Langmuir

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Microcosmic Mechanisms for Protein Incomplete Release and Stability of Various Amphiphilic mPEG-PLA Microspheres

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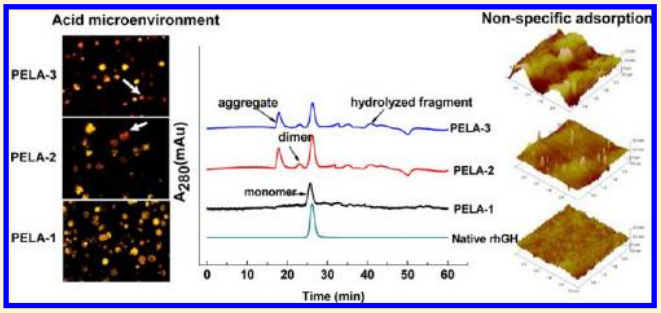
Supporting Information

ABSTRACT: The microcosmic mechanisms of protein (recombinant human growth hormone, rhGH) incomplete release and stability from amphiphilic poly(monomethoxypolyethylene glycol-*co*-D,L-lactide) (mPEG-PLA, PELA) microspheres were investigated. PELA with different hydrophilicities (PELA-1, PELA-2, and PELA-3) based on various ratios of mPEG to PLA were employed to prepare microspheres exhibiting a narrow size distribution using a combined double emulsion and premix membrane emulsification method. The morphology, rhGH encapsulation efficiency, in vitro release profile, and rhGH stability of PELA microspheres during the release were characterized and compared in detail. It was found that increasing amounts of PLA enhanced the encapsulation efficiency of PELA microspheres but reduced both the release rate of rhGH and its stability. Contact angle, atomic force microscope (AFM), and quartz crystal microbalance with dissipation (QCM-D) techniques were first combined to elucidate the microcosmic mechanism of incomplete release by measuring the hydrophilicity of the PELA film and its interaction with rhGH. In addition, the pH change within the microsphere microenvironment was monitored by confocal laser scanning microscopy (CLSM) employing a pH-sensitive dye, which clarified the stability of rhGH during the release. These results suggested that PELA hydrophilicity played an important role in rhGH incomplete release and stability. Thus, the selection of suitable hydrophilic polymers with adequate PEG lengths is critical in the preparation of optimum protein drug sustained release systems. This present work is a first report elucidating the microcosmic mechanisms responsible for rhGH stability and its interaction with the microspheres. Importantly, this research demonstrated the application of promising new experimental methods in investigating the interaction between biomaterials and biomacromolecules, thus opening up a range of exciting potential applications in the biomedical field including drug delivery and tissue regeneration.



1. INTRODUCTION

During the past two decades, biomaterials have received increasing amounts of attention because of their potential uses in many applications such as drug/cell delivery and tissue engineering.[1−6] Among these biomaterials, amphiphilic polymers have garnered considerable interest from their many advantages over hydrophobic polymers such as better compatibility with protein and tissue, a longer blood circulation half-life, and a high capability for noninvasive drug delivery.[7−11] In particular, amphiphilic polymer poly(monomethoxypolyethylene glycol-*co*-D,L-lactide) (mPEG-PLA, PELA) was utilized to improve the protein encapsulation efficiency in microspheres and decrease the burst release of protein from microspheres.[7,12] Compared to conventional hydrophobic polymers PLA and PLGA, PELA acting as a surface modifier can improve the stability of proteins, increase the protein loading efficiency, and decrease the amount of emulsifier used in PELA microsphere preparation. Moreover, the amphiphilic polymers could minimize the contact of protein with the oil/water interface or hydrophobic matrix, reducing the possibility of aggregation and the inactivation of encapsulated protein during encapsulation and release.[13,14] In addition, it has been reported that during the protein release phase hydrophobic PLGA prevented the penetration of water into the center of the microspheres, thus forming an acidic microenvironment as a result of the accumulated acidic products, such as lactic and glycolic acid end groups.[12] The encapsulated proteins can be severely hydrolyzed or aggregated because of the acidic microenvironment. Incorporating a hydrophilic sequence PEG into relatively hydrophobic PLA blocks can promote water uptake and swelling of the microsphere matrix and modulate the diffusion of proteins from the carrier system, resulting in a more stabilizing environment for proteins.[15]

Received: April 26, 2012
Revised: July 21, 2012
Published: August 31, 2012

Although PELA exhibits many general advantages described above, its degree of hydrophilicity, which can be varied by the mass ratio of mPEG to PLA,[15,16] could exert strong effects on protein drug release and stability profiles. Therefore, choosing PELA with appropriate hydrophilicity is critical to the design and preparation of a drug release system. Until now, most researchers studied the effect of materials on drug release on the macroscopic level, such as the drug cumulative release profile, molecular weight decrease, weight loss of microspheres, and medium pH change.[12,15,17] Kim used guanidine hydrochloride (GuHCl)/sodium dodecyl sulfate (SDS) to extract rhGH from PLGA microspheres, concluding that the reason for nonrelease behavior could be the formation of protein aggregates and the surface adsorption of protein within the microspheres.[18] There were a few reports on the direct observation of protein adsorption onto the microspheres matrix. The mechanisms of incomplete drug release from microspheres were studied on the macroscopic level rather than on the microcosmic level. Additionally, maintaining peptide or protein stability during microsphere preparation and release can be a daunting task. Several research groups have developed encapsulation strategies and protective agents that avoid exposing proteins to harsh environments.[4,19,20] Nevertheless, experimental and theoretical work on the effect of material on protein drug stability is still lacking. It remains an open question as to how the nature of the matrix polymer itself affects the incomplete release and stability profile of encapsulated proteins. Therefore, it is desirable to explore the mechanisms on a microcosmic level.

