Characterization and Emulsifying Properties of Block Copolymers Prepared from Lactic Acid and Poly(ethylene glycol)

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ABSTRACT: Block copolymers were prepared by the direct polycondensation of an aqueous lactic acid solution on monomethoxy or dihydroxyl poly(ethylene glycol) (PEG) in the absence of a catalyst. The resulting poly(lactic acid) (PLA)–PEG diblock and PLA–PEG–PLA triblock copolymers were characterized by various analytical techniques, including matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), gel permeation chromatography, and ^{1}H-NMR. The molecular structure between PLA–PEG and PLA–PEG–PLA could be distinguished after the calculation of the repeat unit masses and end-group masses through the MALDI-TOF MS spectra. Interestingly, both copolymers could serve as a hydrophilic emulsifier to stabilize the squalene/water interfaces and yield narrowly distributed oil-in-water nanoparticles. In contrast, the prepolymer PEG failed to stabilize the squalene/water interface under the same homogenization conditions. These features are of great interest for applications as bioactive agent delivery, especially for candidate vaccine antigens and lipophilic anticancer drugs. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 509–516, 2009

Key words: biodegradable; block copolymers; drug delivery systems; MALDI; surfactants

INTRODUCTION

Amphiphilic block copolymers of poly(lactic acid) (PLA) and poly(ethylene glycol) (PEG) have attracted much attention in the sustained delivery of biologically active agents because of their biocompatibility and bioresorbability.[1–7] Attempts have been carried out to investigate the degradability and permeability of PLA/PEG-based micelles,[1] hydrogels,[2–4] microparticles,[4] and nanoparticles.[5–7] Ideally, a bioresorbable delivery vehicle has to degrade and be resorbed *in vivo* at a predefined rate so that the bioactive agents can be either attached at the surface or entrapped within the microenvironment before administration and can be stepwise released at post-administration.[5–7]

Oil-in-water (O/W) emulsions are droplets of oil stabilized by emulsifiers (typically, low-molecular-weight surfactants) in a continuous water phase.[8–10] This type of delivery system is known as an *effective lipophilic drug carrier*, in particular, in applications for the delivery of the anticancer drug paclitaxel.[9,10] Among vaccine delivery systems, there are two squalene-based O/W emulsions that possess significant potential for clinical applications, MF59 (Novartis) and AS03 (GlaxoSmithKline).[11,12] MF59 is stabilized with a combination of a hydrophilic emulsifier Tween80 (polyoxyethylene sorbitan monooleate) and a lipophilic Span85 (sorbitan trioleate),[11] whereas AS03 is stabilized by Tween80 and α-tocopherol.[12] The immunogenicity enhancement of the O/W-formulated vaccines was proposed by a combination of antigen delivery function with strong immune-stimulating activity at the injection site.[11] To enlarge the number of highly safe emulsifiers in the preparation of bioactive delivery vehicles in specific applications, synthetic polymers can be regarded as an interesting alternative to low-molecular-weight surfactants, as the sizes and relative positions of the hydrophilic and lipophilic blocks can be easily tailored and altered by the order of monomer addition and amount of monomer used to thus produce a broad range of surfactant characteristics.[13]

Gel permeation chromatography (GPC) and ^{1}H-NMR have been widely used to characterize synthetic block copolymers.[2,3,5,7,14] GPC allows one to determine the polymer molecular weight (MW) and polydispersity index (M_w/M_n, where M_w is the weight-average molecular weight and M_n is the number-average molecular weight) with respect to a

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series of standards of known MW.[5,14] On the other hand, ^{1}H-NMR is investigated to identify the chemical composition of copolymers.[3,5–7] However, a precise characterization of amphiphilic block copolymers is not very easy because no suitable standards are available for GPC analysis;[5,14] moreover, it is difficult to distinguish on the ^{1}H-NMR spectra whether the recovery samples are of copolymer form or a mixture. So far as the configurational structure is concerned, neither GPC nor ^{1}H-NMR can distinguish the difference between diblock and triblock copolymers. This parameter might be pertinent to the physicochemical properties, in particular, the release profile of entrapped bioactives.[1,2] To determine the rigorous molecular structure and chemical composition of copolymers, it is possible to perform a characterization by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which has dramatically enhanced the analysis capability for copolymers.[5,14,15]

