hornsby. Production of microspheres with surface amino groups from blends of Poly(Lactide-*co*-glycolide) and Poly(epsilon-CBZ-L-lysine) and use for encapsulation. *Biotechnol. Prog.* **15**:763–767 (1999).
6. G. Caponetti, J. S. Hrkach, B. Kriwet, M. Poh, N. Lotan, P. Colombo, and R. Langer. Microparticles of novel branched copolymers of lactic acid and amino acids: preparation and characterization. *J. Pharm. Sci.* **88**:136–141 (1999).
7. K. M. Shakesheff, C. Evora, I. I. Soriano, and R. Langer. The adsorption of poly(vinyl alcohol) to biodegradable microparticles studied by X-ray photoelectron spectroscopy (XPS). *J. Colloid Interface Sci.* **185**:538–547 (1997).

8. S. Schwendeman and C. Cui. Biocompatible polymeric delivery systems having functional groups attached to the surface thereof. US 6326021, 2001, pp. 11.
9. C. Cui and S. P. Schwendeman. Biodegradable microspheres with surface conjugatable groups. *Proc. Int. Symp. Control. Release Bioact. Mater.* **26**:517–518 (1999).
10. C. Cui and S.P. Schwendeman. Surface entrapment of polylysine in biodegradable poly(DL-lactide-*co*-glycolide) microparticles. *Macromolecules* **34**:8426–8433 (2001).
11. C. Cui, V. C. Stevens, and S. P. Schwendeman. Injectable polymer microspheres enhance immunogenicity of a contraceptive peptide vaccine. *Vaccine* **25**:500–509 (2007).
12. R. A. Quirk, W. C. Chan, M. C. Davies, S. J. Tendler, and K. M. Shakesheff. Poly(L-lysine)-GRGDS as a biomimetic surface modifier for poly(lactic acid). *Biomaterials* **22**:865–872 (2001).
13. M. E. Keegan, S. M. Royce, T. Fahmy, and W. M. Saltzman. *In vitro* evaluation of biodegradable microspheres with surface-bound ligands. *J. Control. Release* **110**:574–580 (2006).
14. M. E. Keegan, J. L. Falcone, T. C. Leung, and W. M. Saltzman. Biodegradable microspheres with enhanced capacity for covalently bound surface ligands. *Macromolecules* **37**:9779–9784 (2004).
15. M. Singh, M. Briones, G. Ott, and D. O'Hagan. Cationic microparticles: a potent delivery system for DNA vaccines. *Proc. Natl. Acad. Sci. U. S. A.* **97**:811–816 (2000).
16. J. L. Pearce. Intraocular lenses. *Curr. Opin. Ophthalmol.* **3**:29–38 (1992).
17. M. Amiji and K. Park. Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity. *J. Biomater. Sci. Polym. Ed.* **4**:217–234 (1993).
18. A. Denizli. Heparin-immobilized poly(2-hydroxyethylmethacrylate)-based microspheres. *J. Appl. Polym. Sci.* **74**:655–662 (1999).
19. O. Larm, R. Larsson, and P. Olsson. A new non-thrombogenic surface prepared by selective covalent binding of heparin via a modified reducing terminal residue. *Biomater. Med. Dev. Artif. Organs* **11**:161–173 (1983).
20. B. Pasche, K. Kodama, O. Larm, P. Olsson, and J. Swedenborg. Thrombin inactivation on surfaces with covalently bonded heparin. *Thromb. Res.* **44**:739–748 (1986).
21. C. D. Ebert and S. W. Kim. Immobilized heparin: spacer arm effects on biological interactions. *Thromb. Res.* **26**:43–57 (1982).
22. C. Passirani, G. Barratt, J. P. Devissaguet, and D. Labarre. Interactions of nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate) with the complement system. *Life Sci.* **62**:775–785 (1998).
23. C. Passirani, G. Barratt, J. P. Devissaguet, and D. Labarre. Long-circulating nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate). *Pharm. Res.* **15**:1046–1050 (1998).
24. J. J. Yoon, H. J. Chung, H. J. Lee, and T. G. Park. Heparin-immobilized biodegradable scaffolds for local and sustained release of angiogenic growth factor. *J. Biomed. Mater. Res. A* **79**:934–942 (2006).
25. H. J. Chung, H. K. Kim, J. J. Yoon, and T. G. Park. Heparin immobilized porous PLGA microspheres for angiogenic growth factor delivery. *Pharm. Res.* **23**:1835–1841 (2006).
26. M. C. Chung and N. F. Ellerton. Viscosity at low shear and circular dichroism studies of heparin. *Biopolymers* **15**:1409–1423 (1976).
27. P. K. Smith, A. K. Mallia, and G. T. Hermanson. Colorimetric method for the assay of heparin content in immobilized heparin preparations. *Anal. Biochem.* **109**:466–473 (1980).