In this article, we investigated the influence of polymer hydrophilicity on protein drug incomplete release and stability. Specifically, we designed the PELA copolymer with ratios of mPEG to PLA of 1:9 (PELA-1), 1:14 (PELA-2), and 1:19 (PELA-3) as the polymeric materials and recombinant human growth hormone (rhGH) as the model protein drug. The rhGH-PELA microspheres exhibiting a narrow size distribution were prepared by a double emulsion method combined with a premix membrane emulsification technique, which was found to be promising for good reproducibility and repeatable release behavior. The results obtained according to the technique are credible. The exterior morphologies of microspheres were characterized with scanning electron microscopy (SEM), and in vitro rhGH release profiles of the microspheres were investigated. The contact angle combined with QCM-D and AFM techniques was used to study the interaction between the PELA film and rhGH and to elucidate the mechanism for incomplete protein release. Finally, the structural integrity of released rhGH species was characterized by size exclusion chromatography (SEC). Furthermore, the mechanisms responsible for rhGH stability were examined by monitoring the rhGH aggregation onto PELA and the microenvironment pH change using AFM and CLSM, respectively.

2. MATERIALS AND METHODS

2.1. Materials. mPEG-PLA with number-average molecular weights (M_n) of 20 982 (PELA-1), 39 878 (PELA-2), and 45 912 KDa (PELA-3) and M_w/M_n value of 1.70, 1.56, and 1.58, respectively was supplied by the Dai Gang Company (Shandong, China). The molecular weight of the mPEG block was fixed at 2000 Da. The M_n and M_w/M_n values of PELA were obtained with gel permeation chromatography (GPC, Waters, USA) using polystyrene as a standard. Poly(vinyl alcohol) (PVA-217, degree of polymerization 1700, degree of hydrolysis 88.5%) was kindly provided by Kuraray (Japan). The SNARF-1 dextran fluorescent pH-sensitive dye (M_w = 10 kDa) was from Molecular Probes (USA). rhGH ($M_w \approx$ 22 kDa) was kindly supplied by Pfizer Inc. (USA). The SPG membrane (pore size of the membrane was 18 μm) was provided by SPG Technology Co. Ltd. (Japan). All other reagents were of analytical grade.

2.2. Preparation of Microspheres. Microspheres loaded with rhGH were prepared by two-step procedure reported previously.[21] Briefly, the coarse double emulsions were prepared first, 0.4 mL of an rhGH aqueous solution (32 mg/mL) was mixed with 4 mL of ethyl acetate containing PELA (200 mg) by homogenizer for 20 s in an ice bath to form a W/O primary emulsion, which was further emulsified into an external aqueous phase containing 1% w/v PVA and 0.9% w/v NaCl by magnetic stirring for 60 s at 300 rpm to prepare W/O/W coarse double emulsions. The coarse double emulsions were then poured into the premix reservoir. Second, smaller double emulsions with a relatively uniform size were achieved by extruding the coarse double emulsions through the SPG membrane at a high pressure of 50 kPa. The obtained uniform double emulsions were poured quickly into an 800 mL solution containing 0.9% w/v NaCl (solidification solution) under magnetic stirring for 2 h to solidify the microspheres. The obtained microspheres were collected by centrifugation at 5000 rpm for 10 min, washed with distilled water three times, and then lyophilized for 2 days.

2.3. Characterization of Microspheres. The surface morphology of microspheres was observed with a JSM-6700F (JEOL, Japan) scanning electron microscope (SEM). The volume mean diameter of microspheres was measured by laser diffraction using a Mastersizer 2000 (Malvern, U.K.). Microspheres after being solidified, collected, and washed were dispersed in distilled water and analyzed with a Mastersizer 2000. The uniformity of the microspheres was expressed as the span value, and a smaller span value indicates a more narrow size distribution of microspheres.

2.4. Measurement of the Protein Encapsulation Efficiency. The total encapsulation efficiency of rhGH in PELA microspheres was determined by dissolving 20 mg of the freeze-dried microspheres in 1 mL of 1 M NaOH. The loaded amount of rhGH was determined by micro-bicinchoninic acid (micro-BCA) assay (Pierce, USA). The encapsulation efficiency (EE) was calculated with the following equation

$$\mathrm{EE} = \frac{m}{m_0} \times 100\%$$

where m_0 is the total mass of rhGH added and m is the mass of rhGH loaded in the microspheres.

The loading efficiency (LE) was calculated with the following equation

$$\mathrm{LE} = \frac{a}{b} \times 100\%$$

where a is the mass of rhGH loaded into the microspheres and b is the total mass of loaded microspheres (including the mass of polymer and rhGH).

All analyses were carried out in triplicate (n = 3) and were presented as the mean ± SD. One-way ANOVA (OriginPro, version 8.0) was used to determine the statistical significance, and the difference is considered to be significant when $p < 0.05$.

2.5. In Vitro rhGH Release Measurement. To examine protein release profiles, 20 mg of rhGH-loaded PELA microspheres was suspended in 1 mL of PBS buffer (20 mM sodium phosphate and 0.15 M sodium chloride, pH 7.4). The samples were placed in an incubator (Sukun, SKY-200B) and shaken at 110 rpm, 37 °C. At predetermined time intervals, rhGH released in the medium from microspheres was separated by centrifugation at 2000 rpm for 5 min, and the release medium was replaced by the same amount of fresh buffer. The release medium was used to determine the protein concentration using the micro-BCA method.

2.6. Film Preparation and Determination of Contact Angle. A polymer solution was prepared by dissolving 200 mg of PELA (PELA-1, PELA-2, or PELA-3) in 4 mL of ethyl acetate. The polymer solution was cast on a glass dish and coated with a spin coater (WS-400BZ-6NPP, Laurell Technologies Corporation, USA) at a speed of

dx.doi.org/10.1021/la3017112 | Langmuir 2012, 28, 13984−13992

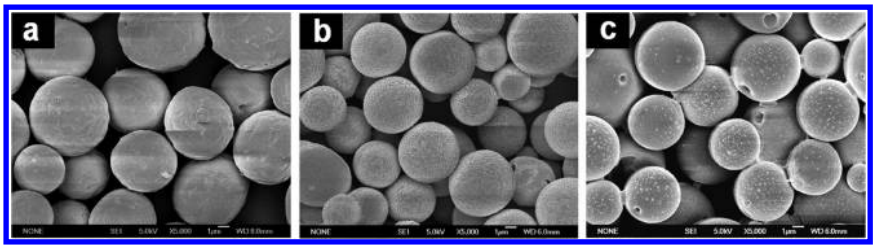


Figure 1. SEM images of microspheres prepared with different PELAs: (a) PELA-1, (b) PELA-2, and (c) PELA-3.

