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Controlled delivery of paracetamol and protein at different stages from core–shell biodegradable microspheres

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ABSTRACT

The core–shell biodegradable microspheres loading human serum albumin (HSA) and paracetamol were fabricated with a hydrophilic alginic acid (ALG) shell and a hydrophobic poly(lactic-*co*-glycolic acid) (PLGA) core. The two model drugs, HSA and paracetamol, were entrapped in the shell and core, respectively. Theses microspheres were characterized in terms of morphology, mean size and size distribution, drug loading efficiency, in vitro degradation and drugs release. The optical microscopy (OM) and transmission electron microscope (TEM) photos revealed directly that the microspheres possessed core–shell structure. The degradation results showed that there were quick drop phases in weight of polymer matrix and in pH of degradation medium after 2 weeks. In vitro release profiles showed that within the initial 20 h, only HSA was almost released from the shell layers, whereas only another drug, paracetamol, was almost released from the cores in the following period.

1. Introduction

Due to the increasing amount of biopharmaceuticals, there is a growing need for advanced drug delivery systems (Langer & Tirrell, 2004). In the traditional drug delivery systems, single layer microspheres have several inherent problems, including the burst effect and difficulty in achieving zero-order release (Jalil & Nixon, 1990). And they also lack the ability to deliver more than one drug or protein in a sequential manner (Pekarek, Dyrud, Ferrer, Jong, & Mathiowitz, 1996). A double-walled microsphere consisting of a core of one polymer surrounded by a coating of the second polymer could work toward solving these problems. In recent years, there were several methods to prepare core–shell microspheres. The first method was to simply encapsulate a therapeutic agent in microspheres using a conventional microencapsulation technique and then to coat the microspheres with a second polymer (Haidar, Hamdy, & Tabrizian, 2008). This coating would reduce the burst effect since no protein or drug would be adsorbed on the surface. However, it involves at least two steps, which brings problems such as quality control and yield. The second method was to prepare core-coated nanoparticles by a self-assembly process from a biodegradable amphiphilic copolymer (Wang, Gao, Ye, Yoon, & Yang, 2006). Nowadays, its disadvantages are mainly to resolve the difficulties in stability of nanoparticles and mass producing. The third method was to create double-walled microspheres in a single step by using the phenomenon of polymer–polymer phase separation, by which the limitations of a single polymer microsphere and present coating technologies could be avoided (Gou et al., 2008; Pekarek, Jacob, & Mathiowitz, 1994a, 1994b).

Polyesters, such as poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA), have been used as medical resorbable sutures for about 40 years. They have also been used widely in the pharmaceutical and biomedical fields as delivery vehicles such as microspheres and nanoparticles and as tissue engineering scaffold as a consequence of their biodegradability and relative biocompatibility (Kulkarni, Moore, Hegyeli, & Leonard, 1971). Polymer properties such as molecular weight (Mw), copolymer composition and crystallinity can be tailored to alter polymer degradation and the consequent drug release profiles. For example, increase in polymer Mw results in longer degradation times and slower drug release (Park, 1994, 1995; Ramirez, Pastoriza, & Herrero-Vanrell, 1999).

Drug release from PLGA microspheres can range from days to months and some methods often involve change in the mechanism of drug release from erosion/diffusion control to diffusion control. For example, use of the polymer with hydrophobicity can lead to diffusion controlled kinetics. This change in the mechanism of release may result in lack of correlation between accelerated release profiles and in vivo release profiles. In particular, where multiple release phases (such as burst release, lag phase and secondary burst phase) occur, these different phases are often lost in accelerated release testing. Correlations have been established for the post burst release phase and these can be augmented by real-time tests

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for the burst phase (Burgess, Crommelin, Hussain, & Chen, 2004; Zolnik & Burgess, 2007; Zolnik, Leary, & Burgess, 2006).

Sodium alginate (SA) is a sodium salt of alginic acid, a naturally occurring non-toxic polysaccharide found in marine brown algae. Alginate has been widely used as food and pharmaceutical additives, such as a tablet disintegrant, a thickening and suspending agent (Kibbe, 2000). It contains two uronic acids, α-L-guluronic and β-D-mannuronic acids, and is composed of homopolymeric blocks and blocks with an alternating sequence (Draget, 2000). Gelation occurs by cross-linking of the uronic acids with divalent cations, such as calcium ion. The primary mechanism of this gelation involves extended chain sequences which adapt a regular twofold conformation and dimerize with specific chelation of calcium ion, namely the so-called 'egg-box' structure (Grant, Morris, Rees, Smith, & Thom, 1973). Each calcium ion takes part in nine co-ordination link with an oxygen atom, resulting in three-dimensional network of calcium alginate. This phenomenon has been applied for preparing an alginate bead (Bstberg, Lund, & Graffner, 1994; Sugawara, Imai, & Otagiri, 1994) and microparticles (Lemoine, Wauters, Bouchendhomme, & Preat, 1998; Li et al., 2008) employed as a delivery system of bioactive agents. The biomolecules could be assembled and entrapped within polyelectrolyte layers which maintaining their bioactivity (Hillberg & Tabrizia, 2006; Thierry, Winnik, Merhi, Silver, & Tabrizian, 2003).