28. K. D. Park, A. Z. Piao, H. Jacobs, T. Okano, and S. W. Kim. Synthesis and characterization of SPUU-PEO-heparin graft-copolymers. *J. Polym. Sci., A, Polym. Chem.* **29**:1725–1737 (1991).
29. A. Martin. *Physical pharmacy: physical chemical principles in the pharmaceutical sciences*, Williams & Wilkins, Baltimore, MD, 1993.
30. D. Grant, W. F. Long, and F. B. Williamson. Examination of cation–heparin interaction by potentiometric titration. *Biochem. Soc. Trans.* **20**:215S, 1992 (1992).
31. J. R. Dunstone. Ion-exchange reactions between acid mucopolysaccharides and various cations. *Biochem. J.* **85**:336–351 (1962).
32. F. Jooyahdeh, J. S. Moore, G. O. Phillips, and J. V. Davies. Polyanions and their complexes. IX. Binding affinities of inorganic ions to heparin. *J. Chem. Soc, Perkin Trans. 2: Phys. Org. Chem.* **12**:1468–1471 (1974).
33. S. Hirano. Molecular structure of heparin as examined by nuclear magnetic resonance, optical rotatory dispersion, and electron microscopy. *Int. J. Biochem.* **3**:677–683 (1972).
34. J. Boyd, F. B. Williamson, and P. Gettins. A physico-chemical study of heparin. Evidence for a calcium-induced co-operative conformational transition. *J. Mol. Biol.* **137**:175–190 (1980).
35. O. L. Johnson, C. Washington, S. S. Davis, and K. Schaupp. The destabilization of parenteral feeding emulsions by heparin. *Int. J. Pharm.* **53**:237–240 (1989).
36. T. Y. Koh and K. R. Bharucha. Intestinal absorption of stable heparinic acid complexes. *J. Lab. Clin. Med.* **80**:47–55 (1972).
37. G. Gatti, B. Casu, G. K. Hamer, and A. S. Perlin. Studies on the conformation of heparin by 1H and 13C NMR spectroscopy. *Macromolecules* **12**:1001–1007 (1979).
38. K. L. Erzinkyan. Spectrophotometric study of the formation reaction of a fibrinogen–heparin complex in the visible region. *Biologicheskii Zhurnal Armenii* **27**:110–113 (1974).
39. T. Matsuda and T. Magoshi. Terminally alkylated heparin. 1. Antithrombogenic surface modifier. *Biomacromolecules* **2**:1169–1177 (2001).
40. G. S. Manning. Counterion binding in polyelectrolyte theory. *Acc. Chem. Res.* **12**:443–449 (1979).
41. G. S. Manning. The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. *Q. Rev. Biophys.* **11**:179–246 (1978).
42. F. Ascoli, C. Botre, and A. M. Liquori. On the polyelectrolyte behavior of heparin. I. Binding of sodium ions. *J. Phys. Chem.* **65**:1991–1992 (1961).
43. D. L. Rabenstein, J. M. Robert, and J. Peng. Multinuclear magnetic resonance studies of the interaction of inorganic cations with heparin. *Carbohydr. Res.* **278**:239–256 (1995).
44. A. L. Stone. Amide cotton effects of heparin. *Nature* **216**:551–553 (1967).
45. B. Chakrabarti and J. W. Park. Glycosaminoglycans: structure and interaction. *CRC Crit. Rev. Biochem.* **8**:225–313 (1980).
46. J. R. Fromm, R. E. Hileman, E. E. Caldwell, J. M. Weiler, and R. J. Linhardt. Differences in the interaction of heparin with arginine and lysine and the importance of these basic amino acids in the binding of heparin to acidic fibroblast growth factor. *Arch. Biochem. Biophys.* **323**:279–287 (1995).
47. S. C. Lee, J. T. Oh, M. H. Jang, and S. I. Chung. Quantitative analysis of polyvinyl alcohol on the surface of poly(D, L-lactide-*co*-glycolide) microparticles prepared by solvent evaporation method: effect of particle size and PVA concentration. *J. Control. Release* **59**:123–132 (1999).
48. S. Y. Lin, K. S. Chen, and H. H. Teng. Functionality of protective colloids affecting the formation, size uniformity and morphology of drug-free polylactic acid microspheres. *J. Microencapsul.* **15**:383–390 (1998).
49. I. Nieduszynski. General physical properties of heparin. In D. A. Lane and U. Lindahl (eds.), *Heparin: Chemical and Biological properties; Clinical Applications*, Edward Arnold, London, 1989, pp. 51–64.
50. J. Mattai and J. C. T. Kwak. Mg and Ca binding to heparin in the presence of added univalent salt. *Biochim. Biophys. Acta* **677**:303–312 (1981).