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PAPER

mPEG-PLA microspheres with narrow size distribution increase the controlled release effect of recombinant human growth hormone†

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The overall goal of this research is to prepare amphiphilic poly(monomethoxypolyethylene glycol-*co*-D,L-lactide) (mPEG-PLA, PELA) microspheres with narrow size distribution for sustained release of recombinant human growth hormone (rhGH) without any exogenous stabilizing excipients. In order to obtain microspheres with narrow size distribution, we used a double emulsion method combined with a premix membrane emulsification technique. The morphology, internal structure, rhGH encapsulation efficiency, *in vitro* release and rhGH stability of PELA microspheres were characterized in detail. The results were compared with those of rhGH encapsulated in poly(D,L-lactic acid) (PLA) and poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres. All microspheres possessed a narrow size distribution and had mean diameters of 2 μm. The encapsulation efficiency of PELA, PLA, and PLGA microspheres were 89.3%, 65.0% and 58.6%, respectively. PELA microspheres showed an initial burst release of 14.2% rhGH, followed by a constant release of 78.3% over a 45-day period. Whereas, the PLA and PLGA microspheres showed higher burst levels of 22.3% and 26.2%, followed by release of 49.1% and 60.5%, respectively. In addition, the PELA microspheres maintained much better integrity of rhGH than PLA and PLGA microspheres. These results suggested that PELA is an excellent polymer for encapsulating rhGH. We propose that the mPEG-PLA block copolymer acts in a similar way to a surfactant and that it may orientate itself on the biphasic interface, thereby minimizing the contact of protein with the oil/water interface for protein stabilization. Furthermore, the microcosmic mechanisms responsible for rhGH encapsulation and rhGH stability were elucidated using a confocal laser scanning microscope. These results are promising for the construction of a sustained release system for rhGH using PELA microspheres.

1. Introduction

Human growth hormone (HGH), a single polypeptide chain of 191 amino acid residues with a molecular mass of 22 kDa, is a somatotropic hormone secreted from the anterior pituitary gland.[1] Since the introduction of recombinant human growth hormone (rhGH) in 1985, it has been used in children to treat growth retardation and applied in growth hormone replacement therapy for adults.[2,3] Unfortunately, rhGH therapy still suffers the burden of daily injection, which leads to poor patient compliance.[4] In this respect, development of a sustained-release rhGH formulation over a period of 1 week to 1 month would be a significant improvement of the current daily treatment.

Microspheres have been considered as being amongst the most promising delivery systems, because they not only prolong the half-life of a drug, but also potentially favor an increase in the bioavailability of a drug by controlling the release rate of the drug from the matrix.[5–9] Currently, the best known rhGH sustained delivery systems available have been designed for monthly and bi-weekly treatment of growth hormone deficient patients employing poly(D,L-lactic-*co*-glycolic acid) (PLGA) and poly (D,L-lactic acid) (PLA) as polymer materials.[10,11] Albeit promising, the microsphere-related research area was still in its infancy when these polymer materials were developed. The most common problem of the rhGH-PLGA/PLA formulation was the high initial burst with little further release.[12] This problem mostly occurred in *in vitro* release and has been attributed to preferential location of rhGH within or near the surface of microspheres and nonspecific adsorption onto the hydrophobic matrix, respectively. Furthermore, the double emulsion method used for the rhGH encapsulation seemed to provide unsatisfactory rhGH profiles with insufficient rhGH activity after release.[12,13] Because

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an aqueous protein solution was apt to be dispersed into an organic polymer solution during water/oil (W/O) emulsification, using an homogenizer or sonicator, the exposure of protein to the W/O interface might affect stability.[14] In addition, the hydrophobic nature of the PLGA and PLA matrix and acidification arising from degradation of the polymer matrix can easily lead to protein aggregation and inactivation, and these aggregates may result in a serious immunogenic response.[15,16] Therefore, it is highly demanding but challenging to develop new formulation strategies to stabilize the protein during microencapsulation, storage, and release under physiological conditions.

Poly(ethylene glycol) (PEG), has been considered to be one of the most promising polymers to prevent protein adsorbing to hydrophobic matrices and improve biocompatibility with a blood contact compound.[17,18] Compared with PLA and PLGA, it was found that the diblock copolymer poly(monomethoxypoly ethylene glycol-*co*-D,L- lactide) (mPEG-PLA, PELA) yielded a more stable interfacial layer at the oil and water interface, and thus was more suitable for minimizing the contact of protein with the O/W interface.[19,20] Besides, this stable interfacial layer can inhibit coalescence of inner droplets and external water phase when the microencapsulation was prepared from W/O/W emulsions. Consequently, microspheres synthesized by PELA would self-stabilize the protein. Moreover, inserting hydrophilic PEG into a relevantly hydrophobic PLA block can promote the water uptake and swelling of the microsphere matrix, and modulate diffusion of acidic degradation products from the carrier system. Therefore the acidic microenvironment would be minimized, which could reduce the possibility of aggregation of the encapsulated protein during encapsulation and release.[19–21]