In the paper, the biodegradable microspheres with core–shell structure were designed to alleviate burst effect of drug in the cores, realize zero-order release patterns, and in particular controlled deliver two kinds of drugs at different stages, which could resolve some problems of single layer microspheres. The strategy can be designed as Fig. 1. The two kinds of drugs can be released at different stages from core–shell microspheres by controlled degradation of microspheres matrix. Due to the complication of disease and advance of medical technology, disease cure is demanded to use different drugs in different period in order to obtain a more effective therapy. Therefore, the drug delivery system loaded only one drug or multiple drugs which were released simultaneously, could not meet their requirement. Here, the cores, namely PLGA microspheres, were prepared by solvent evaporation method, and the shells, calcium alginate layer was adsorbed on the surface of PLGA microspheres through a physical adsorption (Haidar et al., 2008). Human serum albumin (HSA) employed as macromolecule model drug, was efficiently entrapped within PLGA cores and subsequently microencapsulated into calcium alginate layer as well as paracetamol used as micromolecule model drug. The PLGA microspheres coated by calcium alginate layer (Ca-ALG/PLGA) were used to deliver the two kinds of drugs at different stages in a controlled manner.

2. Materials and methods

2.1. Materials

The copolymer poly(D,L-lactic-*co*-glycolic acid) (PLGA) with lactide/glycolide molar ratio of 75/25 was synthesized in our lab and the average molecular weight was 10,000 g mol^{-1} according to the results of gel permeation chromatograph (GPC). Polyvinyl alcohol (PVA, M_n of 130,000 g mol^{-1}, degree of hydrolysis 88) was purchased from Shanghai Petrochemical Industry Company (China). Human Serum Albumin (HSA) with the isoelectric point (IEP) of 4.7 was purchased from Aventis Behring Gmbh (Germany). Paracetamol was purchased from Kangquan pharmacy, Zhejiang, China. Sodium alginate and calcium chloride anhydrous were both purchased from Chengdu Kelong Chemical Reagent Company (China). All the chemicals used in this research were the analytical reagent grade from the commercial market without further purification.

2.2. Fabrications of paracetamol-loaded PLGA microspheres

PLGA microspheres were prepared by single emulsion method (O/W) based on solvent evaporation (Deng, Zhou, Li, Zhao, & Yuan, 2001). Firstly, 0.3 g PLGA was dissolved in 5 ml methylene chloride and mixed with some amount of paracetamol under stirring, formed the stable organic phase (O). Secondly, the resultant solution was added dropwise into 100 ml of PVA solution (W) and emulsified for 40 min using overhead stirrer from 500 to 1100 rpm. Thirdly, 50 ml of a 6% isopropanol solution was poured into the emulsion and stirred about 2 h in fuming cupboard. After the solvent evaporated completely, the microspheres were collected by centrifuge (Avanti™ J-30I, BECKMAN COULTER, USA) at 8000 rpm. The resultant microspheres were rinsed with distilled water and centrifuged three times, then lyophilized overnight and stored at 4 °C in a desiccator.

2.3. Preparation of HSA-loaded core–shell microspheres

The above paracetamol-loaded PLGA microspheres and HSA were simultaneously dispersed in sodium alginate solution under gentle stirring. The concentration of sodium alginate solution was varied from 0.5 to 2 wt.%. These PLGA microspheres adsorbing sodium alginate containing HSA were collected by centrifuge at speed of 8000, 10,000 and 12,000 rpm, respectively. The resultant microspheres were re-dispersed and added dropwise into 100 ml of calcium chloride solution (1.5 wt.%) by syringe (Pongjanyakul & Puttipipatkhachorn, 2007), and stirred for about 30 min. Finally, calcium alginate layer was formed by cross-linking sodium alginate with Ca^{2+} on the surface of PLGA microspheres (Haidar et al., 2008).

2.4. Drug encapsulation efficiency

For the determination of paracetamol encapsulation efficiency, 10 mg microspheres were dissolved in 1 ml dichloromethane (DCM) and kept at room temperature for about 30 min. After dissolution of microspheres, 10 ml phosphate-buffer saline (PBS) (pH 7.4) was added and the mixture was shaken vigorously for 2 min. The mixture was left to stand at room temperature for 1 h before the aqueous layer was drawn out. The aqueous solution was then filtered. Paracetamol content in the filtered solution was analyzed using an UV–visible spectrumphotometer (UV-2550, Shimadzu, Japan) at absorbance of 243 nm (Shi et al., 2003).

