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Preparation of PLGA Microspheres with Different Porous Morphologies*

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Abstract Poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres were prepared by emulsion solvent evaporation method. The influences of inner aqueous phase, organic solvent, PLGA concentration on the morphology of microspheres were studied. The results showed that addition of porogen or surfactants to the inner aqueous phase, types of organic solvents and polymer concentration affected greatly the microsphere morphology. When dichloromethane was adopted as organic solvent, microspheres with porous structure were produced. When ethyl acetate served as organic solvent, two different morphologies were obtained. One was hollow microspheres with thin porous shell under a lower PLGA concentration, another was erythrocyte-like microspheres under a higher PLGA concentration. Three types of microspheres including porous, hollow core with thin porous shell (denoted by hollow in brief) and solid structures were finally selected for *in vitro* drug release tests. Bovine serum albumin (BSA) was chosen as model drug and encapsulated within the microspheres. The BSA encapsulation efficiency of porous, hollow and solid microspheres was respectively 90.4%, 79.8% and 0. And the ultimate accumulative release was respectively 74.5%, 58.9% and 0. The release rate of porous microspheres was much slower than that of hollow microspheres. The experiment results indicated that microspheres with different porous structures showed great potentials in controlling drug release behavior.

Keywords: PLGA; Porous microsphere; Morphology; BSA; Tissue engineering.

INTRODUCTION

Microspheres with outstanding performance have shown wide applications in tissue engineering. They offer large surface area but reduced space, which are the distinct advantages for cultivating cells in large scale. Cell-seeded microspheres can be injected directly into damaged sites for repair, and also be connected together to form implants. Many factors could affect the use of microspheres in tissue engineering. As for cell culture, not only the hydrophilicity, surface charge and functional groups of microspheres have influences on cell response[1, 2], but also the surface topographies and inner porous structures of microspheres affect cell attachment, growth, uptake of nutrients and release of metabolites[3]. To be an ideal drug delivery vehicle, the composition, molecular weight, hydrophilicity[4, 5], the size and amount of pores for microspheres are important factors for controlled drug release[6, 7]. It has been known that porous microspheres have a faster release than solid microspheres[7]. Addition of trehalose or nonionic surfactants[8], and coating of chitosan or gelatin will both

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slower the drug release rates of microspheres.

Emulsion solvent evaporation is a commonly used technique to fabricate polymer microspheres. The process involves the elimination of organic solvent from the emulsion droplets and thus the precipitation of polymers, finally forming polymer microspheres[9, 10]. The requirements of the adopted organic solvent include capacity of dissolving polymers, a certain degree of solubility with water and a relatively low boiling point. Then, the organic solvent can diffuse into the water, evaporate into the atmosphere, and finally be removed. The conventional organic solvents are dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate, or the mix of them. Besides, methyl dichloroacetate was reported as an unconventional organic solvent[11]. It resulted in immediately hardened microspheres and the almost complete encapsulation efficiency. Our previous work reported the preparation of polylactide (PLA) microspheres using methylene chloride, chloroform, toluene and ethyl acetate as organic solvents, respectively. The resulting microspheres fabricated by ethyl acetate displayed a hollow structure with porous thin shell, while the others exhibited a similar porous structure. And further study showed that the removal rate of organic solvents played the most important role in determining the morphological structure of microspheres[9].

Poly(D,L-lactide-*co*-glycolide) (PLGA) is an excellent biocompatible biodegradable material and has been widely used in the fields of cell cultivation[12–14], drug delivery[15–19] and scaffolds[20, 21]. In this study, we prepared the porous PLGA microspheres by emulsion solvent evaporation method. Microspheres with different porous structures were obtained *via* regulating inner aqueous phase, types of organic solvents and polymer concentrations. The formation mechanism of microspheres with different porous structures was explained. In addition, three different microspheres with porous, hollow and solid structures were investigated as microcarriers for drug release.

EXPERIMENTAL

Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA75/25, the molar ratio of lactide to glycolide in copolymer is 75/25, M_w = 66000–107000 kDa) and poly(vinyl alcohol) (PVA, 87%–89% hydrolyzed, M_w = 85000–120000 kDa) were purchased from Sigma-Aldrich. Ammonium bicarbonate (NH_4HCO_3) with analytical grade was purchased from Shanghai Sihewei Chemical Co. Ltd. Dichloromethane (CH_2Cl_2) and ethyl acetate were purchased from Beijing Chemical Co. Ltd. Bovine serum albumin (BSA) protein was biological grade and purchased from Beijing Xinjingke Biotechnology Co. Ltd. All reagents in our experiments were used as received.