The particle size of microspheres is also a crucial parameter in drug release. Uniformity of microspheres can correlate highly with process reproducibility, high bioavailability, and repeatable release behavior. However, conventional processes for preparing double emulsions have usually involved mechanical stirring, homogenization or sonication techniques.[22] In these cases, the size distributions of microspheres obtained were very broad and the particle sizes were difficult to control. Although selective centrifugation has been considered for obtaining microspheres with a narrow size distribution,[23] this process was tedious and inefficient, and expensive protein drugs could be wasted. Here, a double emulsion method combined with a premix membrane emulsification technique was used to prepare rhGH-PELA microspheres with a narrow size distribution. The primary emulsions (W/O) prepared by homogenization were emulsified into an external aqueous phase by magnetic stirring to prepare large size W/O/W double emulsions. The coarse double emulsions were further extruded through a Shirasu Porous Glass (SPG) membrane under a relatively high pressure to form uniform-sized emulsions. Finally, the microspheres with a narrow size distribution were obtained by solvent extraction.

In all, the objective of this study was to explore a new rhGH microencapsulation formulation using PELA as a polymer material to self-stabilize entrapped protein drug and improve drug release behavior. For comparison, we also prepared PLA and PLGA microspheres loaded with rhGH. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to compare the exterior and interior structures of the microspheres. *In vitro* rhGH release profiles of the three microspheres were also investigated and explained in detail. Structural integrity and conformation of released rhGH species were characterized by size exclusion chromatography (SEC), native-PAGE and circular dichrosim (CD) spectroscopy, respectively. We further elucidated the microcosmic mechanisms responsible for rhGH encapsulation and rhGH stability using CLSM.

2. Experimental section

2.1 Materials

PLA and PLGA with a molecular weight Mw ~20 000 Da were purchased from the Institute of Medical Instruments (Shandong, China). PELA with a molecular weight Mw ~ 20 000 Da was supplied by the Dai Gang Company (Shandong, China), in which the mPEG block has a molecular weight of 2000 Da. Poly (vinyl alcohol) (PVA-217, degree of polymerization 1700, degree of hydrolysis 88.5%) was provided by Kuraray (Japan). The fluorescent dye Cyanine-5 (Cy5), fluorescent pH sensitive dye SNARF-1® dextran (MW = 10 kDa) were from Molecular Probes (USA). rhGH (Mw ~22 kDa) was kindly supplied by Pfizer (USA). SPG membrane (pore size of the membrane was 5.2 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 a two-step procedure.[24] Briefly, the coarse double emulsions were prepared at first, 0.4 mL rhGH aqueous solution (32 mg mL^{-1}) was mixed with 4 mL ethyl acetate containing PELA (200 mg) by homogenizing for 30 s in an ice bath to form a primary emulsion. The W/O 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 coarse double emulsions. The coarse double emulsions were then poured into a premix reservoir. Secondly, double emulsions with smaller and relatively uniform size were achieved by extruding the coarse double emulsions through the SPG membrane under a high pressure of 110 kPa. The obtained uniform double emulsions were poured quickly into 800 mL of a solution containing 0.9% w/v NaCl (solidification solution) under magnetic stirring for 5 min to solidify the microspheres. The obtained microspheres were collected by centrifugation at 5000 rpm for 10 min and washed with distilled water three times and then lyophilized for 2 days.

As control, rhGH was loaded into PLA and PLGA microspheres using the same methods above.

2.3 Characterization of microspheres

The surface morphology of the microspheres was observed using a JSM-6700F (JEOL, Japan) scanning electron microscope. The volume-mean diameter of the microspheres was measured by laser diffraction using a Mastersizer 2000 (Malvern, UK). Microspheres after being solidified, collected and washed were dispersed in distilled water and analyzed using the Mastersizer 2000. The uniformity of the microspheres was expressed as a span value, the size distribution of the microspheres became narrower the lower the value of the span.

2.4 Measurement of protein encapsulation efficiency

The total encapsulation efficiency of rhGH in PELA, PLA, and PLGA microspheres were 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 by 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 by the following equation:

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

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

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

2.5 Confocal laser scanning microscopy (CLSM) imaging of microspheres

Cy5 was used as a fluorochrome to label rhGH for observing the distribution of rhGH within the microspheres by CLSM. The process for covalently attaching Cy5 to rhGH is as follows: a solution of Cy5 (5 mg mL^{-1}) was slowly added into rhGH solution. Then, 2 mL of a 4 mg 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) solution were added to the above suspension and mixed. After an incubation period of 12 h at 4 °C with magnetic stirring, unbound Cy5 was separated by ultrafiltration (MilliporeUFC901024). The Cy5-rhGH loaded microspheres were added into phosphate buffered saline (PBS) to form a suspension. In order to observe the process of inner droplets escaping to external water phase *in situ*, the Cy5-rhGH loaded emulsions after being pressed through an SPG membrane were added into a Petri dish immediately and then observed by CLSM. For further observing the interior structure of PELA microspheres, the PELA was dissolved in ethyl acetate with Nile Red and then the material was used to prepare microspheres. The suspensions containing microspheres in the Petri dish were observed by a TCS SP2 CLSM (Leica) and the transmitted image was taken.