In this article, we report the synthesis and characterization of PEG-bearing PLA diblock and triblock copolymers, PLA–PEG and PLA–PEG–PLA, prepared by the direct polycondensation of an aqueous lactic acid solution on monomethoxy or dihydroxyl PEG. Unlike the block copolymers reported in the literature, which were prepared in the presence of cytotoxic catalyst-containing heavy metals,[5,6,14,15] in this study, no catalyst was added during polymerization, which increased the confident biocompatibility in the final material. The resulting copolymers were characterized by MALDI-TOF MS, GPC, and ^{1}H-NMR. In contrast to micelles or nanospheres in which the polymer provides a matrix or a vehicle to encapsulate the bioactive agents, here we wanted to demonstrate whether the amphiphilic polymer could play an auxiliary role (as a surfactant) to stabilize the oil/water interface so that the bioactive candidates could be either surface attached or encapsulated within the core oil. The emulsifying properties were investigated by the homogenization of a polymer aqueous solution and oily squalene. Stability, size distribution, and *in vitro* release with ovalbumin (OVA) as model protein were performed to identify the resulting emulsion. For the sake of biocompatibility, squalene was selected as the core oil because it has a low toxicity and is used in clinical trials.[11,12,16]

EXPERIMENTAL

Polymer synthesis

Lactic acid was purchased as a 85–90% aqueous solution from TEDIA (Fairfield, OH). Poly(ethylene glycol) 2000 monomethyl ether ($MePEG_{2000}$) and poly(ethylene glycol) 2000 ($diOH–PEG_{2000}$) were purchased from Fluka (Buchs, Switzerland). These materials were used without further purification. All solvents were analytical grade.

The PLA–PEG diblock copolymer was synthesized through the polycondensation of lactic acid on $MePEG_{2000}$ in the absence of any catalyst. Briefly, 10 g of $MePEG_{2000}$ and 10 g of an aqueous lactic acid solution were placed in a round-bottom bottle. We performed the polymerization by simply distilling out water from lactic acid at 140°C for 24 h using a system composed of a Rotavapor R-210 (Buchi Labortechnik AG, Flawil, Switzerland) and vacuum pump V-700 (Buchi Labortechnik AG). The products were recovered by precipitation in an excessive amount of ethanol. They were further purified twice by successive dissolution/precipitation cycles with acetone as a solvent and ethanol as a nonsolvent to eliminate low-molecular-weight byproducts. The yield was about 25 wt %. The PLA–PEG–PLA triblock copolymer was synthesized according to the same procedure, with $diOH–PEG_{2000}$ used instead of $MePEG_{2000}$. The product was recovered by precipitation in cold ethanol (<10°C), and the yield was about 30 wt %.

Measurements

MALDI-TOF MS was performed on a Waters MALDI micro MX mass spectrometer (Milford, MA) equipped with a nitrogen laser (337 nm). All spectra were recorded in the reflection mode with an acceleration voltage of 12 kV. The irradiation targets were prepared from 0.1% trifluoroacetic acid (Riedel-de Haën, Seelze, Germany) in an acetonitrile/water mixture at a ratio of 50/50 (v/v) with α-cyano-4-hydroxy cinnamic acid (Sigma, Steinheim, Germany) as the matrix and sodium trifluoroacetate (Na-TFA; Fluka) as the dopant. The sample solutions were then spotted on a MALDI sample plate and air-dried before analysis. GPC was performed with a setup composed of an isocratic pump (Waters high-performance liquid chromatography (HPLC) model 510), a refractive index detector (Waters 410 differential refractometer), and two columns connected in series, one PLgel 5-μm mixed-C column (100-Å pore size, 7.5 × 300 mm, Polymer Laboratories, Ltd., Shropshire, United Kingdom), and one PLgel 3-μm column (100-Å pore size, 7.5 × 300 mm). The mobile phase was tetrahydrofuran, and the flow rate was 0.8 mL/min. Data were expressed with respect to polystyrene standards (Polysciences, Inc., Warrington, PA). ^{1}H-NMR spectra were recorded at room temperature with a Varian VXR 300-MHz spectrometer (Varian, Palo Alto, CA) with dimethyl sulfoxide-d_6 (Aldrich, Steinheim, Germany) and tetramethylsilane as the solvent and shift reference, respectively.