Techniques

Emulsification was carried out by T10 basic homogenizer (IKA Labortechnik Company). The surface and inner morphologies of microspheres were characterized by scanning electron microscope (JEOL JSM-6700F, Japan). The size of microspheres was obtained by measuring diameters of 150 microspheres using optical microscope (Leica DMLP, Germany). The concentration of BSA was measured by UV spectrophotometer (ShimadzuUV-1601, Japan).

Preparation of Microspheres and Drug-Loaded Microspheres

PLGA microspheres were prepared by a modified double emulsion solvent evaporation method. Briefly, adequate NH_4HCO_3 was dissolved in 1.25 mL deionized water to form the inner aqueous phase W_1. The organic phase was achieved by dissolving PLGA into 4 mL organic solvent. The outer aqueous phase W_2 was 150 mL PVA aqueous solution with a concentration of 0.1 *W*/*V*%. The inner aqueous phase was added into the organic phase and emulsified to form the primary emulsion (W_1/O). The primary emulsion was immediately put into the outer aqueous phase under vigorously stirring to generate the W_1/O/W_2 emulsion. The stir lasted at ambient temperature to evaporate the organic solvent and solidify the microspheres. The preparation of drug-loaded porous microspheres was similar to the above one, except 50 mg BSA was added into W_1. The solid microspheres were prepared by O/W emulsion solvent evaporation method. For the preparation of drug-loaded solid microspheres, 50 mg BSA was directly mixed with the organic phase.

In-vitro Drug Release

50 mg lyophilized drug-loaded microspheres were suspended in 5 mL phosphate buffer and incubated in the shaker at 37 °C and 100 r/min. At pre-determined intervals (0.5, 1, 2, 4, 8, 12, 16, 20, 24 h), 2.5 mL supernate was taken out and replaced by 2.5 mL fresh phosphate buffer. The concentration of BSA in the supernate was measured *via* UV spectrophotometer at the absorbance of 280 nm. Each experiment was repeated in triplicate and the average value was adopted.

RESULTS AND DISCUSSION

Influence of Inner Aqueous Phase on Morphology

Figure 1 shows the morphology of PLGA microspheres prepared by different inner aqueous phases. It can be observed that the inner and surface of microspheres differ greatly in morphologies. When the inner aqueous phase was NH_4HCO_3 solution, microspheres with average diameter of (229.2 ± 38.5) μm were obtained, whose surface was distributed by little pores with average diameter of 5 μm, and the inner exhibited a homogeneous porous structure (Fig. 1a). When extra 2 wt% PVA was added into the NH_4HCO_3 solution, the inner of microspheres still kept a homogeneous porous structure but the pore size decreased. Pores on the surface of microspheres sharply decreased both in size and amount. But the diameter of microspheres remarkably increased to (326.0 ± 83.2) μm, as shown in Fig. 1(b). Solid microspheres with the smallest diameter of (138.4 ± 31.0) μm were generated without the addition of inner aqueous phase, the surface and inner of microspheres were smooth and compact (Fig. 1c).