2.6 *In vitro* rhGH release measurement

To examine protein release profiles, 20 mg of rhGH loaded PELA microspheres were suspended in 1 mL 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 shook at 110 rpm, 37 °C. rhGH released in the medium from microspheres was separated by centrifugation at 2000 rpm for 5 min. At pre-determined time intervals, the release medium was replaced by the same amount of fresh buffer each day. The release medium was used to determine protein concentration using Micro-BCA method.

2.7 Size exclusion chromatography (SEC)-HPLC and native-PAGE analysis

The amount of aggregates in the released rhGH in the medium was quantified by SEC. The released rhGH samples from the microspheres were analyzed using an aqueous size exclusion column (Amersham Superdex 75 series column (SEC, 10 mm ID × 300 mm L, GE, Co., USA)): 0.5 mL min^{-1} of 100 mM phosphate buffer with 100 mM of NaCl was used as a mobile phase, with absorption at 280 nm for eluted sample detection. The ratio of the monomeric and dimeric rhGH was the area percentage of the peak representing the monomeric and dimeric rhGH as measured by SEC-HPLC. The calculation formula was:

$$\text{Ratio of monomeric and dimeric rhGH (\%)} = \frac{A_\mathrm{m} + A_\mathrm{d}}{A_\mathrm{m} + A_\mathrm{d} + A_\mathrm{a} + A_\mathrm{f}} \times 100\%$$

where A_m, A_d, A_a and A_f are the peak areas of the monomeric, dimeric, aggregate and fragmentary rhGH, respectively.

Native-PAGE was performed using a Bio-Rad Mini-Protean II apparatus (Bio-rad, USA) to investigate the aggregation and hydrolysis behaviors of rhGH. After centrifuging samples (2000 rpm, 4 °C, 5 min), 80 μl of each sample was mixed with 20 μl of the non-reducing buffer (Tris-HCl (pH 6.8, 0.05 M), glycerol (3 mM), bromophenol blue (0.01% w/v)). Gels were prepared discontinuously with stacking and running gel of 4% and 10% polyacrylamide (acrylamide : methylene acrylamide = 29 : 1), respectively. Electrophoresis was carried out at a constant voltage mode at 80 V for stacking and at 120 V for running using a Bio-Rad power supply in Tris/glycine buffer. After running the gels, they were stained using silver staining.

2.8 Circular dichroism (CD) analysis

In order to see whether a conformational change of released rhGH occurred, far CD spectra were taken at 25 °C using a Jasco 810 spectropolarimeter (Jasco, Japan) equipped with a temperature control unit. For far-UV CD spectra, 5 μM rhGH samples were loaded into a 0.1 cm path-length quartz cell. Far-UV CD spectra were obtained by averaging five scans in the 250–200 nm wavelength range.

2.9 Confocal imaging of microclimate pH inside microspheres

A quantitative ratiometric method based on laser scanning confocal microscopic imaging was employed to monitor the microclimate pH change inside the microspheres prepared using different polymers. Microspheres were prepared by a double emulsion and membrane emulsification technique. In brief, 0.4 mL of 32 mg mL^{-1} rhGH with 3–4 mg mL^{-1} SNARF-1 dextran (fluorescent pH-sensitive dye) in PBS solution were added to 4 mL ethyl acetate containing PELA, PLA and PLGA (200 mg), respectively, followed by homogenization for 30 s in an ice bath to form a primary emulsion. The other procedures were the same as detailed in section 2.2. Ten milligrams of the microspheres were suspended in 1 mL of PBS buffer (pH = 7.2)

under mild agitation at 37 °C. At predetermined times, the release media was removed after mild centrifugation (3 min, at 2000 rpm) and fresh buffer was added to maintain sink conditions. At the same time, a small amount of microspheres was removed to obtain confocal images for 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 ratio image I_{640}/I_{580} was correlated with pH.

3. Results and discussion

3.1 Characterization of microspheres

Uniform-sized PELA microspheres were successfully prepared by a double emulsion method followed by a premix membrane emulsification technique. For comparison, we also used a similar process to prepare PLA and PLGA microspheres. As shown in Fig. 1, the three types of microspheres all possessed a smooth surface and regular sphericity. PELA, PLA and PLGA microspheres had mean diameters of 2.07, 2.66 and 2.51 μm, respectively, and span values of 0.708, 0.863 and 0.776, respectively. Notably, they exhibited narrow size distributions around 2 μm, which were promising for good reproducibility and repeatable release behaviour.