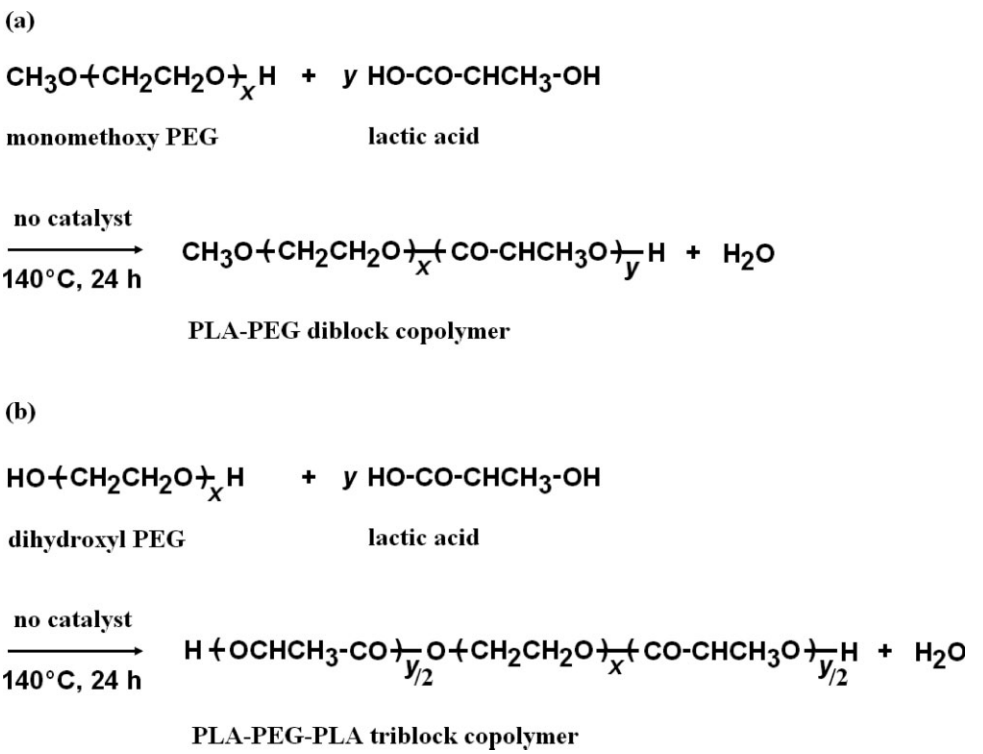

Figure 1 Synthesis schemes for the (a) PLA–PEG diblock copolymer and (b) PLA–PEG–PLA triblock copolymer.

Polymer-stabilized emulsions

The polymer aqueous solution [120 mg of polymer dissolved in 0.8 mL of phosphate-buffered saline (PBS)] and 1.1 mL of squalene oil (Sigma, Steinheim, Germany) were emulsified with a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland) under 6000 rpm for 5 min. The emulsified formulations served as stocks for further physicochemical characterizations.

To mimic the usual storage conditions and the postadministration stage, we determined the stability by placing each formulation at 4 and 37°C and then noting the visual aspects. We investigated the size distribution of the emulsions by redispersing them in PBS and measuring by the laser light-scattering technique using a Brookhaven 90 plus particle size analyzer (Brookhaven Instruments Limited, New York). *In vitro* release experiments were performed with the inverted dialysis tube method.[16] Formulations containing OVA (albumin from chicken egg white, Grade V, Sigma) formulations (3 mg/0.3 mL) were first placed in a dialysis chamber (cutoff = 0.2 μm, Pall Life Sciences, Ann Arbor, MI), and then, the device was immersed in a 50-mL centrifuge tube containing 2 mL of PBS and left to stand at 37°C. At different time intervals, 100 μL of sample was aspirated from the medium outside of the chamber and then replaced with 100 μL of PBS buffer. The OVA release was regularly determined by the bicinchinonic acid method (BCA protein assay kit, Pierce, Rockford, IL).

RESULTS AND DISCUSSION

The schematic diagrams of the synthesis and chemical structure of the block copolymers are shown in Figure 1. The PLA–PEG diblock copolymer was synthesized by the polycondensation of lactic acid in the presence of monomethoxy of PEG, which resulted in a copolymer composed of hydrophilic block PEG and lipophilic block PLA. Similarly, the triblock copolymer PLA–PEG–PLA was obtained from the polymerization of lactic acid in the presence of dihydroxyl PEG. In general, PLA compounds are synthesized by the ring-opening polymerization of lactide (a cyclic diester of lactic acid)[2,3,5] or the polycondensation of lactic acid.[6,17] Although the latter is a reasonably low-cost, straightforward method for synthesizing polymers bearing PLA segments, this route generally leads to oligomers with low-molar-mass chains.[6,17] The molecular characteristics of the resulting copolymers are summarized in Table I.