8000 rpm for 40 s. The PELA film was air dried first and completely dried in a vacuum oven.

The contact angle reflects the hydrophilic−hydrophobic characteristic of the microspheres matrix. Static contact angle measurements were performed on the PELA films using the OCA 20 system (Dataphysics Instruments GmbH, Germany). Drops of deionized water (1 μL) were deposited on film surfaces. At least three drops per sample were analyzed.

2.7. Quartz Crystal Microbalance with Dissipation (QCM-D) Measurement. The quartz crystal microbalance (QCM-D) technique was used to measure the real-time macromolecule adsorption in liquid-phase research applications.[22,23] Investigating the protein adsorption on the PELA film can reflect the interaction between PELA microspheres and rhGH. The general QCM-D principle is to apply an ac voltage in the megahertz range across an AT-cut piezoelectric quartz crystal and to record the resonance frequency of the crystal. Au-coated sensor crystals (Q-Sense AB) were immersed in a 5:1:1 mixture of deionized (Milli-Q) water, NH_3 (25%) and H_2O_2 (30%) for 5 min at 70 °C, rinsed thoroughly in deionized (Milli-Q) water, and exposed in a UV/ozone chamber for 15 min. A polymer solution was prepared by dissolving 200 mg of PELA (PELA-1, PELA-2, and PELA-3) in 4 mL of ethyl acetate. The polymer solution was cast on a Au-coated sensor crystals and coated with a spin coater at a speed of 8000 rpm for 40 s. The PELA film was air dried first and completely dried in a vacuum oven. The QCM-D experiments were conducted at 22 °C and coupled to a peristaltic pump using a flow rate of 300 μL/min. When the adsorption of rhGH reached saturation at a particular concentration, degassed PBS buffer (20 mM sodium phosphate and 0.15 M sodium chloride, pH 7.4) was injected into the chamber to desorb rhGH using a flow rate of 150 μL/min until balance was achieved.

2.8. Atomic Force Microscope (AFM) Measurement. The topographical changes in the PELA film surface can reflect the adsorption abilities of inner water phase rhGH within PELA microspheres (interface of inner water/PELA). After protein adsorption and subsequent desorption by QCM-D, the topographical changes in the PELA film surface were investigated by atomic force microscopy (Multimode AFM, Veeco Co, Ltd., USA) at 25 °C. All of the AFM images were obtained in the Scanasyst-in-air mode with a silicon nitride cantilever at a scanning speed of 1.0 Hz and a scanner with maximum ranges of 3 μm in the x and y directions and 3.5 nm in the z direction. By scanning a film surface with the probe tip at a constant interaction by controlling the piezo scanner, a 3D sample surface can be imaged. A commercial AFM image-processing software package, Nanoscope 8.1 from Veeco, was used. Roughness data were obtained from a minimum of three separate images obtained from different regions on each sample.

2.9. Size Exclusion Chromatography (SEC)-HPLC. The number of aggregates of released rhGH in the medium was quantified by size-exclusion chromatography using a column of 10 mm i.d. × 300 mm L (Amersham Superdex 75 series column, GE, Co., USA). Phosphate buffer (0.5 mL/min of a 100 mM solution) with 100 mM NaCl was used as a mobile phase, with absorption at 280 nm for eluted sample detection. The fraction of monomeric and dimeric rhGH was the area percentage of the peak representing monomeric and dimeric rhGH as measured by SEC-HPLC. The calculation equation was

$$\text{fraction of monomeric and dimeric rhGH}(\%) = \frac{A_m + A_d}{A_m + A_d + A_a + A_f} \times 100\%$$

where A_m, A_d, A_a, and A_f are the peak areas of monomeric, dimeric, aggregate, and rhGH fragments, respectively. The fractions of monomeric and dimeric rhGH in the total amount of rhGH represented the integrity of the protein because they showed bioactivity.[24]

2.10. Confocal Imaging of the Microclimate pH inside Microspheres. A quantitative ratiometric method based on laser scanning confocal microcosmic imaging was employed to monitor the microclimate pH change inside microspheres prepared with different polymers. Microspheres were prepared by double emulsion and membrane emulsification techniques. In brief, 0.4 mL of 32 mg/mL rhGH with 3 to 4 mg/mL SNARF-1 dextran (fluorescent pH-sensitive dye) in PBS solution was added to 4 mL of ethyl acetate containing PELA (200 mg) by homogenizer for 20 s in an ice bath to form a primary emulsion. The other procedures were the same as in method 2.2. Ten milligrams of microspheres was suspended in 1 mL of PBS buffer (pH 7.2) under mild agitation at 37 °C. After a predetermined interval, the release medium was removed after mild centrifugation (3 min at 2000 rpm) and fresh buffer was added to maintain the sink conditions. At the same time, a small number of microspheres were removed to obtain confocal images for the microclimate pH change. The fluorescent dye that was encapsulated in the microspheres was excited at 488 nm by an Ar/He laser, and two images at different wavelengths (580 and 640 nm) were taken. The two images were overlapped to observe the microclimate pH change, and the I_{640}/I_{580} ratio image was correlated with pH.[25] The larger value of ratio image I_{640}/I_{580} indicates a microenvironment that is close to alkalinity.