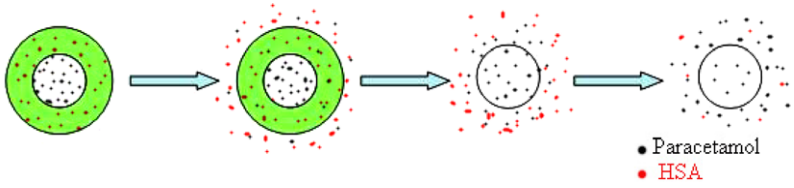

Fig. 1. Schematic representation of the drug release from the core–shell microspheres.

Table 1
The effect of agitating speed on properties of PLGA microspheres.

Stirring speed (rpm)	Particle size ± SD (μm)	Encapsulation efficiency of paracetamol (%)
500	12.2 ± 1.5	86.45 ± 2.23
700	9.0 ± 1.1	90.32 ± 2.67
900	8.91 ± 2.1	89.63 ± 2.12
1100	6.45 ± 1.3	67.79 ± 2.36

HSA content in the filtered solution was also analyzed. For the determination of HSA encapsulation efficiency, we measured its content in the sodium alginate solution without adsorbed on PLGA microspheres with an UV–visible method at absorbance of 278 nm. The encapsulation efficiency (EE) was calculated using Eq. (1) (Calvo, Remunan-lopez, Villa-Jato, & Alonso, 1997; Douglas & Tabrizian, 2005):

$$EE = \frac{drug_{total} - drug_{super}}{drug_{part}} \quad (1)$$

where $drug_{total}$ is the initial amount of drug; $drug_{super}$ is the amount of un-adsorbed drug.

2.5. Characterizations

Optical microscopy (OM) observation was performed with a DXM1200C (Nikon) to observe the core–shell structure. The samples of OM were prepared by dropping one or two solution droplets containing microspheres on a microscope slide and air-dried before measurement.

The surface morphology of the microspheres was examined by scanning electron microscope (SEM, QUANTA 200, FEI, USA). Several solution droplets containing microspheres were placed on the SEM sample stage. The samples were sputter coated with gold after lyophilized overnight.

To observe the core–shell structure within microspheres matrix, transmission electron microscopy observation was performed with a HITACHI H-700H (TEM, Japan) at the acceleration voltage of 150 kV. The samples of TEM were prepared by dropping the microspheres suspension on a carbon-coated copper grid and then air-dried before measurement.

The mean size and size distribution were determined by laser diffraction particle size analyzer (LA-9200, HORIBA, Japan). Microspheres were re-suspended in distilled water by ultrasonic to prevent aggregation. Then, the resultant microspheres emulsion was poured into the sample tank of particle size analyzer.

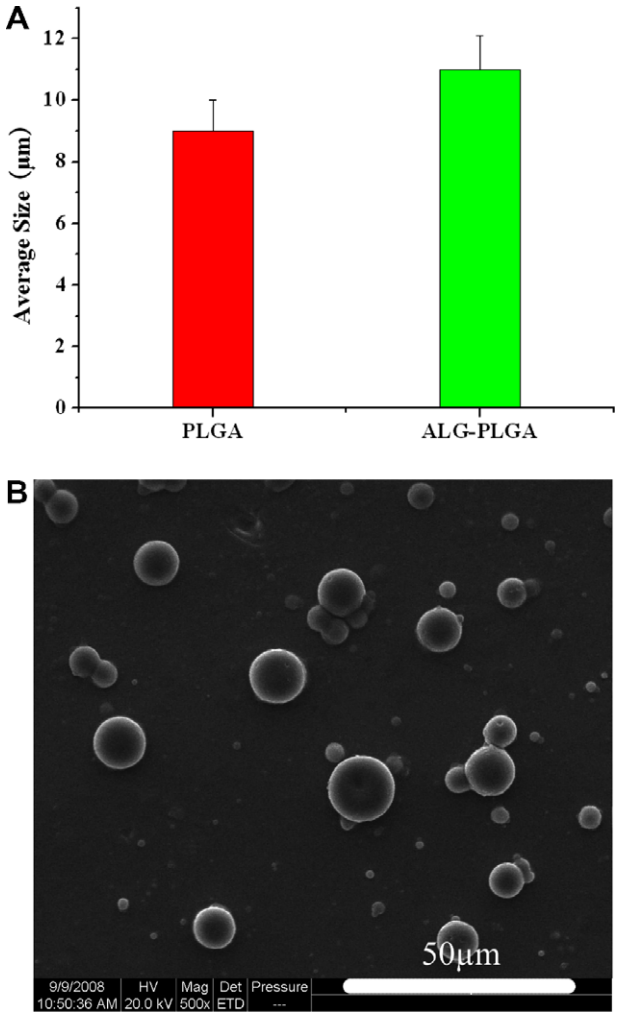

Fig. 2. (A) Average particle size change with the adsorption of each polyelectrolyte layer. The left value is for bare PLGA microspheres and the right value is for the ALG/PLGA microspheres. Values are reported as mean ± standard error. (B) The morphological of the core–shell microspheres detected by SEM, bars = 50 μm.