Characterization of the PLA/PEG block copolymers by MALDI-TOF MS

Mass spectrometry is used to measure the real MW of synthetic polymers.[5,14,15] With this technique, the molecular structure and chemical composition of

TABLE I
Molecular Characteristics of the Block Copolymers of PEG and Lactic Acid Initiated by PEG

Polymer	MALDI-TOF MS[a]		GPC[b]		^{1}H-NMR[c]
	M_n	M_w/M_n	M_n	M_w/M_n	M_n
$MePEG_{2000}$	1970	1.05	2650	1.10	2000
PLA–PEG	2370	1.03	3360	1.08	2150
diOH–PEG_{2000}	1840	1.04	2700	1.08	2000
PLA–PEG–PLA	2240	1.04	3520	1.07	2200

[a] Data obtained by MALDI-TOF MS with α-cyano-4-hydroxy cinnamic acid as the matrix and Na-TFA as a dopant.
[b] Data obtained by GPC with respect to polystyrene standards from Polysciences.
[c] $M_n = M_{n\mathrm{PEG}} + M_{n\mathrm{PLA}} = 2000 + 72 \times 2000/44 \times ([\mathrm{LA}]/[\mathrm{OE}])$, where [LA]/[OE] was determined from the integrations of the signals due to the PEG blocks at 3.6 ppm and to the PLA blocks at 1.5 ppm on the ^{1}H-NMR spectra.

copolymers can be accurately studied. Figure 2 presents the MALDI-TOF MS spectra of PLA–PEG and the corresponding $MePEG_{2000}$. The $MePEG_{2000}$ spectrum was well resolved [Fig. 2(a)], and the peaks were separated by 44 mass units, which corresponded to the MW of the PEG monomer [oxyethylene (OE) units = 44.03 g/mol]. The subsidiary peaks were assigned to the isotopes of elements. The MW of $MePEG_{2000}$ ranged from 1200 to 2800 g/mol with $M_n = 1970$ and $M_w/M_n = 1.05$. After the condensation (140°C, 24 h, no catalyst) of the lactic acid aqueous solution in the presence of $MePEG_{2000}$, the MW distribution of the resulting polymer shifted to 1600–3200 g/mol with $M_n = 2370$ and $M_w/M_n = 1.03$ [Fig. 2(b)], which indicated the chain extension of the lactyl (LA) monomer onto the macroinitiator $MePEG_{2000}$. No signal characteristics of $MePEG_{2000}$ were detected on the MALDI-TOF MS spectra of PLA–PEG, which indicated that PLA-free $MePEG_{2000}$ species were removed during purification. On the spectra of $MePEG_{2000}$ and PLA–PEG, the number of OE units and LA units could be uniquely determined (x and y, respectively) from the MW of the major peaks. Each major peak in the mass spectrum corresponded to a polymer species (molecular structure proposed in Fig. 1) that had OE units and LA