3.2 rhGH encapsulation efficiency

High encapsulation efficiency has been considered to be an important requirement for the successful fabrication of microspheres containing protein. The encapsulation efficiency of rhGH in the microspheres was affected greatly by the polymer type. As shown in Fig. 2, the encapsulation efficiencies of PLA and PLGA microspheres were 65.0%, and 58.6%, respectively. The loading efficiencies of rhGH in PELA, PLA and PLGA microspheres were 5.72%, 4.16% and 3.75%, respectively. Once using PELA as material, we found the encapsulation efficiency significantly increased to 89.3%. A possible explanation for such a distinguishable difference in encapsulation efficiency was that PELA might have generated a more 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 led to the loss of encapsulated protein during the fabrication process.

Furthermore, in order to confirm the above conclusion, we observed, by CLSM, the distribution of rhGH within the microspheres and the dynamic process of rhGH loss during the fabrication of the microspheres . As shown in Fig. 3a, the rhGH labeled by Cy5 were effectively encapsulated and uniformly

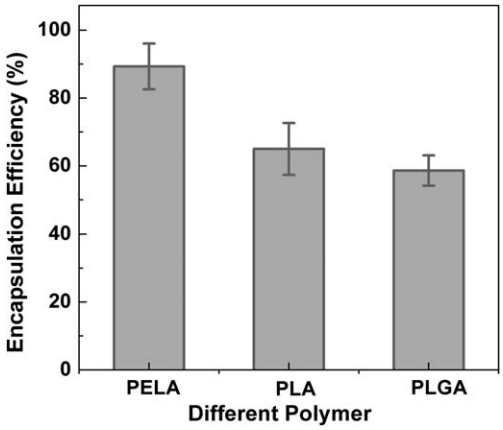

Fig. 2 Effect of polymer composition on encapsulation efficiency of rhGH. Results are expressed as mean ± SD ($n = 3$). Statistical significant differences ($p < 0.05$) exist between all the series.

dispersed in the whole microspheres. This is because when PELA was employed to prepare the double emulsion, the hydrophilic mPEG sequences extended into the water phase whereas the hydrophobic PLA sequences spread into the oil phase. The polymer itself forms an interfacial layer, thereby stabilizing the primary emulsion. The internal structure of the PELA microspheres, the polymer being labeled with Nile Red, is displayed in Fig. 3d, e. The PELA microspheres are shown to have a porous honeycomb-like interior due to the stable primary emulsion, which is beneficial for increasing encapsulation efficiency.

In contrast, the rhGH was distributed in a small portion of the PLA microspheres and the Rose Bengal color of the external water phase indicated the large amount of rhGH (labeled by Cy5) that had escaped outside (Fig. 3b). For verification, we observed the movement of rhGH-containing droplets in the PLA capsules *in situ* by CLSM. It showed that inner droplets in the PLA microspheres had moved to the O/W interface, and were separated from the external water phase by a thin oil film. When the oil film was evacuated, the inner droplets then merged with the external water phase rapidly and dissolved. The unstable primary emulsion led to the loss of rhGH and low encapsulation efficiency. Similar phenomena occurred in the PLGA microspheres (Fig. 3c).

3.3 *In vitro* release profiles of rhGH

The ideal release profile should consist of a low initial burst release followed by a sustained release phase. The extent of burst release depended on the polymer composition and other factors.

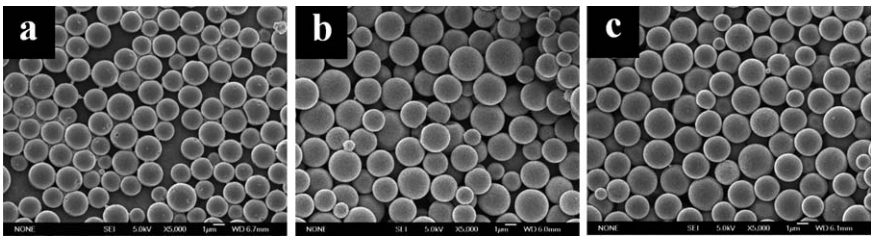

Fig. 1 SEM images of microspheres prepared using different polymers: (a) PELA, (b) PLA and (c) PLGA.