2.6. In vitro degradation

Pre-weighed microspheres were placed individually in test tubes containing 10 ml of 0.1 M PBS at pH 7.4. The tubes were kept in a thermostated incubator (Haerbin Dongming Medical Equipment Company, China) which was maintained at 37 °C and 100 cy-

Table 2
The optimization of experimental parameters to prepare core–shell microspheres.

Number	Studies variables[a]	Particle size ± SD (μm)	Encapsulation efficiency of HSA (%)	Dispersion homogeneity [b]
1	A0.5 B8000	9.1 ± 1.7	–	++
2	A0.5 B10000	9.7 ± 2.5	17.48	+
3	A0.5 B12000	9.2 ± 3.1	16.83	+
4	A1.0 B8000	10.6 ± 1.5	37.59	+
5	A1.0 B10000	9.7 ± 1.6	43.73	+
6	A1.0 B12000	10.4 ± 3.7	60.38	+
7	A1.5 B8000	10.7 ± 1.2	57.72	++
8	A1.5 B10000	11.0 ± 1.4	61.83	++
9	A1.5 B12000	11.6 ± 1.6	53.69	+
10	A2.0 B8000	11.8 ± 2.1	54.17	–
11	A2.0 B10000	12.0 ± 2.3	60.74	–
12	A2.0 B12000	12.6 ± 3.5	59.67	–

[a] A = ALG concentration (Wt.%); B = centrifuge speed.
[b] –, un-disperse; –, partial-disperse; +, disperse; ++, stable-disperse.

cles per minute. The degradation samples were washed by distilled water and collected at predetermined intervals. Then, the resultant samples were dried to constant weight. The degradation medium was collected to characterize the pH change during the degradation process. The morphological change of microspheres surfaces can be characterized by SEM. Mass loss was detected by comparing the weight remained with the initial weight. The pH change determined by detecting the supernate of degradation medium using pH meter (Shanghai Leici Instrument Company, China) at 25 °C.

2.7. In vitro drug release

Pre-weighed microspheres were suspended in test tubes containing PBS. The test tubes were placed in a thermostated incubator and continuously agitated with the same condition as the in vitro degradation test. At predetermined intervals, 1.0 ml of supernatant was collected and 1.0 ml of fresh PBS was added to the test tube. Concentration of HSA and paracetamol released was determined by using an UV–visible spectrumphotometer at absorbance of 278 nm and 243 nm, respectively.

3. Results and discussion

3.1. Drug encapsulation efficiency

In this research, single emulsion method was chosen to prepare PLGA microspheres. Paracetamol powder was directly dissolved in the oil phase (O) consisting of PLGA/methylene chloride (MC) solution. PVA aqueous solution was used as an outer continuous aqueous phase (W). The agitating speed of the dispersive process was a key factor to affect the morphology of microspheres. Therefore, we studied the impact of different agitating speeds on particle size and encapsulation efficiency of paracetamol, as shown in Table 1. It exhibited that with the increase of stirring speed, the particle size decreased gradually. However, in lower stirring speed, the higher encapsulation efficiency of drug could be achieved. And 90% of loading efficiency at the speed of 700 rpm was realized. The reason is that the higher agitating speed which provided the shearing force would destroy the spherical structure of microspheres, which led some drug encapsulated in microspheres matrix to diffusing into outer aqueous phase. Therefore, as circled in Table 1, the optimized fabrication parameters could be achieved: agitating speed of 700 rpm.

For alginate (ALG) layers adsorbed on the surface of PLGA microspheres by a physical interaction, it is very important to study the effects of alginate concentration and the centrifugal speed on the core–shell microspheres size and their dispersion homogeneity (Li & Zhu, 2003) and HSA encapsulation efficiency. As shown in Table 2, we could find that the microspheres size was only influenced by alginate concentration, and with the increase of its concentration, the size also increased. These data also indicated that the core–shell structure was formed. The centrifugal speed could influence the microspheres dispersion homogeneity and morphology. The microspheres would be irregular and conglutinate one another if the centrifugal speed was too low, and they could be destroyed at a higher speed. Both the HSA encapsulation efficiency and dispersion homogeneity of microspheres got to the best state at alginate concentration of 1.5% and centrifugal speed of 10,000 rpm. Whether the best state could be obtained or not depended to a considerable extent on the adsorbed strength and the viscosity of alginate. HSA-loading ALG could be adsorbed on the