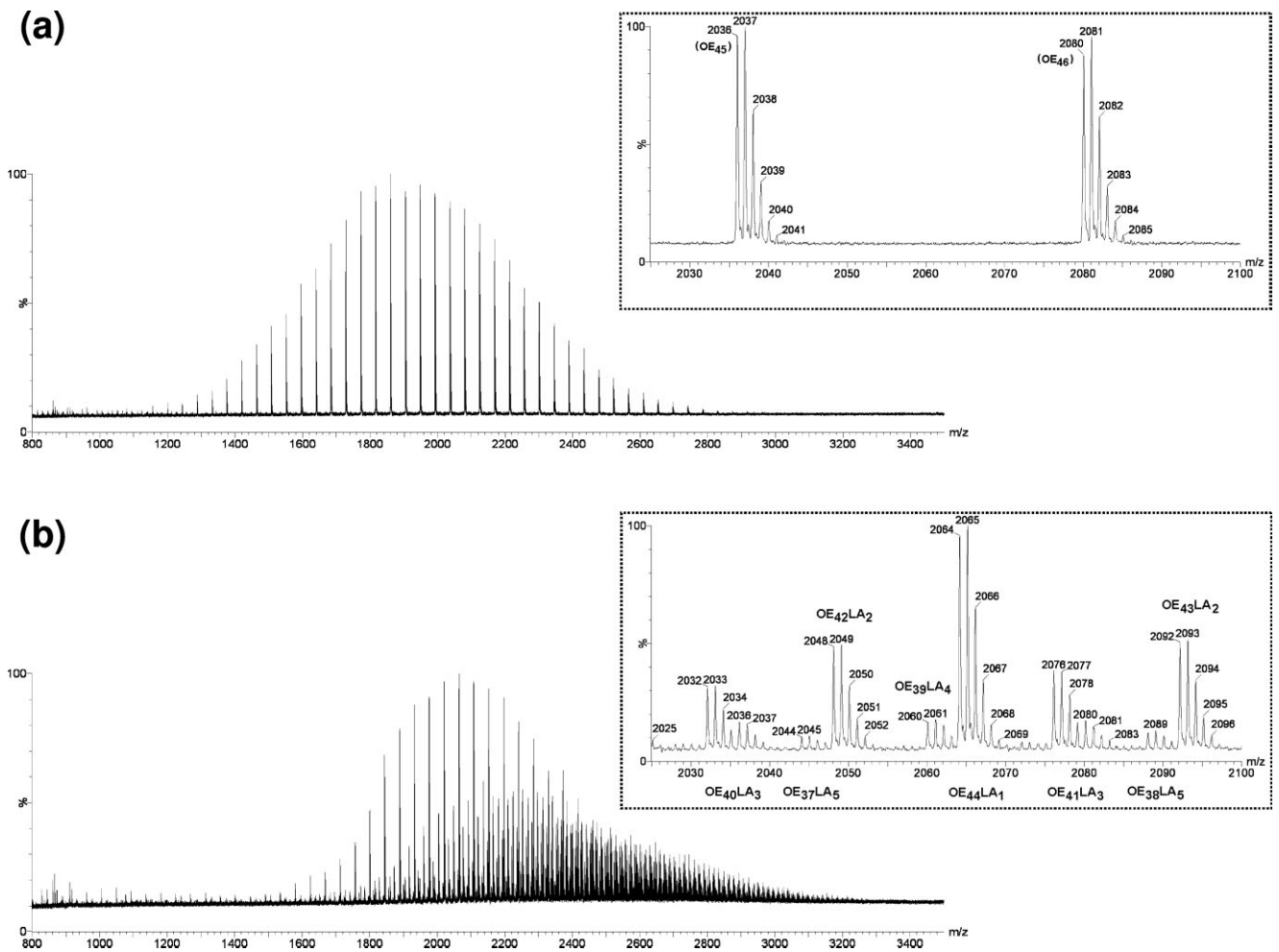

Figure 2 MALDI-TOF MS spectra of (a) $MePEG_{2000}$ and (b) PLA–PEG.