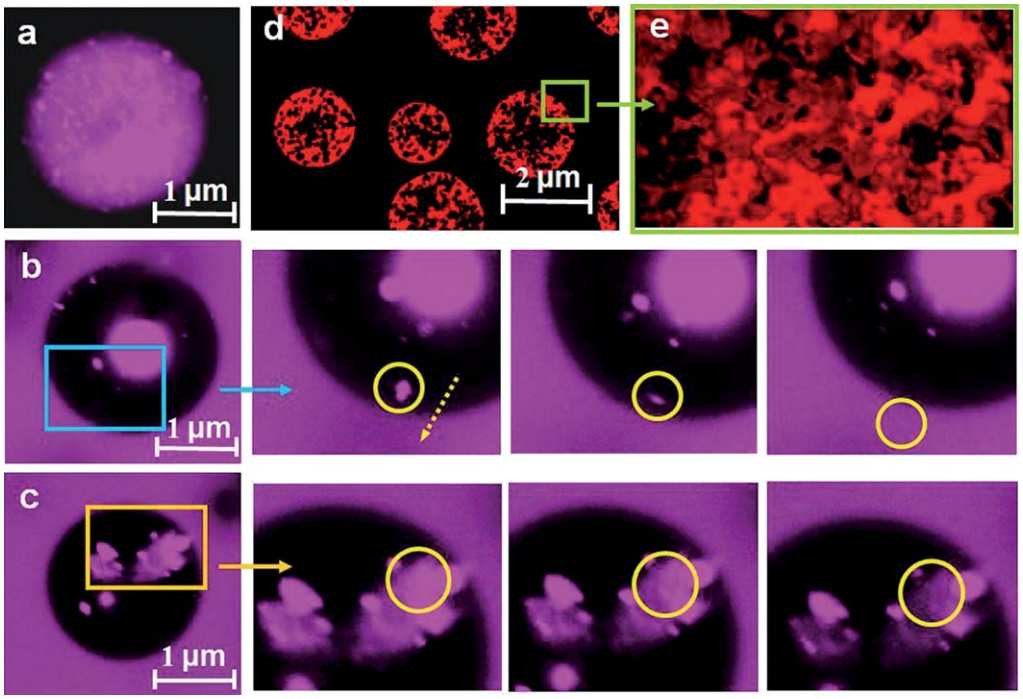

Fig. 3 CLSM images of (a) PELA, (b) PLA, (c) PLGA microspheres loaded with rhGH labeled by Cy5 (Rose Bengal color) showing the merging of the inner droplets into the external water phase in PLA and PLGA microspheres, (d) PELA matrix labeled by Nile Red (red color) indicating the interior structure of the microspheres, (e) three-dimensional structure of PELA microspheres.

As shown in Fig. 4, PLA and PLGA microspheres showed a burst release of 22.3% and 26.2%, respectively, over the first 5 h, in comparison to 14.2% for PELA microspheres. PELA microspheres showed a total of 78.3% rhGH release within 45 days, whereas PLA and PLGA microspheres produced about 49.1% and 60.5% rhGH release, respectively.

Drug release from the microspheres is influenced mainly by two principal mechanisms: (1) diffusion of the drug from the surface of the microspheres during the initial release phase, and (2) release of the drug upon the erosion of the polymer matrix during the sustained release phase. The microspheres prepared by a conventional W/O/W process often showed a high initial burst release followed by multiphasic slow release.[25] The strong burst effect 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, which were formed by coalescence between the inner droplets and external water phase during the microspheres solidification process.[26–28] PELA microspheres showed a lower initial release because the polymer itself may 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. The release rate of protein during the second phase was principally correlated to the erosion of polymeric matrix by hydrolysis in water. During this phase, protein release depended to a large extent on the properties of the polymer. The addition of hydrophilic sequences into the hydrophobic PLA can enhance water penetrability and consequently change the degradation rate of the polymer. Meanwhile, we monitored the surface morphology of these three types of microspheres with time (Fig. 5). Although at 20 days, the difference of the sphere surface between the three microspheres was not very significant, we could observe a distinct diversity of the sphere surface after 40 days. At first glance, PLA and PLGA microcapsules seemed to degrade faster than PELA microcapsules after 40 days in solution, which was expected to generate more rhGH release. However, it was found that PLA and PLGA microspheres release much less rhGH (49.1% and 60.5%) than PELA microspheres (78.3%). A possible explanation was nonspecific adsorption with high affinity, leading to incomplete release. It was reported that incomplete release was frequently encountered for many proteins entrapped in PLA or PLGA microspheres, this was principally due to nonspecific protein adsorption occurring within the microspheres.[29,30] Increased release rate from PELA microspheres could be a result of the mPEG sequences preventing contact of the protein with

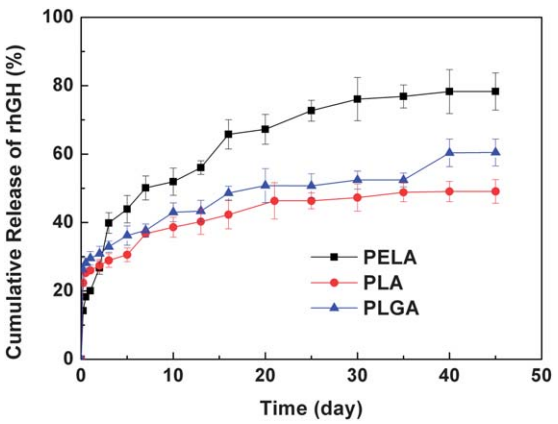

Fig. 4 Cumulative release of rhGH from the three microspheres.