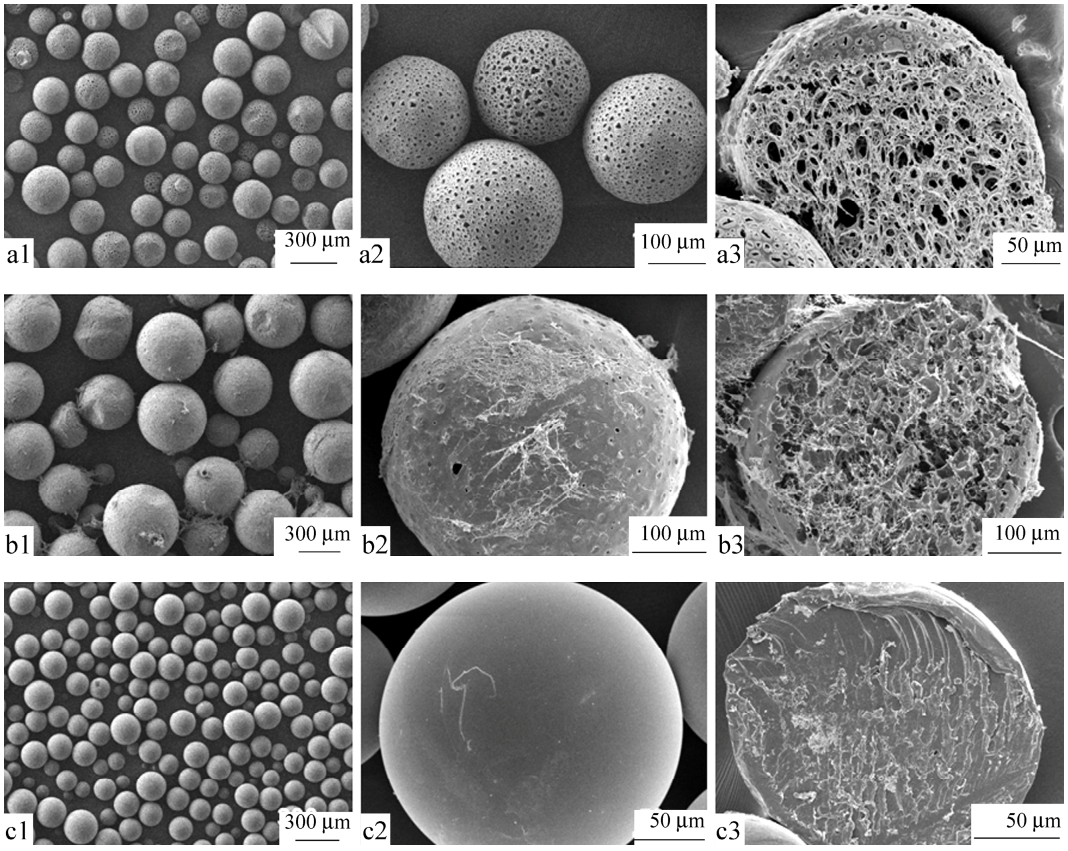


Fig. 1 SEM photographs of microspheres prepared with different inner aqueous phases: (a1–a3) NH_4HCO_3 solusion, (b1–b3) NH_4HCO_3/PVA solusion and (c1–c3) no inner phase (The polymer concentration in dichloromethane was 62.5 mg/mL.)

The inner aqueous phase dispersed in the form of droplets inside the organic phase after emulsification. With the removal of organic solvents, the aqueous droplets developed into holes and extended the volume of microspheres. The NH_4HCO_3 played dual roles, one was to stabilize emulsion and prevent the aqueous droplets from aggregation during the removal of organic solvent for maintaining the porous structure of microspheres. Another was to generate pores when NH_4HCO_3 decomposed to release gases. The gases were able to pass through the membranes between inner holes to make them open, and further escape from the surface of microspheres, leaving little pores on the surface. In the presence of additional PVA, the volume of microspheres obviously expanded. It occurred probably due to that the inner aqueous droplets increased in number but decreased in volume in the same emulsification condition since PVA was a sort of surfactant. The decreased volume of inner aqueous droplets led to the decreased size of inner holes of microspheres. The increased number of inner aqueous droplets resulted in the increased viscosity of primary emulsion, which led to the larger microspheres.

Influence of Organic Solvent on Morphology

Types of organic solvents had a great effect on microsphere morphology. The microspheres with homogeneous porous structure were prepared when dichloromethane was used as solvent (Fig. 1a). When ethyl acetate was used as solvent, the erythrocyte-like products with one or more pits were obtained (Fig. 2a). The inner of microspheres showed a porous structure but the surface kept smooth. When the mix of dichloromethane and ethyl acetate (with the volume ratio of 2:8) was used as solvent, the products possessed the traits of both above (Fig. 2b). These products were still spherical particles with smooth surface and insufficient porous core.

The different solvent removal rate was supposed to make a great difference in the morphology of microspheres[9]. The solubility in water and the boiling point of organic solvent were two key factors determining the rate of solvent removal. The high solubility in water of ethyl acetate makes it quick to remove from microdroplets into outer aqueous phase, which drives the still unstable inner events of microdroplets out and leads to the coalescence of some inner aqueous droplets. After the removal of ethyl acetate, erythrocyte-like microspheres formed. While for the mix of dichloromethane and ethyl acetate, the slow removal rate of organic solvent caused the formation of PLGA microspheres with porous structure.