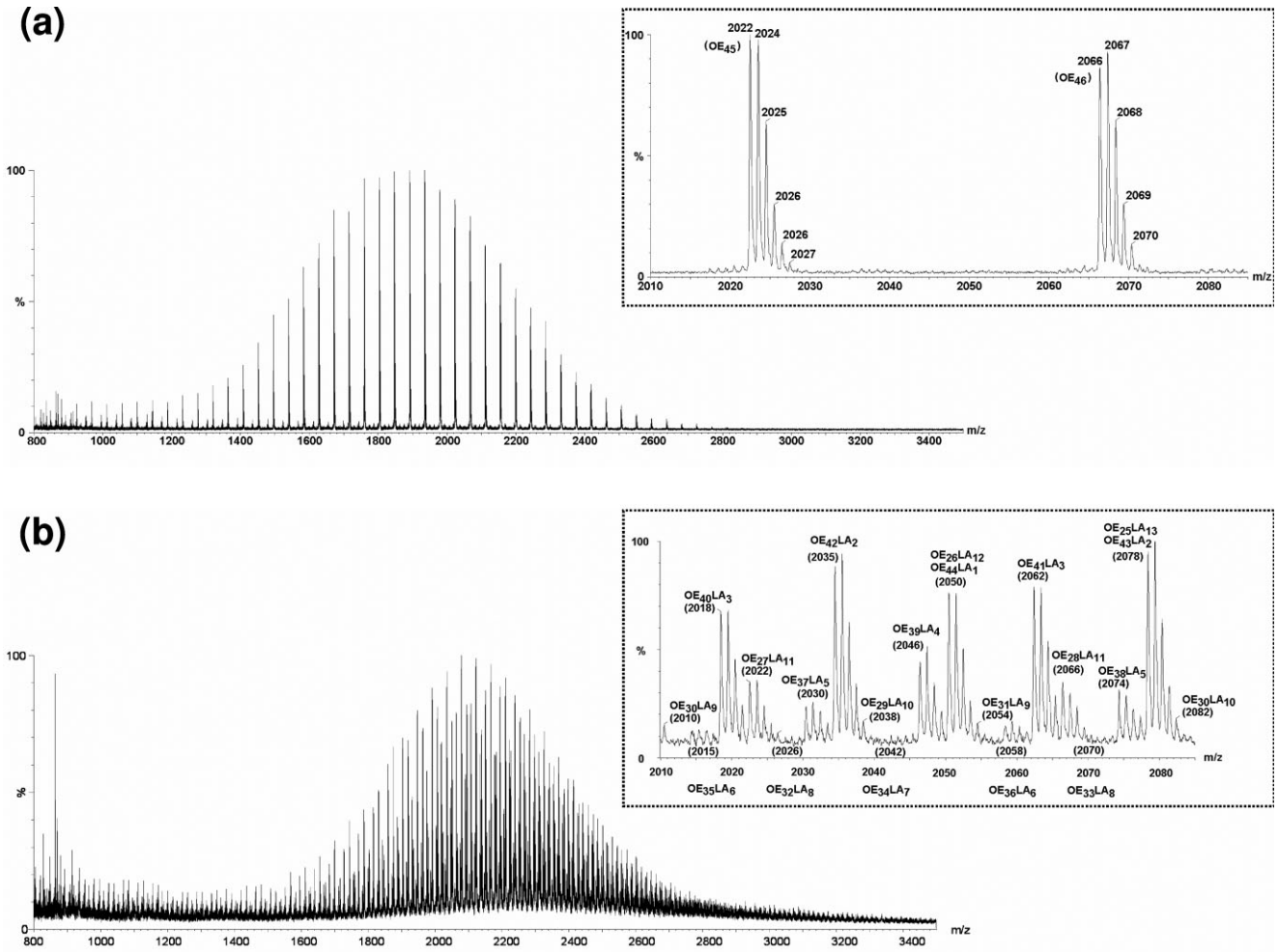

Figure 3 MALDI-TOF MS spectra of (a) diOH–PEG_{2000} and (b) PLA–PEG–PLA.

units (MW = 72.06) in addition to the end groups (one methyl and one hydroxyl, MW = 32.03) and a Na^+ ion (MW = 22.99, due to Na-TFA):

$$MW_{MePEG2000} = x(44.03) + 32.03 + 22.99$$

$$MW_{PLA-PEG} = x(44.03) + y(72.06) + 32.03 + 22.99$$

For example, the major five polymer species between 2030 and 2100 m/z in the spectra of PLA–PEG [Fig. 2(b)] were represented as follows:

$$MW_{PLA-PEG} = 2032, x = 40, y = 3$$

$$MW_{PLA-PEG} = 2048, x = 42, y = 2$$

$$MW_{PLA-PEG} = 2064, x = 44, y = 1$$

$$MW_{PLA-PEG} = 2076, x = 41, y = 3$$

$$MW_{PLA-PEG} = 2092, x = 43, y = 2$$

To substantiate the speculation of correlation between the MW and molecular architecture of copolymers, the same method were employed for the analysis of the triblock copolymer PLA–PEG–PLA. Each major peak in the mass spectrum, as shown in Figure 3, corresponded to a polymer species that had OE units, LA units, the end groups of one hydrogen and one hydroxyl, and Na^+ ion:

$$MW_{diOH-PEG2000} = x(44.03) + 18.02 + 22.99$$

$$MW_{PLA-PEG-PLA} = x(44.03) + y(72.06) + 18.02 + 22.99$$

For example, the major five polymer species between 2010 and 2080 m/z in the spectra of PLA–PEG–PLA [Fig. 3(b)] were represented as follows:

$$MW_{PLA-PEG-PLA} = 2018, x = 40, y = 3$$

$$MW_{PLA-PEG-PLA} = 2035, x = 42, y = 2$$

$$MW_{PLA-PEG-PLA} = 2050, x = 44, y = 1 \text{ or } x = 26, y = 12$$

$$MW_{PLA-PEG-PLA} = 2062, x = 41, y = 3$$

$$MW_{PLA-PEG-PLA} = 2078, x = 43, y = 2 \text{ or } x = 25, y = 13$$

After calculating the repeat unit masses and end-group masses through the MALDI spectra, we could distinguish the molecular structure between the diblock and triblock copolymers. The hydrophilic–lipophilic balance (HLB) value of the nonionic PLA/PEG diblock or triblock copolymer was expressed according to Griffin's method as follows:[18]