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Microspheres. PELA microspheres with narrow size distributions were successfully prepared by the double emulsion method followed by a premix membrane emulsification technique. As shown in Figure 1, microspheres with different molecular weights all showed regular sphericity. PELA-1, PELA-2, and PELA-3 microspheres had mean diameters of 6.41, 6.25, and 6.06 μm, respectively, and span values of 0.885, 0.921, and 0.951, respectively (Figure S1 in Supporting Information). Importantly, they possessed narrow size distributions of around 6 μm, which contributed to good process reproducibility and repeatable drug release behavior.

3.2. Encapsulation Efficiency of rhGH. It has been reported that the encapsulation efficiency depended on the properties of polymer used as a microsphere matrix.[26,27] To investigate the influence of the molecular weight of PELA on the rhGH encapsulation efficiency, we determined the loading and encapsulation efficiencies of PELA microspheres with different M_n values. As shown in Figure 2, the encapsulation efficiencies of PELA-1, PELA-2, and PELA-3 microspheres were 81.0 ± 6.9, 85.7 ± 5.7, and 90.2 ± 8.5%, respectively. The loading efficiencies of rhGH in PELA-1, PELA-2, and PELA-3

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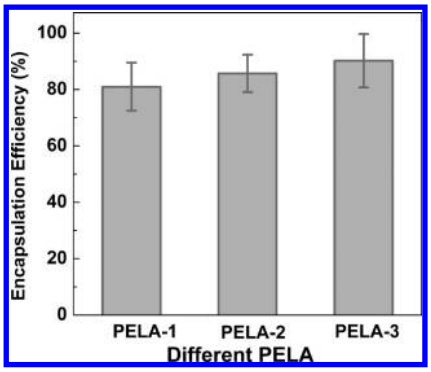

Figure 2. Effect of polymer properties on the encapsulation efficiency of rhGH. Results are expressed as the mean ± SD (n = 3). Statistically significant differences (p < 0.05) exist among all components of the series.

microspheres were 5.18 ± 0.44, 5.48 ± 0.36, and 5.57 ± 0.53%, respectively. It was shown that the higher molecular weight correlated with a higher encapsulation efficiency. When the molecular weight increased, the viscosity of the oil phase increased accordingly, which could enhance the stability of the internal water phase (the rhGH solution entrapped in the primary emulsion during microsphere preparation). Therefore, a higher molecular weight of PELA resulted in a higher encapsulation efficiency. However, all PELA microspheres achieved encapsulation efficiencies above 80%, indicating that PELA could generate a stable interfacial layer at the oil and water interface and prevented the protein in the inner droplets from merging into the external water phase, which prevented the loss of encapsulated protein during the fabrication process.

3.3. Effect of PELA Hydrophilicity on Incomplete Release Profiles. For a controlled drug release product, a low burst release and a constant release are both highly desired, especially for those diseases such as diabetes, pediatric hypopituary dwarfism, and Parkinson's[28−31] strictly needing plasma drug concentrations in the ranges of narrow therapeutic requirements for the duration of treatment. As shown in Figure 3, PELA-1, PELA-2, and PELA-3 microspheres showed burst releases of 23.1, 18.3, and 10.7% over the first 5 h, respectively. It has been reported that the strong burst effect (>30%) observed in PLA or PLGA microspheres was a result of the diffusion of rhGH located near the microsphere surface through the channels and inner pores,[29,31] which were formed by

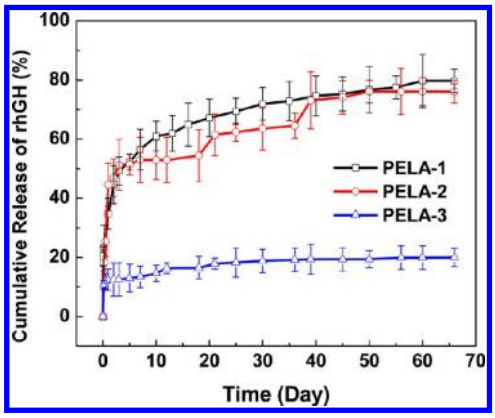

Figure 3. Cumulative release profiles of rhGH from three microspheres.

coalescence between the inner droplets and external water phase during the microsphere solidification process.[1,32,33] In this study, all PELA microspheres exhibited a relatively low burst releases (<25%) because the polymer itself could form an interfacial layer and acted as a stabilizer, thereby stabilizing the primary emulsion and preventing coalescence between the inner droplets and external water phase.

With respect to cumulative release, PELA-1 microspheres showed a total of 79.8% rhGH release within 66 days, whereas PELA-2 and PELA-3 microspheres released about 76.1 and 20.1% rhGH, respectively, during the same period. It is particularly interesting that a significant plateau phase of 40 days (day 26 to day 66) was observed for the PELA-3 microspheres. At first glance, PELA-1 and PELA-2 microspheres showed similar cumulative release profiles at day 66, but PELA-2 microspheres provided an initial release of protein followed by a plateau phase of 13 days (day 5 to day 18) and a subsequent continuous release phase of 27 days (day 28 to day 45), and finally a second plateau phase occurred for 20 days (day 46 to day 66). However, the lower-molecular-weight PELA-1 microspheres yielded a 2 month continuous release of rhGH without a plateau phase. The reason for the difference among the above release profiles was mainly due to the varied erosion rate of the polymeric matrix by hydrolysis in water. Generally, the hydrolysis of polymer depended to a large extent on its property. The rate of polymer erosion increased with a decrease in molecular weight. In addition, with the decrease in molecular weight as well as high ratios of mPEG, water penetrability would be enhanced and consequently the degradation rate of the polymer could be accelerated. Meanwhile, we monitored the surface morphology of these PELA microspheres at different release times (Figure 4). At 50