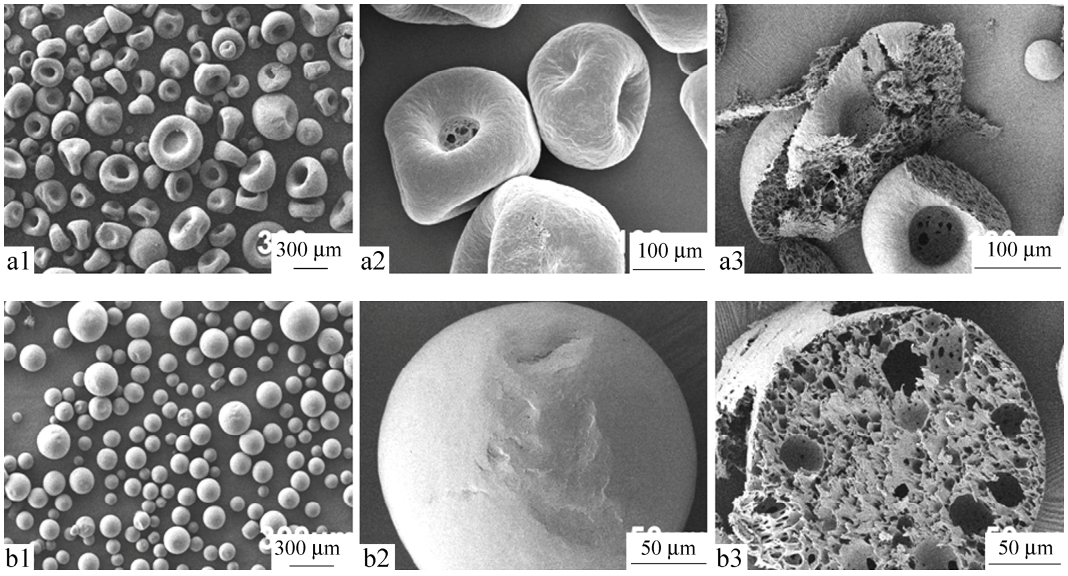


Fig. 2 SEM photographs of microspheres prepared by ethyl acetate (a1–a3), and mixed solvent (b1–b3) (The polymer concentration of organic phase (O) was 62.5 mg/mL and the inner aqueous phase was 1.25 mL NH_4HCO_3 solution.)

Influence of Polymer Concentration on Morphology

A series of experiments with various polymer concentrations using ethyl acetate as organic solvent were conducted to explore the influence of polymer concentration on microspheres morphology. It can be seen from optical photographs in Fig. 3 that only two kinds of morphologies were observed. Higher polymer concentration led to the formation of erythrocyte-like microparticles, while lower polymer concentration resulted in the formation of hollow structure microspheres. The critical polymer concentration of transition of two morphologies was found between 46.9 mg/mL and 43 mg/mL. The erythrocyte-like microparticles had an average diameter of (195.7 ± 61.6) μm while the hollow microspheres of (169.2 ± 36.6) μm. SEM results showed that the products took on an erythrocyte-like structure at the polymer concentration of 62.5 mg/mL (as seen in Fig. 2a). When the polymer concentration reduced to 46.9 mg/mL, hollow microspheres with a thin porous shell were achieved (as seen in Fig. 3b). When organic solvent was dichloromethane, morphology of microspheres did not change with the variation of polymer concentration, maintaining a homogeneous porous structure (as seen in Fig. 1a). It is the viscosity of organic phase changed with the polymer concentration, which affects greatly the shape and diameter of microspheres.