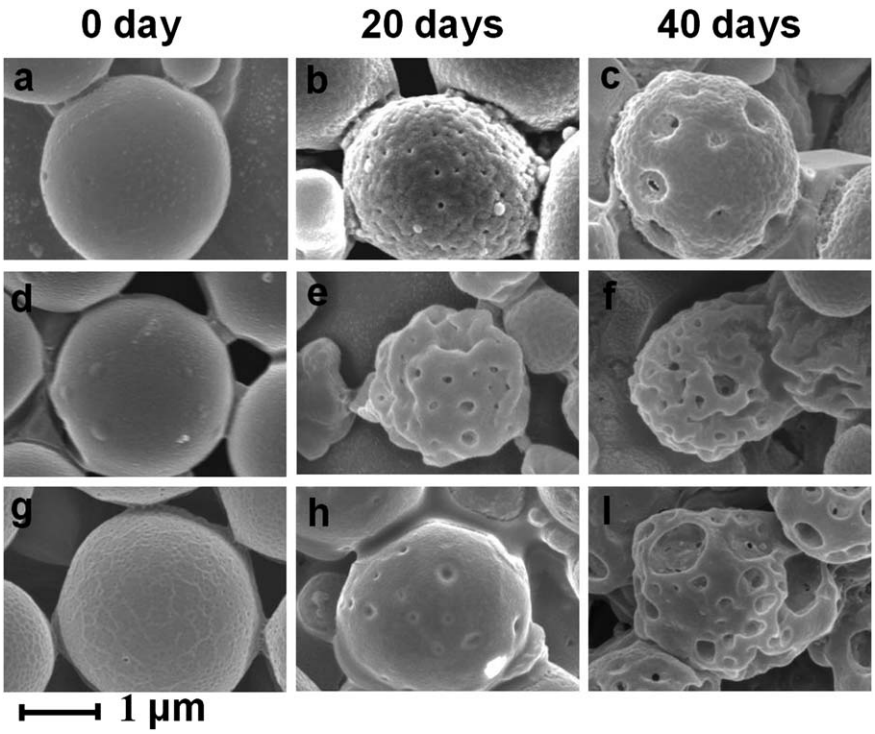

Fig. 5 SEM micrographs of rhGH-loaded (a, b, c) PELA, (d, e, f) PLA, and (g, h, i) PLGA microspheres at different release times.

the hydrophobic matrix. As a result, the PELA microspheres with a minimal initial release were more favorable than PLA and PLGA microspheres for rhGH delivery

3.4 Stability of rhGH released from microspheres

Another task pharmaceutic researchers have to face is how to maintain stability of protein during drug delivery. We next asked whether our system can maintain the stability of rhGH. Aggregation is often the main degradation pathway, resulting in loss of bioactivity.[31] Thus, SEC-HPLC was used to characterize the integrity of rhGH. As shown in Fig. 6a, the rhGH released from PELA basically maintained its monomeric and dimeric form ranging from 90% to 100%. However, PLA and PLGA microspheres released the monomeric and dimeric rhGH floating around 70% and 80%, respectively. The result indicated that the aggregates released from PLA and PLGA microspheres increased more quickly during the release, compared with that of PELA microspheres. This was because mPEG-PLA block copolymer possessed a function similar to surfactants which had an intrinsic characteristic of oriented localization on the biphasic interface. The function can effectively minimize the contact of protein with oil/water interfaces and decreases the protein aggregates. As shown in Fig. 6b, the majority of rhGH released from the PELA microspheres maintained its monomeric form, while a large part of the rhGH released from the PLA and PLGA microspheres were aggregates. In order to confirm the structural integrity of the rhGH released from the different microspheres, Native-PAGE measurement was employed. It can be seen from Fig. 7 that the native rhGH and rhGH released from PELA microspheres showed one and the same peak of molecular weight 22 KDa. It was indicated that nonmolecular hydrolysis and covalent aggregation occurred during the preparation and release process. However, at day 20 and day 40, protein bands appeared at the position of much higher molecular weight for PLA and PLGA microspheres, which suggested that the fraction of aggregation rhGH formed. In addition, rhGH released from PLA and PLGA microspheres both exhibited trace hydrolyzed fragments at the position of lower molecular weight. The encapsulated rhGH was severely hydrolyzed due to an acidic microenvironment generated from polymer degradation. These results agreed well with the data listed in Fig. 6a, confirming that the state of rhGH in the PELA microspheres maintained monomer rhGH well.

Further physical characterization of the released rhGH was carried out to assess the protein's secondary structure. CD spectra of the control rhGH and released rhGH from PELA microspheres during the release period are shown in Fig. 8. The spectra were essentially identical, with minima at 221 nm and 210 nm and a maximum at 191 nm, which indicated that the protein had a high α-helical content. All the released rhGH samples maintained their structural integrity as judged from the shape of the far-UV CD spectra.