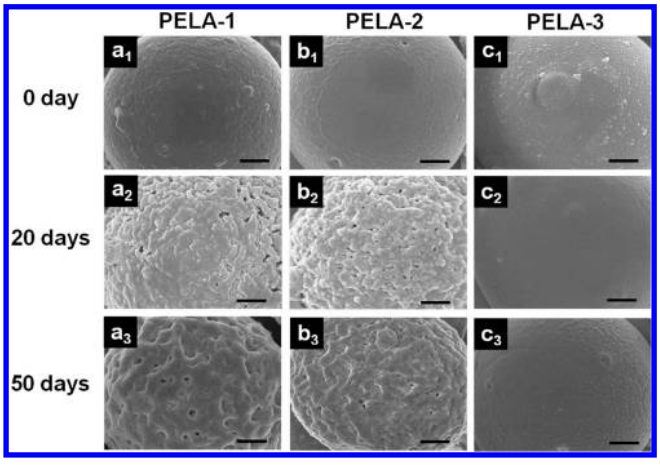

Figure 4. SEM micrographs of rhGH-loaded (a_1−a_3) PELA-1, (b_1−b_3) PELA-2, and (c−c $_3$) PELA-3 microspheres at different release times. The scale bar is 1 μm.

days, a distinct diversity of the sphere surface among the three microspheres was very obvious. Remarkable macroporous structures were observed on the surfaces of PELA-1 microspheres, whereas PELA-2 microspheres displayed tiny pores and PELA-3 microspheres remained almost as smooth a surface as the original one, indicating that PELA-1 microspheres degraded faster than others in solution.

PELA-2 and PELA-3 showed little difference in M_n (39 878 and 45 912 Da), but the release profiles exhibited a significant difference. We proposed that another important reason for the

dx.doi.org/10.1021/la3017112 | Langmuir 2012, 28, 13984−13992

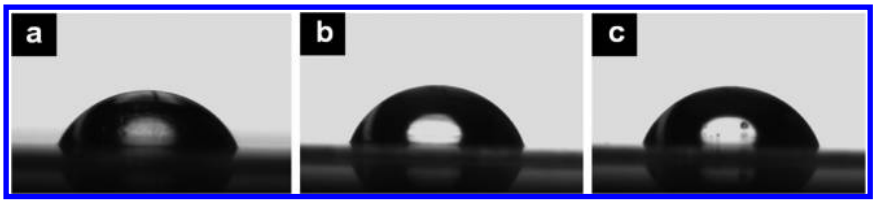

Figure 5. Contact angle (CA) of a water droplet on different PELA films: (a) PELA-1 with a CA of 65.0 ± 0.5°, (b) PELA-2 with a CA of 69.2 ± 0.9°, and (c) PELA-3 with a CA of 71.1 ± 0.7°.

incomplete release of rhGH was the nonspecific adsorption of rhGH with high affinity. It was reported that many proteins entrapped in hydrophobic matrixes, such as PLA and PLGA, frequently suffered from incomplete release primarily because of nonspecific protein adsorption within the microspheres.[18,34] With the aim of investigating the effect of polymer hydrophilicity on rhGH adsorption on a microcosmic level, we carried out contact angle tests, QCM-D, and AFM analysis on a PELA film. The water contact angle measurement was used to evaluate the hydrophilicity of polymers (Figure 5). Water contact angles of 65.0 ± 0.5, 69.2 ± 0.9, and 71.1 ± 0.7° were obtained for PELA-1, PELA-2, and PELA-3 films, respectively. The smaller water contact angle of PELA-1 could be attributed to the mPEG chain being prone to stretch out compared to the others, leading to more hydrophilic, lower water contact angles. Moreover, the amounts of rhGH adsorbed on the surfaces of different PELA films were detected by QCM-D. QCM-D can monitor two parameters: frequency (f) and dissipation (D). By detecting the frequency under an ac voltage across the electrodes, one can measure the mass changes in films. A decrease in frequency means an increase in film mass. Meanwhile, by measuring the dissipation of an adsorbed film, one can characterize the structural variations in thin viscoelastic films.[23] The enhancement of dissipation indicates that the structure of the film became rough. Figure 6 shows the final changes in frequency (Δf) and dissipation (ΔD) for the PELA film after the adsorption and subsequent desorption of rhGH. As shown in Figure 6a, PELA-1 resulted in a decrease in frequency (Δf) of 59 Hz and an increase in dissipation (ΔD) of 35.2. For PELA-2 and PELA-3 (Figure 6b,c), larger decreases in frequency (130 and 231 Hz, respectively) and larger increases in dissipation (48.8 and 43.2, respectively) were observed. These results demonstrated that PELA-2 and PELA-3 can adsorb more rhGH than PELA-1, suggesting that the ratio of mPEG to PLA influenced the contact angle and rhGH adsorption of PELA. When the ratio of mPEG to PLA was high (PELA-1), namely, a hydrophilic mPEG chain occupied a relative higher mass ratio, it gave rise to a low contact angle, low rhGH adsorption, and fast desorption. The results agreed well with the release profiles in Figure 3. Furthermore, we observed the topographical changes after protein desorption by QCM-D. Figure 7 and Table 1 show that the amount of rhGH adsorbed on the surface of PELA-1 was smaller than the others. From the results on rhGH adsorption to PELA, it can be concluded that the incomplete release was at least partially due to nonspecific protein adsorption within the hydrophobic matrix. Therefore, a higher ratio of mPEG could minimize the protein contact with the hydrophobic matrix, thus increasing the release rate and promoting complete release from the PELA microspheres.