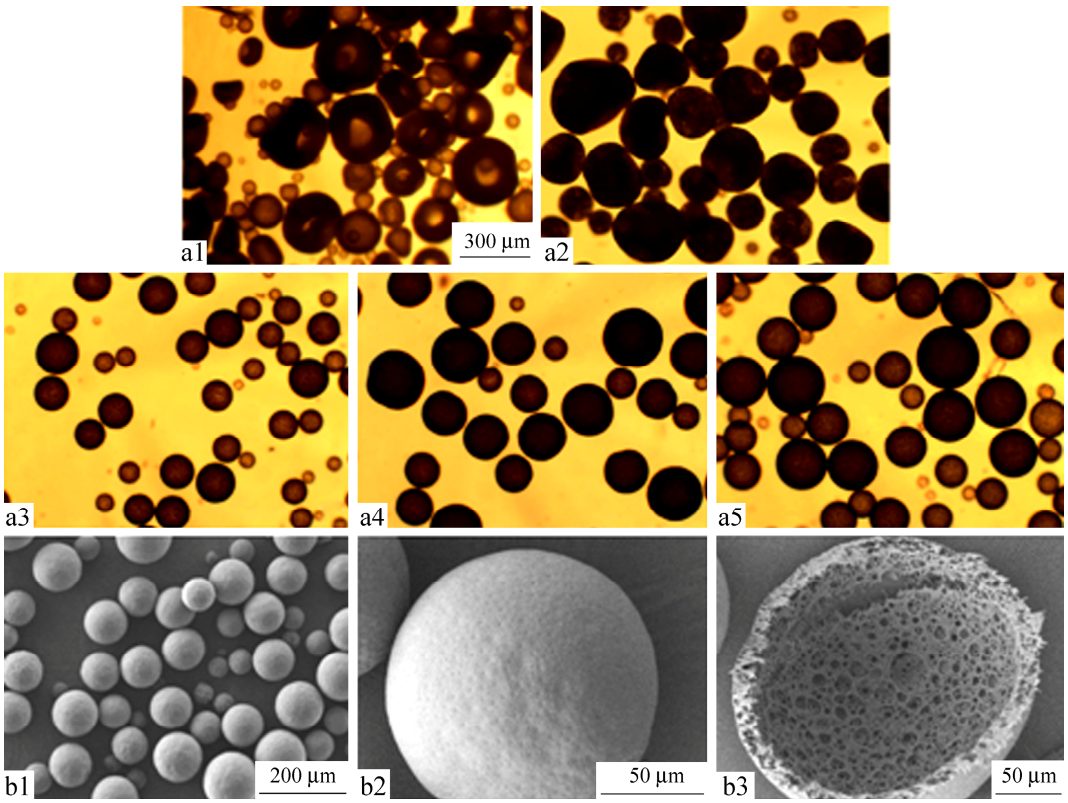


Fig. 3 Optical microscope photographs of microspheres with different polymer concentrations. (a1) 62.5 mg/mL, (a2) 46.9 mg/mL, (a3) 43 mg/mL, (a4) 39.1 mg/mL, (a5) 31.3 mg/mL. (b1–b3) SEM photographs of hollow microspheres with the polymer concentration of organic phase (O) 31.3 mg/mL (Organic solvent was ethyl acetate and the inner aqueous phase was 1.25 mL NH_4HCO_3 solution.)

Formation Mechanism of Microspheres with Different Morphology

The preparation process of microspheres through emulsion solvent evaporation method involves phase separation. Polymer precipitates to form microspheres during phase separation. The morphology of microspheres is dependent strongly on the thermodynamics and kinetics of phase separation. In this work, reaction system altered along with the variation of organic solvents' boiling point, viscosity, solubility and compatibility with polymers and so on. All of these complex factors gave birth to the following phenomena. In the presence of

dichloromethane, the inner aqueous droplets remained stable all along and dispersed homogeneously inside the oil drops, forming microspheres with homogeneous porous structure after the organic solvent was totally eliminated. When ethyl acetate acted as organic solvent, the system was no longer stable and the inner aqueous droplets aggregated extensively in two forms. One was to aggregate toward the core of oil drops thus producing the hollow microspheres. Another was toward off the core and the bigger coalescent aqueous droplet broke through the surface of oil drops, leading to pits on the surface of microparticles, as shown in Fig. 4.

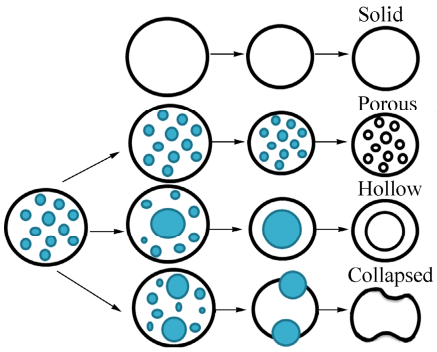


Fig. 4 Schematic of formation mechanism of microspheres with different morphologies

***In-vitro* BSA Release from Microspheres**

Three kinds of microspheres with different morphologies (porous microspheres, hollow microspheres with a thin porous shell, solid microspheres) were selected for drug release. Figure 5 shows the SEM photographs of drug-loaded microspheres. Compared to original microspheres as shown in Fig. 1(a), Fig. 1(c) and Fig. 3(b), drug-loaded microspheres showed enlarged diameters (except solid ones) and kept similar morphology. The average diameters of drug-loaded microspheres with porous, hollow and solid structure were (271.7 ± 49.7) μm, (238.8 ± 72.7) μm and (139 ± 30.1) μm, respectively. Furthermore, the cross-section results of microspheres in Fig. 5(a3, b3, c3) indicated that the fibrous BSA homogeneously distributes in the small inner holes of the porous microspheres, while there are a lot of floccules of BSA assembled inside the hollow microspheres.