In addition, the generation of an acidic microenvironment within PLA and PLGA microspheres due to accumulation of acidic degradation products is known to induce the aggregation and hydrolyzed fragments of encapsulated protein molecules.[12,32,33] Herein, we used dextran-SNARF-1® conjugate, a long-wavelength fluorescent pH indicator, to monitor the microclimate pH change.[34] The emission spectrum of SNARF-1 dextran undergoes a pH-dependent wavelength shift, thus allowing the ratio of the fluorescence intensities from the dye at two emission wavelengths to be used for more accurate determinations of pH. When the strongest fluorescence emission of

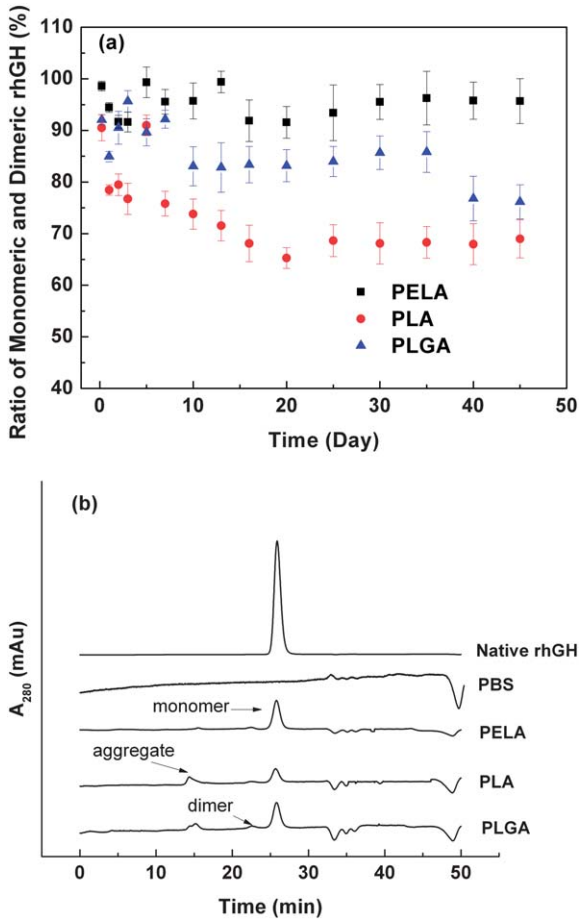

Fig. 6 (a) Release ratio of monomeric and dimeric rhGH from the three microspheres. (b) Size exclusion chromatography (SEC-HPLC) of rhGH after recovery from PELA, PLA, and PLGA microspheres on the 21st day.

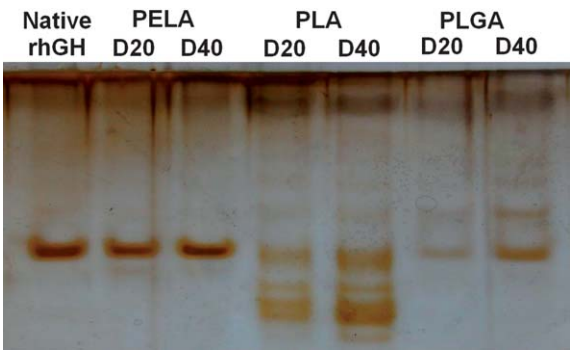

Fig. 7 Native-PAGE analysis of released rhGH from PELA, PLA and PLGA microspheres incubated for different time periods (D20: 20 days; D40: 40 days).

dye was near 580 nm, the dye showed red light and the microclimate pH was closed to 6.0. While the strongest fluorescence emission was near 640 nm, the dye showed green light and the microclimate pH was close to 9.0.[35] The dye exhibited was yellow, indicating that the microclimate pH was close to neutral pH range. As shown in Fig. $9a_1$, at the early stage (7 days after

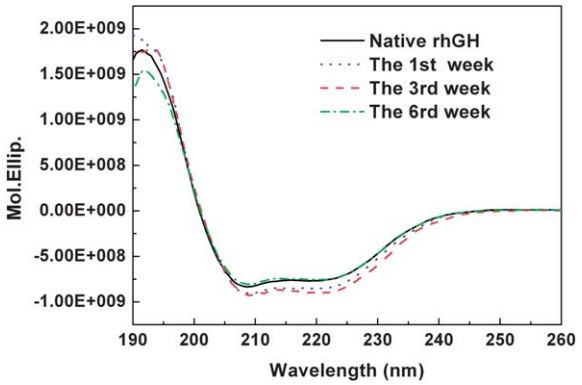

Fig. 8 CD spectra of rhGH released from PELA microspheres at different times.