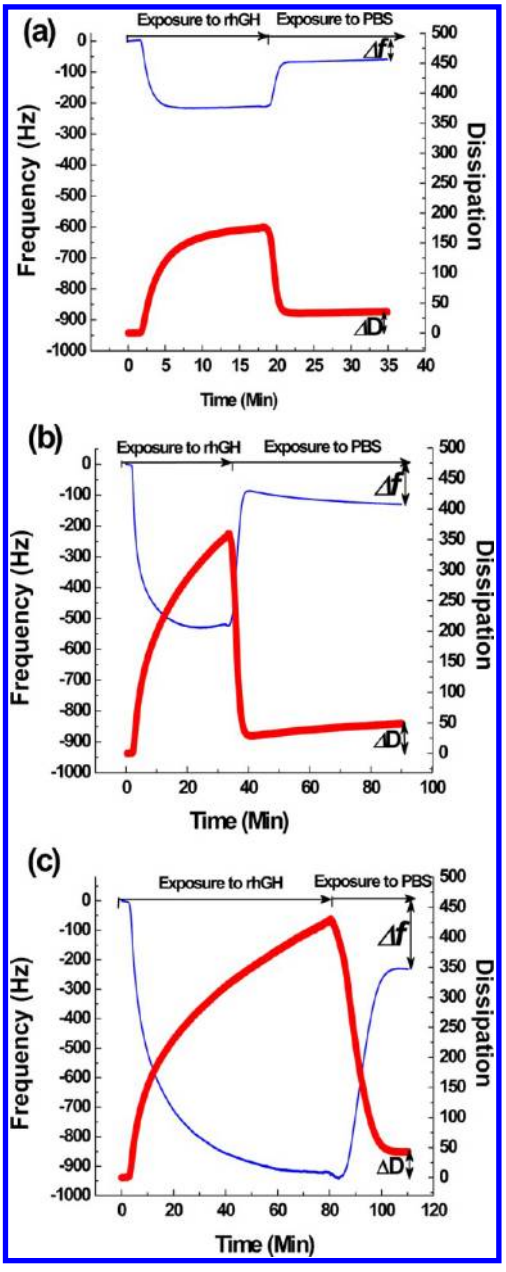

Figure 6. Diagram of frequency (thin line) and dissipation (thick line) vs time for exposure of the (a) PELA-1, (b) PELA-2, and (c) PELA-3 surfaces to rhGH solution, followed by exchange of the protein solution with PBS buffer solution. Δf and ΔD represent the changes in frequency and dissipation, respectively, when desorption becomes saturated.

3.4. Effect of PELA Hydrophilicity on rhGH Stability. During matrix erosion of PLA and PLGA microspheres, most protein and peptide drugs are subjected to an unfriendly microenvironmental impact due to the generation of acidic monomers. Consequently, major research efforts have been undertaken to investigate the impact of polymer composition and processing techniques on the stability of protein drugs.[35]

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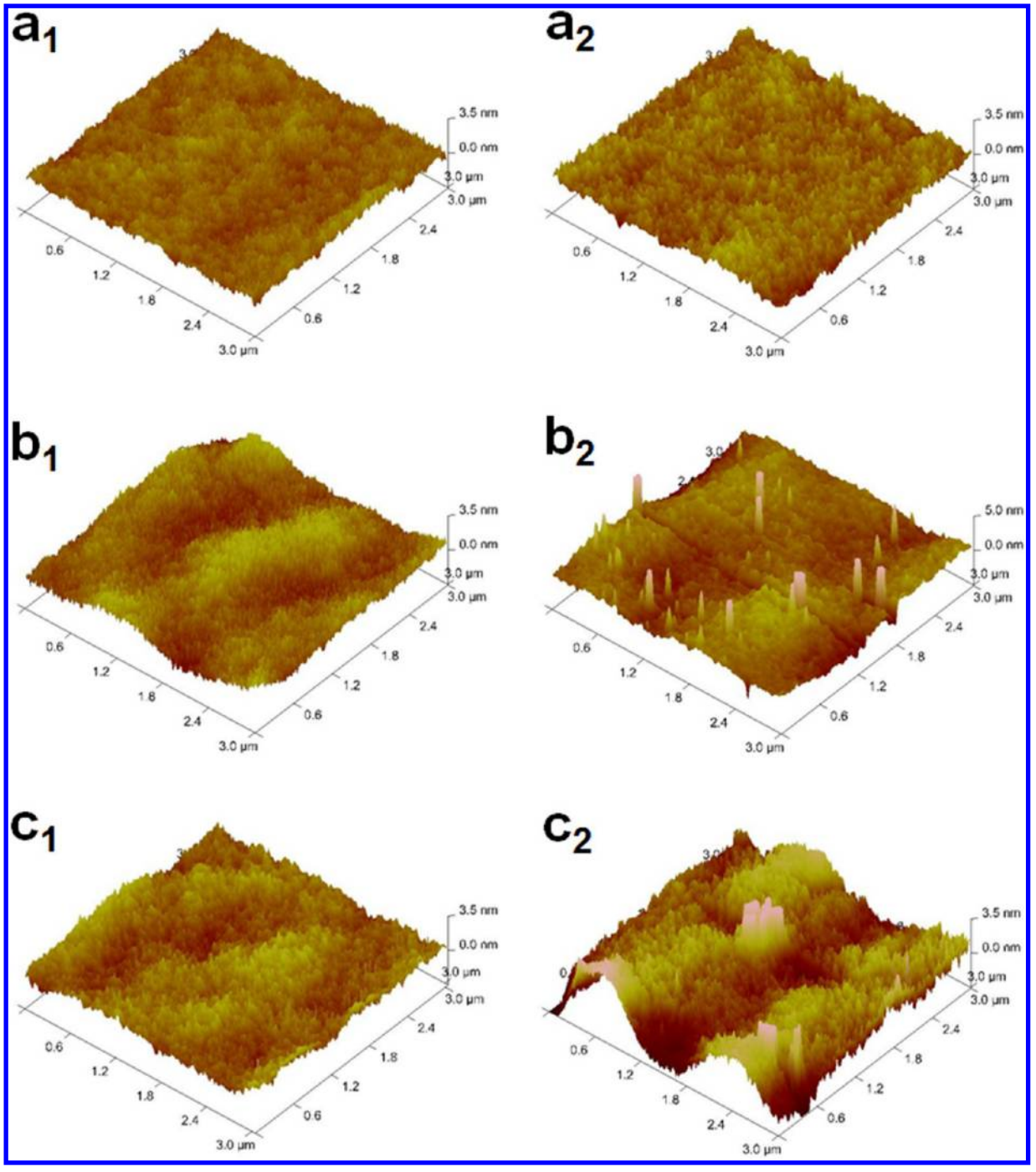

Figure 7. AFM images of the different PELA films before and after the adsorption of rhGH: (a_1) PELA-1, (b_1) PELA-2, and (c_1) PELA-3 films before adsorption; (a_2) PELA-1, (b_2) PELA-2, and (c_2) PELA-3 films after the adsorption and subsequent desorption of rhGH.