For solid microspheres, the amount of encapsulated BSA was near to 0 mg due to little BSA dissolved in dichloromethane. For porous microspheres and hollow microspheres, Table 1 lists their production ratio, BSA encapsulation efficiency and loading capacity. It was found that these two microspheres had high BSA encapsulation efficiency especially the porous microspheres. The production ratio did not reach to 100% on account of the following three factors: the primary emulsion could not be completely added into the outer aqueous phase because viscous residues stuck to the wall of vessels; some polymers entangled on the agitator blade; some microspheres were wasted in the step of wash. The sticking to the wall of vessels occupied the most of loss when dichloromethane was used as organic solvent. When ethyl acetate served as solvent, the main loss included not only the sticking to the wall of vessels but also the entanglement on the agitator blade. That was why the production ratio and the BSA encapsulation efficiency of hollow microspheres prepared by ethyl acetate were lower than that of porous microspheres prepared by dichloromethane.

Table 1. The production ratio, BSA encapsulation efficiency and loading capacity of porous and hollow microspheres

	Porous microspheres	Hollow microspheres
PLGA/BSA[a] (mg/mg)	249.6/50	151.5/50
Obtained microspheres[b] (mg)	253.2	137.4
Loaded BSA (mg)	45.2	39.8
Production ratio (%)	84.51	68.19
BSA encapsulation efficiency (%)	90.4	79.6
Loading capacity (%)	17.85	28.97

[a] The originally added amount of PLGA and BSA, the amount of PLGA differed in porous and hollow microspheres because the morphology is affected by polymer concentration of organic phase; [b] The obtained amount of drug-loaded microspheres