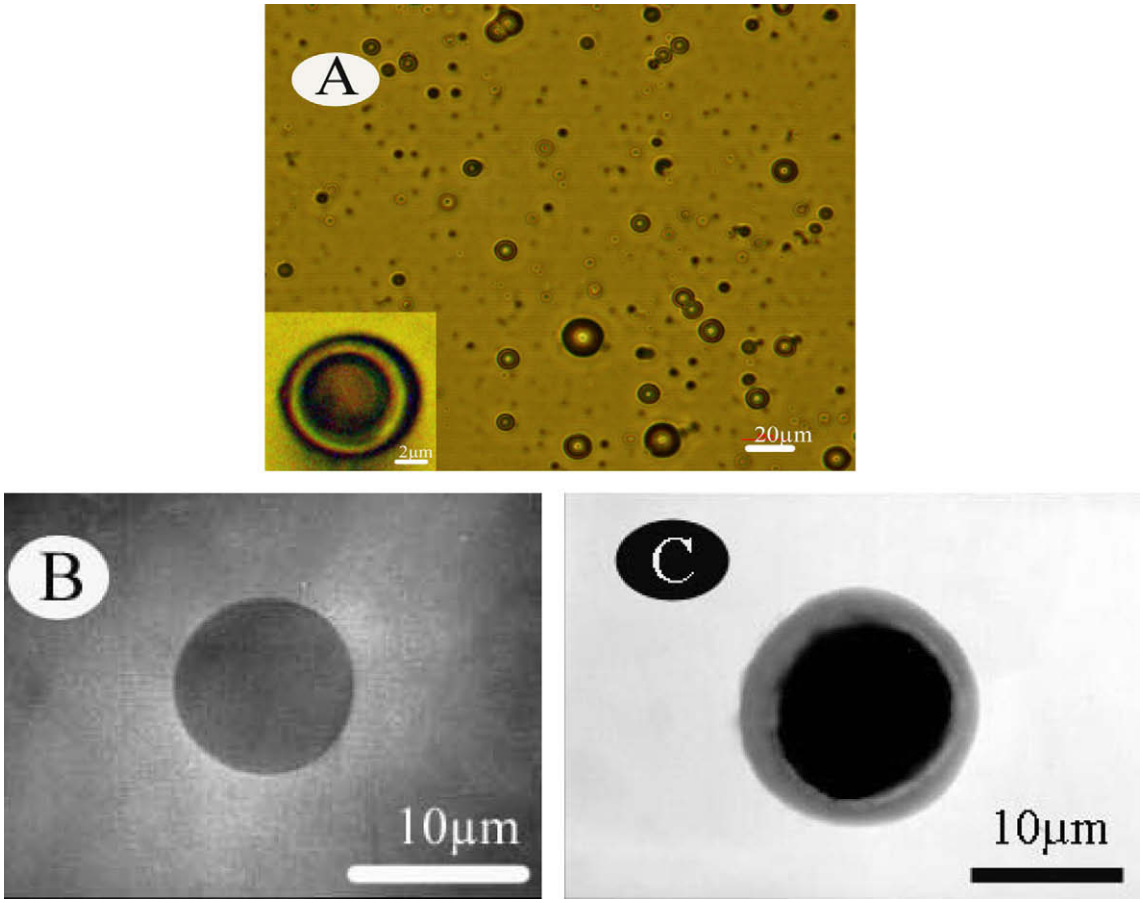

Fig. 3. The morphological of the core–shell microspheres detected by OM (A) and TEM (C), and the bare PLGA microspheres detected by TEM (B).

surface of PLGA microspheres while ALG solution held the enough viscosity caused by suitable concentration. If the concentration was too low, little ALG could be adsorbed. Thus, HSA could not be loaded. On the contrary, if the concentration was too high, PLGA microspheres were difficult in dispersing into ALG solution due to its high viscosity. Calcium alginate layer formed by cross-linking sodium alginate with Ca^{2+} further solidified the shell of the microspheres. Thus, the delivery system consisted of a paracetamol-loaded PLGA core and HSA-loaded Ca-ALG shell could be obtained. Therefore, the optimized fabrication parameters could be achieved: alginate solution concentration of 1.5%, PVA solution concentration of 3%, agitating speed of 700 rpm, and centrifugal speed of 10,000 rpm. Under these optimized fabrication parameters, we obtained that the mean size of initial PLGA microsphere was 9.0 ± 1.0 μm and 11.0 ± 1.4 μm for PLGA microspheres coated with calcium alginate layers. The mean thickness of shell was 1 μm.

3.2. Characterizations

3.2.1. Particle size and size distribution

The build-up of shell of the microspheres was accompanied by an increase in particle size, as determined by laser diffraction particle size analyzer. The mean size of initial PLGA microsphere was 9.0 ± 1.0 μm versus 11.0 ± 1.4 μm for PLGA microspheres coated with calcium alginate layers (shown in Fig. 2A). It was noteworthy that alginate had been adsorbed on the surface of PLGA microspheres. Of course, it can be concluded that the core–shell structure of microspheres was formed.

3.2.2. Morphologies of the microspheres

Fig. 2B exhibited the typical SEM images of drugs-loaded Ca-ALG/PLGA microspheres. We can see that all the microspheres took on a good dispersion and all were well spheric shaped. The result indicated that the presence of two drugs and Ca-ALG gelation did not affect the morphologies of the neat PLGA microspheres (images not shown).