Table 1. Surface Average Roughness Analysis of Different PELA Films

average roughness (nm)	PELA-1	PELA-2	PELA-3
before adsorption	0.443 ± 0.028	0.808 ± 0.042	1.14 ± 0.06
after adsorption–desorption	0.827 ± 0.059	7.54 ± 0.34	15.8 ± 0.9

Nevertheless, the effects of the properties of the matrix polymer itself on drug stability and the associated micromechanism have not been fully clarified. Our first strategy was to determine the integrity of rhGH in terms of aggregation and hydrolysis by SEC-HPLC and explain the results by combining AFM and QCM-D because aggregation and the molecular hydrolysis of rhGH are the main pathways resulting in poor stability.[36] As shown in Figure 8a, the size exclusion chromatogram of rhGH released from PELA-1 microspheres yielded a single peak, unlike the rhGH released from PELA-2 and PELA-3 microspheres that showed several peaks. For 21 kDa PELA microspheres, the single peak suggested that rhGH maintained its monomeric form well. For PELA-2 and PELA-3 microspheres, the peaks appearing near 18, 24, and 40 min represented the aggregates, dimers, and hydrolyzed fragments, respectively. Further detailed calculations of the fraction of

dx.doi.org/10.1021/la3017112 | *Langmuir* 2012, 28, 13984–13992

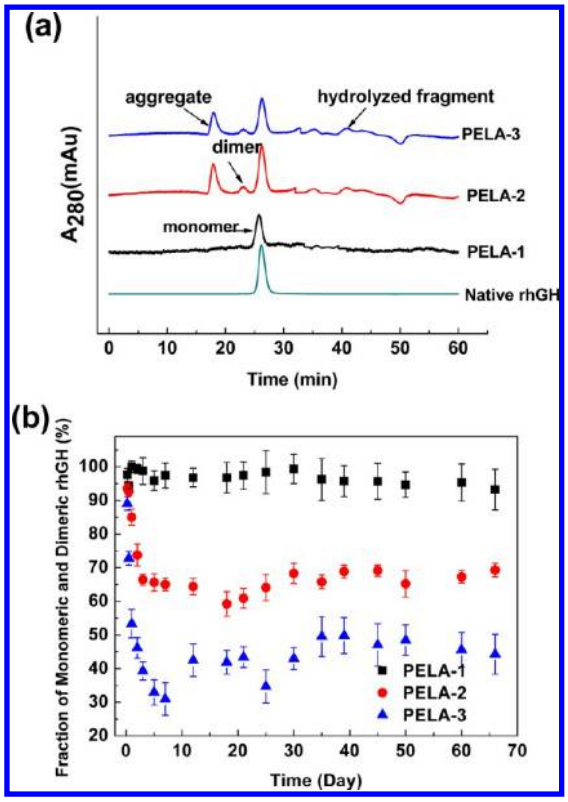

Figure 8. (a) Size exclusion chromatography (SEC-HPLC) of rhGH after recovery from PELA-1, PELA-2, and PELA-3 microspheres on the second day. (b) Fraction of monomeric and dimeric rhGH released from three microspheres.

monomeric and dimeric rhGH are shown in Figure 8b. rhGH released from the PELA-1 microspheres basically maintained its monomeric and dimeric forms, ranging from 90 to 100%. This indicates that minimal aggregation occurred during the preparation and release process. In contrast, the total amounts of monomeric and dimeric forms of rhGH released from the PELA-2 and PELA-3 microspheres were around 65 and 40%, respectively. We further explored the role of polymer properties in maintaining protein stability by AFM. Figure 7 shows three AFM images with significant differences in microsurface topographies. The microsurface of PELA-1 after rhGH adsorption and desorption (Figure $7a_2$) was relatively smooth, with only some tiny peaks present. In comparison, Figure $7b_2$ shows a rougher microsurface with more intensive, sharper peaks, which are even broader in Figure $7c_2$. Further data estimated by AFM showed that the average surface roughness (R_a) values of PELA-1, PELA-2, and PELA-3 before adsorption were about 0.443 ± 0.028, 0.808 ± 0.042, and 1.14 ± 0.06 nm, respectively (Table 1).

The enhancement of film surface roughness is associated with an increased contact angle and hydrophobicity of polymer. After adsorption and subsequent desorption, the R_a values of PELA-1, PELA-2, and PELA-3 were about 0.827 ± 0.059, 7.54 ± 0.34, and 15.8 ± 0.9 nm, respectively. The significant increases in R_a for PELA-2 and PELA-3 (from 0.808 to 7.54 nm and from 1.14 to 15.8 nm, respectively) suggested that rhGH formed aggregates, which was consistent with Figure 8b. The results confirmed that the higher ratios of mPEG, which increase the hydrophilicity of copolymer PELA, effectively minimize the contact of protein with the matrix, resulting in less protein aggregation.