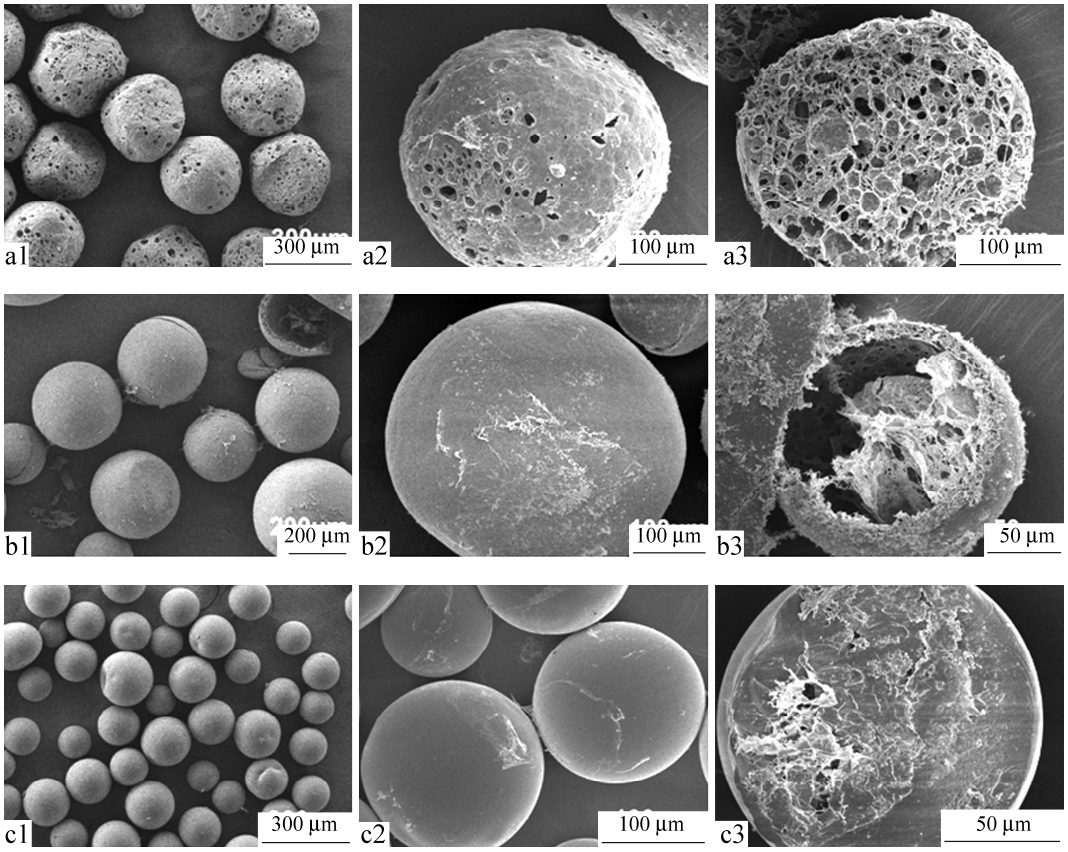


Fig. 5 SEM photographs of drug-loaded microspheres with different morphologies of porous (a1–a3), hollow (b1–b3) and solid (c1–c3)

Figure 6 illustrates the in-vitro drug release profiles. The release profile of solid microspheres was a horizontal line which indicated none BSA release. While the release of porous microspheres and hollow microspheres lasted for 24 h. It was found that the BSA release from porous microspheres was almost linear within 15 h, and the ultimate cumulative release reached 74.5%. For hollow microspheres an initial burst release by 5 h was observed in the profile, and the ultimate cumulative release was 58.9%, which was lower than that of porous microspheres. The ultimate cumulative release from both porous and hollow microspheres was far from 100%, which was because that a certain amount of amphiphilic BSA was embedded in PLGA matrix or in closed pores, making it hard to diffuse completely out from microspheres.

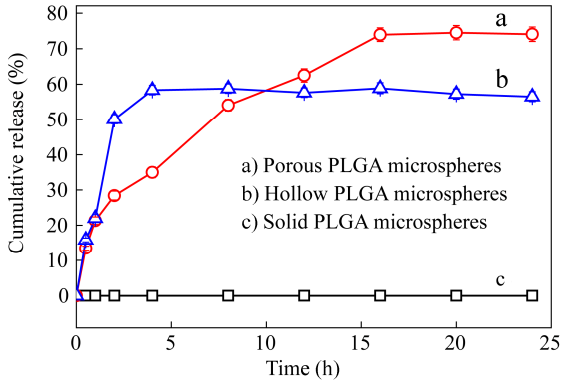


Fig. 6 Release profiles of BSA from PLGA microspheres with different morphologies

BSA released prevailingly by diffusion since PLGA microspheres did not show obvious degradation in buffer solution by one month. Compared to the release profiles of BSA from hollow microspheres, the release rate of BSA from porous microspheres was much slower in spite of many pores distributed in microspheres. It could be due to the factor that the pores inside microspheres acted as separated compartments connected merely by the holes with smaller size, which prevented BSA from diffusing outward directly and quickly. As for hollow microspheres with thin porous shell, BSA gathered in the hollow core could easily pass through the thin porous shell (the average thickness was 10–20 μm) and resulted in rapid release of BSA, which was driven by the large concentration gradient of BSA between the inner and outer of microspheres. That was why the initial burst release by 5 h turned up.

Therefore, the porous microspheres showed advantages over the hollow microspheres in properties of both BSA encapsulation efficiency and controlled release behavior.

CONCLUSIONS

In summary, this work reported the successful preparation of porous PLGA microspheres by means of emulsion solvent evaporation. The microspheres with different porous morphologies were obtained by changing experimental parameters. Microspheres with homogeneous porous structure were generated using dichloromethane as organic solvent no matter the variation of polymer concentration. When ethyl acetate used as organic solvent, the microspheres displayed a feature of erythrocyte-like structure at higher polymer concentrations, while hollow structure at lower polymer concentrations. The drug loading results showed that the BSA encapsulation efficiency of solid microsphere prepared by single emulsion evaporation method was close to 0, while that of porous and hollow microspheres could reach high up to 90.4% and 79.6%, respectively. The BSA release from porous microspheres showed a slower and linear rate, which was better than that from hollow microspheres. The ultimate accumulative release of porous, hollow and solid microspheres was respectively 74.5%, 58.9% and 0. Therefore, the encapsulation of BSA in PLGA microspheres and its release from these microspheres could be well controlled by the porous structure of microspheres.

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