The core–shell structure was one of the most important properties of Ca-ALG/PLGA microspheres researched in this paper. Thus, OM and TEM were employed to evaluate the core–shell structure, as shown in Fig. 3. By observing the OM pictures in Fig. 3A, we could find that the core–shell structure is very clear due to the different kinds of materials presented in core and shell layer. Compared with Fig. 3B of bare PLGA microspheres, we could further find that the microsphere exhibited a uniform structure, whereas the Ca-ALG/PLGA microsphere shown in Fig. 3C took on a distinct

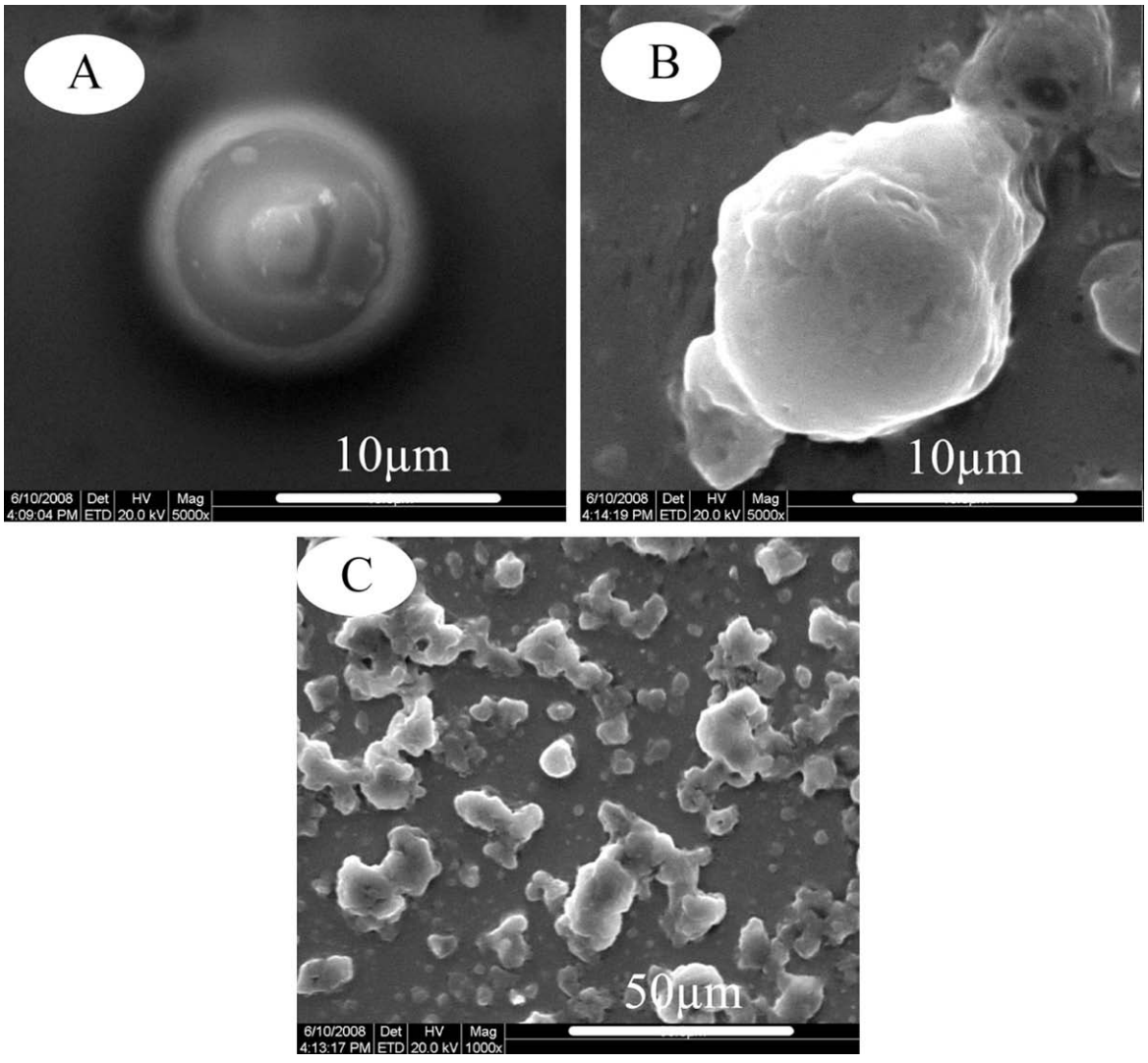

Fig. 4. The SEM photos of the core–shell microspheres (A–C) incubated at 1, 3, 5 weeks, respectively.

two-phase structure due to polymer–polymer phase separation. The colour of inner core is darker than that of coated layer. The reason is that the core, PLGA microsphere is solid whereas the shell, Ca-ALG gel is transparent. The photos further confirmed that the microspheres have core–shell structure indeed.