In addition, the acidic microenvironment created inside the microspheres during polymer degradation is known to induce the aggregation and fragments of encapsulated protein molecules.[37] Our second strategy was to use dextran-SNARF-1 conjugate, a long-wavelength fluorescent pH indicator, to monitor the microenvironment pH change and to investigate the influence of polymer hydrophilicity on protein stability. When the strongest fluorescence emission of the dye is near 580 nm, the dye shows red light and the local pH is close to 6.0. When the strongest fluorescence emission is near 640 nm, the dye shows green light and the local pH is close to 9.0.[38] Therefore, if the dye is yellow, the local pH is close to neutral. As shown in Figure $9a_2$, in an early stage (2 weeks after

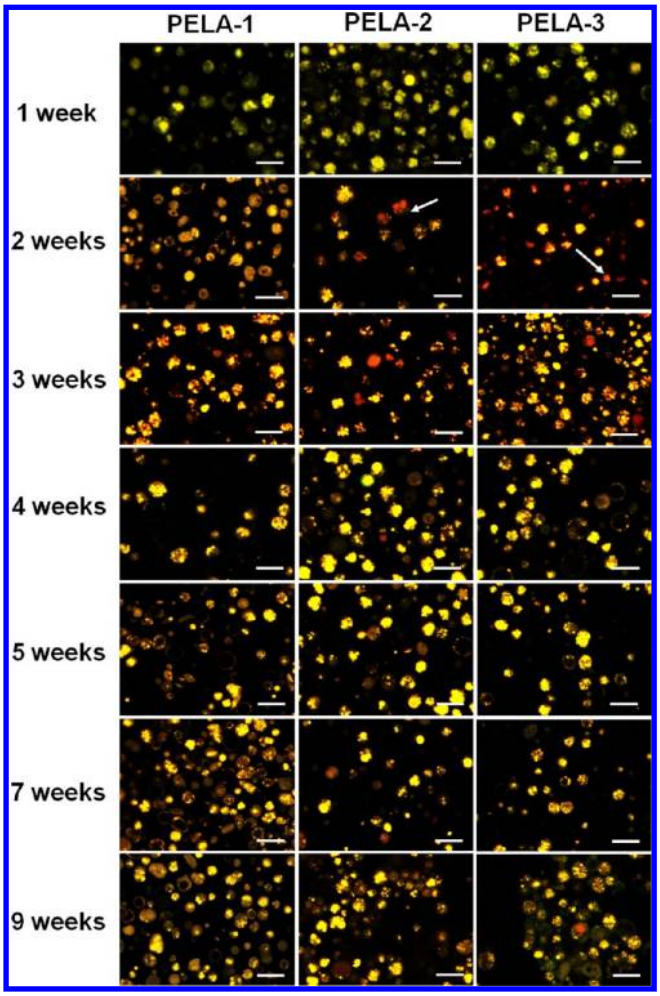

Figure 9. CLSM images of pH-sensitive dye-loaded PELA-1 (a_1–a_7), PELA-2 (b_1–b_7), and PELA-3 (c_1–c_7) microspheres at different incubation times (1–5, 7, and 9 weeks). The scale bar is 10 μm.

incubation), the PELA-1 microspheres were mostly orange and a few spots in the PELA-2 and PELA-3 microspheres became red (Figure $9b_2$,c_2, arrows indicated). These results suggested that the microenvironments of PELA-2 and PELA-3 microspheres were more acidic than that of PELA-1 microspheres. Furthermore, by calculating the ratio of fluorescence intensities measured at two wavelengths (I_{640}/I_{580}), we can directly compare the microenvironment pH change for different microspheres. As shown in Figure S2 (Supporting Information), the values of I_{640}/I_{580} in PELA-1 microspheres were always higher than those in the other two types of microspheres

dx.doi.org/10.1021/la3017112 | Langmuir 2012, 28, 13984–13992

during the release process. The results suggested that rhGH encapsulated in PELA-1 microspheres experienced a less-acidic microenvironment, resulting in a smaller adverse impact on drug stability. The results corresponded well to those in Figure 8a, where PELA-1 microspheres released mostly monomeric rhGH and a large part of the rhGH released from PELA-2 and PELA-3 microspheres consisted of aggregates and hydrolyzed fragments from the the acidic microenvironment. Because PELA-1 possessed the highest ratio of hydrophilic mPEG, which can be hydrated more easily in the releasing medium, its microspheres could swell more readily and the water-soluble acids produced by polyester hydrolysis could diffuse out more easily. The result confirmed that except for the aggregation caused by nonspecific adsorption, the acidic microenvironment inside the microspheres also led to rhGH hydrolysis and aggregation. The combined QCM-D, AFM, and CLSM analysis strongly indicated that the hydrophilic characteristic of PELA significantly affected the stability of encapsulated rhGH during release. Increases in polymer hydrophilicity thus seem to improve the stability of encapsulated rhGH greatly.

4. CONCLUSIONS

The microcosmic mechanisms for protein incomplete release and stability profiles from amphiphilic copolymer PELA with various hydrophilic properties have been investigated in detail. The results indicate that when the ratio of hydrophilic mPEG to PLA increased, the total release of rhGH increased from 20.1 to 79.8% within 65 days. It is clear that increasing the PELA hydrophilicity can modulate the protein release behavior. The incomplete release of rhGH can be attributed at least partially to the adsorption of rhGH onto the microsphere matrix. A Combination of contact angle with QCM-D and AFM techniques can be used to elucidate the mechanism on a microcosmic level. Additionally, increasing the ratio of mPEG to PLA can greatly improve the stability of rhGH during the preparation and/or release process. The improved stability can be attributed to several factors: a reduction in the adsorption of rhGH on the matrix, the minimization of rhGH aggregation, and a friendlier microenvironment. The pH difference in the microenvironments among the three types of polymers is due to differences in the hydrophilicity, hydrolytic rate, water diffusion in and out of the matrix, and the relative amount of acids generated within the matrix. Thus, rhGH encapsulated in the most hydrophilic PELA-1 microspheres experienced a less-acidic microenvironment as shown by CLSM. Overall, these results suggest that the PELA hydrophilicity played an important role in protein incomplete release and stability, thus offering an alternative to adjust these key parameters in the preparation of successful sustained protein formulations. This work also demonstrates the utility of several novel methods in the investigation of potential interactions between biopharmaceuticals and macromolecules on a microcosmic level.

■ ASSOCIATED CONTENT

Supporting Information

Particle size distribution of PELA microcapsules with different molecular weights. Ratios of fluorescent intensity I_{640}/I_{580} of different microspheres during incubation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Pfizer Inc. for kindly providing the material, and we are grateful for the financial support of the National Natural Science Foundation of China (20820102036) and the 973 project (2009CB724700).

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