$$\mathrm{HLB}_{\mathrm{PLA/PEG}} = 20(W_{\mathrm{PEG}}/W_{\mathrm{PLA/PEG}})$$

where $W_{\mathrm{PEG}}/W_{\mathrm{PLA/PEG}}$ is the weight ratio of the hydrophilic portion of the main-chain polymer and was obtained from $M_{n\mathrm{PEG}}/M_{n\mathrm{PLA/PEG}}$. The most lipophilic portion had an HLB number approaching 0, and the most hydrophilic portion had a number of about 20. According to this equation, high HLB values ($\mathrm{HLB}_{\mathrm{PLA–PEG}} = 16.6$ and $\mathrm{HLB}_{\mathrm{PLA–PEG–PLA}} = 16.4$) were obtained, which indicated that the two copolymers had high affinities for water. However, there was no significant difference between the copolymers initiated by MePEG_{2000} or $\mathrm{diOH–PEG}_{2000}$.

MWs determined by GPC and ^{1}H-NMR

GPC is a separation technique based on the molecular hydrodynamic volume. By comparison with the standard curve of known MW species, the relative MW of the samples could be easily calculated. As shown in Table I, the average MW increased after the introduction of lactic acid chains onto the prepolymer PEG. The GPC traces of PLA–PEG and PLA–PEG–PLA exhibited monomodal distributions and reflected rather narrow MW distributions, which indicated the absence of residual low-molecular-weight species. ^{1}H-NMR data revealed that these low-molecular-weight species consisted of unreacted lactic acid and/or LA-rich species. The M_n values calculated from GPC were higher than those calculated from MALDI-TOF MS and ^{1}H-NMR (Table I). This finding could be assigned to changes in the hydrodynamic volume of the hydrophilic PEG and/or PLA blocks as compared with that of the polystyrene standards.

The LA units/OE units molar ratio or [LA]/[OE] was determined from the integrations of the proton resonances due to PEG blocks at 3.6 ppm and to PLA blocks at 1.5 ppm on the ^{1}H-NMR spectra.[5–7] The single peak at 3.3 ppm assigned to the hydrogens of methyl groups was also detected on the NMR spectra of MePEG_{2000} and PLA–PEG.[6,7] The MW of the copolymers was determined according to the following relationship:

$$\begin{aligned} M_n(\mathrm{NMR}) &= M_{n\mathrm{PEG}} + M_{n\mathrm{PLA}} \\ &= 2000 + 72 \times 2000/44 \times ([\mathrm{LA}]/[\mathrm{OE}]) \end{aligned}$$

where 44 and 72 are the MWs of the OE and LA repeat units, respectively, and 2000 is the average MW of PEG indicated by the supplier.

TABLE II
Physicochemical Characteristics of the Squalene Emulsions Based on PLA–PEG and PLA–PEG–PLA

Component		HLB[a]	Emulsion type	Particle size (nm)[b]
Aqueous phase	Oily phase			
PLA–PEG/PBS	Squalene	16.6	O/W	343 ± 67
PLA–PEG–PLA/PBS	Squalene	16.4	O/W	331 ± 68

[a] $\mathrm{HLB}_{\mathrm{PLA/PEG}} = 20(M_{n\mathrm{PEG}}/M_{n\mathrm{PLA/PEG}})$.
[b] Each value represents the mean of three experiments (Mean ± Standard deviation).

Emulsifying properties of the amphiphilic block copolymers

To demonstrate whether PLA–PEG and PLA–PEG–PLA could be used as the emulsifier, the polymer aqueous solution was homogenized with squalene oil, which resulted in an isotropic emulsified formulation. The emulsions remain stable for a few weeks when they were stored at 4°C. After 2 weeks, 5% of water disassociated, but beyond this, no further water disassociation from the emulsion occurred. The isotropic emulsion could be reformed by vortex mixing. Little difference was observed between the PLA–PEG- and PLA–PEG–PLA-stabilized emulsions. Homogenization with MePEG_{2000} or $\mathrm{diOH–PEG}_{2000}$ failed to stabilize the squalene/water interface; this indicated that even PEG bore only short PLA units in the main-chain polymer of PLA–PEG or PLA–PEG–PLA, which could have had amphiphilic behavior.