3.3. In vitro degradation

The bare PLGA microspheres as drug carrier have been widely investigated from studying preparation and characterization of microspheres, in vitro degradation and drug release, to studying in vivo functions. Therefore, here we only compared the bare PLGA microspheres with the core–shell microspheres from in vitro degradation and drug release. Fig. 4 showed the morphological change of the core–shell microspheres during incubation. The morphology change of microspheres at degradation time of 1, 3, 5 weeks was shown in Fig. 4A–C, respectively. By contrast with the morphology of initial microspheres shown in Fig. 2B, the microspheres almost maintained spheric shape at the first week due to the shell layer Ca-ALG falling off from PLGA core surface gradually (shown in Fig. 4A). The phenomenon also confirmed that the calcium alginate layer coated on bare PLGA microspheres successfully. The shell could be efficient to inhibit water penetrating into PLGA core and further to postpone its degradation. After 3 weeks degradation, the microspheres lost their sphere structure as shown in Fig. 4B, which indicated that water had penetrated into PLGA cores and induced PLGA degradation. In the following degradation, it should be the same as that of conventional PLGA microspheres, and so the process was not discussed again.

Fig. 5A showed the decrease of medium pH versus incubation time. Within the initial 1 week degradation, the pH decrease proceeded slowly. The reason may be that calcium alginate layer retarded PLGA core from degrading, and in this period it was mainly the degradation of calcium alginate, and also the degrada-

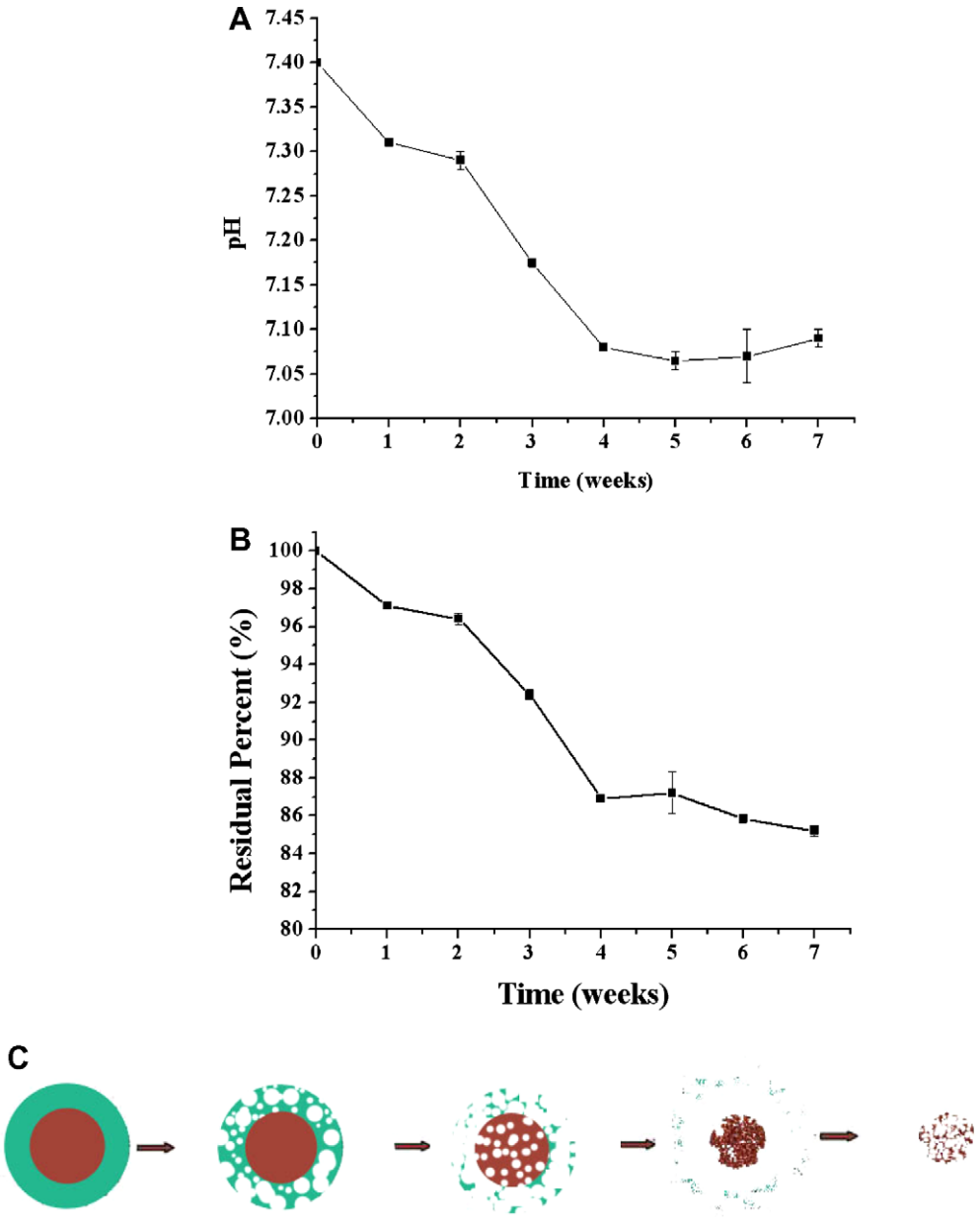

Fig. 5. (A) The pH value of degradation medium versus incubation time; (B) The percent residual weight of the core–shell microspheres incubated in PBS at 37 °C; and (C) Schematic representation of the in vitro degradation of calcium alginate layer and PLGA core.