incubation), the PELA microspheres were mostly yellow, while a small part in PLA and PLGA microspheres became an orange color (Fig. $9b_1$, c_1, arrows indicated). This suggests that the microenvironment of PLA and PLGA microspheres were more acidic than that of the PELA microspheres. With increasing incubation time, the PLA and PLGA microspheres became steadily more acidic. It can be seen that after incubation for 14 days, more and more PLA microspheres became an orange color, and a similar phenomena occurred in the PLGA microspheres. In contrast, PELA microspheres still remained yellow. After 21 days, all the PLA and PLGA microspheres became orange or even red in color while the PELA microspheres remained a yellow color, which implied that the microclimate pH in PLA and PLGA microspheres were acidic (pH 6.0). While PELA microspheres still kept a neutral microenvironment. By calculating the ratio of the fluorescence intensities measured at two wavelengths (I_{640}/I_{580}), we can directly compare the microclimate pH change of different microspheres. As shown in Table 2 (†in ESI), the values of I_{640}/I_{580} in PELA microspheres were all higher than those in the other two microspheres during the release process. The results suggest that the rhGH encapsulated in the PELA microspheres were in a less acidic microenvironment. The results correspond well to those of Fig. 6a, PELA microspheres released mostly the monomeric and dimeric rhGH, implying that less aggregates and hydrolysis of rhGH were formed. This is because the water-soluble acids generated by the polyester hydrolysis were easy to diffuse out by the swelling structure of the microspheres, due to the hydration of the hydrophilic mPEG sequences in the releasing medium. On the other hand, the strongest fluorescence emission of PLA and PLGA microspheres occured at 580 nm after 21 days, indicating that they were under the detection limit at pH 6.0, thus we didn't observe the microclimate pH when a pH-sensitive dye was used after 21 days. However, the microclimate pH change trend in different microspheres has been elucidated clearly by comparing their color during the drug release.

Combined with the SEC-HPLC, native-PAGE, CD and CLSM results, it can be concluded that the structure and conformation of rhGH were not adversely affected by PELA microspheres, which concluded that the integrity of the protein's structure was maintained well during the encapsulation and release.

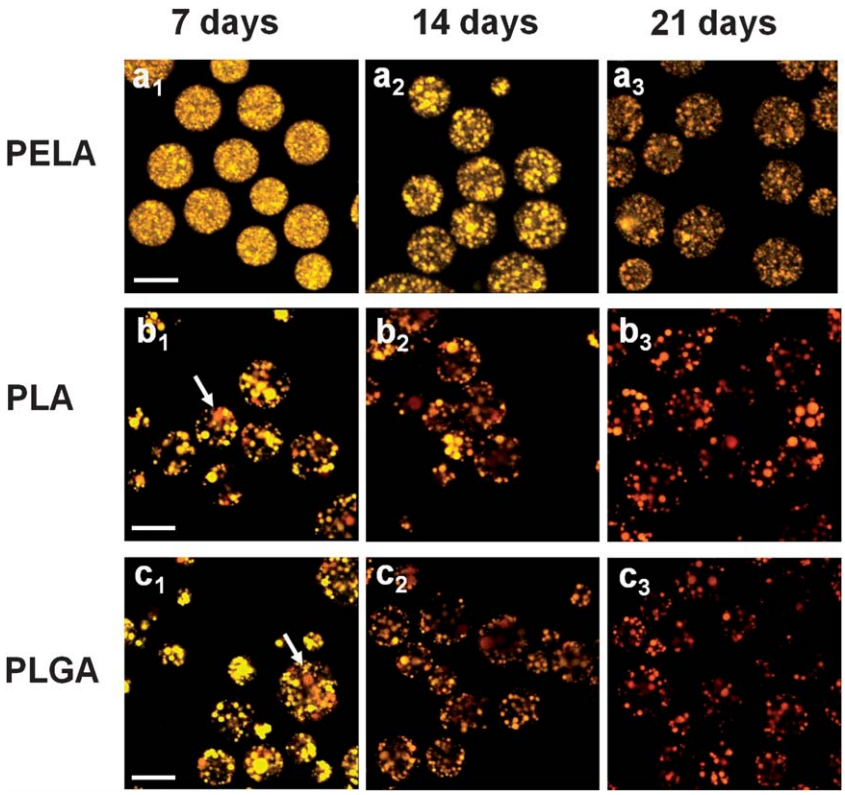

Fig. 9 CLSM images of pH-sensitive dye loaded PELA (a1, a2, a3), PLA (b1, b2, b3), and PLGA (c1, c2, c3) microspheres at different incubation times (7 days, 14 days and 21 days), scale bar: 2 μm.

4. Conclusion

rhGH-PELA microspheres with narrow size distribution have been successfully prepared by combining a double emulsion method with a premix membrane emulsification technique. Compared with PLA and PLGA microspheres, PELA microspheres exhibited advantages of high encapsulation efficiency, low initial burst, sustained release, and stability of rhGH. All these results strongly suggest that PELA would be a more suitable candidate for preparation of a sustained rhGH delivery system.

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