The size distribution of the emulsions and *in vitro* OVA release were measured to identify the dispersion type of the resulting emulsion and to understand the effect of the copolymer in the emulsification process. The size distribution of the emulsions was investigated by their redispersal in PBS and measurement with a particle size analyzer. Typically, a droplet of water-in-oil (W/O) emulsion remained floating on the water surface;[16] the particle size was undetected with light-scattering technology. On the other hand, the O/W emulsion droplet could only stand for seconds in the aqueous phase and then diffused into the water. The dynamic light-scattering pattern showed that PLA–PEG or PLA–PEG–PLA was a suitable emulsifier for the squalene/water emulsions and yielded narrowly distributed nanoparticles in PBS (Table II). Figure 4 shows the cumulative release of OVA from different formulations. Initially, a fast release was observed in the case of nonformulated OVA, from which more than 80% of loaded OVA was released into the outside PBS medium within the first 50 h. PLA–PEG/squalene or PLA–PEG–PLA/squalene emulsion allowed

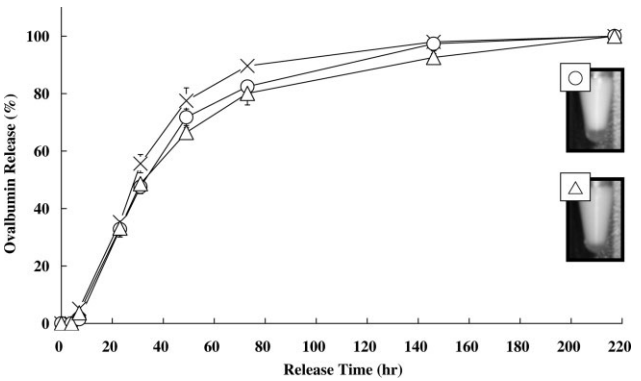

Figure 4 *In vitro* OVA release profile from the squalene emulsions based on PLA–PEG and PLA–PEG–PLA. OVA-containing formulations (3 mg/0.3 mL) were placed in a dialysis chamber in a centrifuge tube containing 2 mL of PBS and left to stand at 37°C. The release was regularly monitored by the BCA method read by an ultraviolet–visible spectroscopy instrument at 562 nm with calibration curves obtained from standard BSA solutions. The data are presented as the mean with standard errors of three samples: (-×-) nonformulation, (-○-) PLA–PEG/squalene, and (-△-) PLA–PEG–PLA/squalene.

a slight delay, but the protein was quickly released. The visual aspect showed that the emulsions remained stable; only 5% of water disassociated at the bottom over 200 h at 37°C. It is known that surfactants as emulsifiers can be defined by their HLB value,[16,19] which gives information on their relative affinity for both aqueous and oily phases. A lipophilic emulsifier renders a W/O emulsion with a high affinity for the oily phase, whereas a hydrophilic emulsifier renders an O/W emulsion with a high affinity for the aqueous phase. However, these are strongly influenced by the optimization of the surfactant system and the emulsification process.[16] Here, light-scattering and *in vitro* release data indicated that polymers with high HLB values rendered stable O/W emulsions. Moreover, no significant difference was found between PLA–PEG- and PLA–PEG–PLA-stabilized emulsions.

Mostly, degradable aliphatic polyesters used for vaccine or protein delivery have been in the form of injectable microspheres or implant systems.[20–22] Such systems require complicated fabrication processes with organic solvents; this may cause denaturation when antigens (viruses or proteins) are to be encapsulated. Moreover, the systems require polymers with high MWs (generally >50,000 Da), which require severe polymerization conditions (extreme temperature and pressure and toxic catalysts). In this study, the stable squalene/water emulsions were obtained with PEG-containing PLA oligomers as emulsifiers without the addition of any other stabilizer; the bioactive candidates could be either surface attached or encapsulated within the core oil. The obtained emulsions had a high affinity for water so that nanoparticles were obtained after they were redispersed into PBS. Moreover, no catalyst was required for the preparation of the designed polymers. Last but not least, the emulsified formulation developed here was free of organic solvents. These features are of great interest for the local delivery of bioactive agents, especially for applications in candidate vaccine delivery and anticancer treatments.

CONCLUSIONS

PLA/PEG diblock and triblock copolymers with high HLB values were synthesized by the direct polycondensation of an aqueous lactic acid solution on monomethoxy PEG or dihydroxyl PEG in the absence of a catalyst. MALDI-TOF MS data allowed us to calculate the repeat unit masses and end-group masses so that the molecular structure between the diblock and triblock copolymers could be distinguished. The obtained copolymers could serve as a hydrophilic emulsifier and rendered stable O/W emulsified nanoparticles when the polymer aqueous solution was homogenized with squalene oil. However, little difference was found in the physiochemical characteristics, such as the stability, particle size, and emulsion type between PLA–PEG- and PLA–PEG–PLA-stabilized emulsions. Further investigations are under way to examine the potential of these formulations as delivery systems for prophylactic and therapeutic vaccine candidates and anticancer drugs.

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