tion of small amounts of PLGA. Calcium alginate in PBS solution achieved ion exchange with Na ions to form the soluble alginate. After 2 weeks degradation, almost all the calcium alginate layer fell off the surface of cores and consequently lost the protective effect (Pongjanyakul & Puttipipatkhachorn, 2007). So the degradation rate of microspheres became faster. In the following period, PLGA cores began to degrade, which resulted in a rapid decline of pH value of the medium (Zolnik & Burgess, 2008). The result is very consistent with Fig. 4. This phenomenon could also be observed in the mass lose of the microspheres, as shown in Fig. 5B. These results were also in agreement with above mentioned results. In summary, the degradation could be divided into two steps that the first was the degradation of shell within the first week and the second was the degradation of PLGA cores in the following period. In order to understand the mechanism of the core–shell microspheres, here a schematic diagram of the in vitro degradation of calcium alginate shell and PLGA core was given in Fig. 5C.

3.4. In vitro drug release

Fig. 6 displays the percent release of protein and paracetamol from the core–shell microspheres and the paracetamol from the bare PLGA microspheres against incubation time. Seen from the figure, 91.63% of HSA was released at first 40 h versus 13.12% of paracetamol adhered to the interface between the PLGA microspheres and alginate layer was released from the microspheres, and about 50% of paracetamol was released from the bare PLGA microspheres. It was well known that the drug release profile depended to a considerable extent on microspheres matrix degradation. Based on above analysis, the degradation of the core–shell microspheres included two phases, i.e. Ca-ALG degradation and then PLGA cores degradation. HSA release mechanism was the same as diffusion of macromolecules through a hydrogel. So its release should be very fast. Alginate layer effectively prevented the release of paracetamol from PLGA cores. However, paracetamol released from PLGA cores was more persistent and within 20 days, only 73% paracetamol released. Based on above analysis to degradation of the microspheres, we can understand that the faster degradation of Ca-ALG in PBS solution induced HSA burst release. The reason that paracetamol released from PLGA cores was identical with conventional PLGA microspheres. In our designed system, the extent of paracetamol burst release could be avoided, and a nearly zero-order release pattern for drug-loaded PLGA cores can be achieved. It indicated that HSA and paracetamol could release from the core–shell microspheres at different stages and simultaneously a sustained drug release from PLGA microspheres could also be achieved due to the presence of the shell layer. The result was very consistent with our previous expectation shown in Fig. 1.

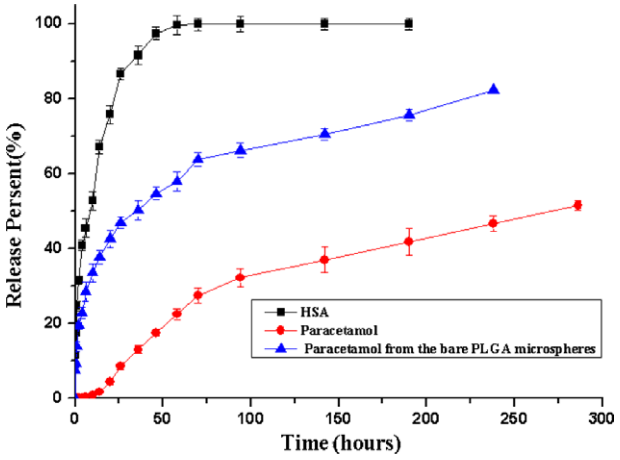

Fig. 6. Percent release of HSA and paracetamol from the core–shell microspheres and paracetamol from the bare PLGA microspheres incubated in PBS at 37 °C.

4. Conclusions

In this study, by combination of polymer–polymer phase separation and an O/W single emulsion method, hydrophilic HSA and hydrophobic paracetamol were encapsulated in the Ca-ALG shell layer and PLGA cores, respectively. The TEM and OM photos confirmed that the microspheres had obvious core-coated structure. High encapsulation efficiency of HSA and paracetamol in the core–shell microspheres was achieved. In vitro degradation indicated the degradation could be divided into two steps: the first was the degradation of shell within the first week and the second was the degradation of PLGA cores in the following period. HSA and paracetamol could release from the core–shell microspheres at different stages, and a nearly zero-order release of drug from PLGA microspheres could be achieved. Therefore, the novel core–shell microspheres may have potential application as a carrier for drug delivery system.

Acknowledgements

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