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Preparation of Porous Polylactide Microspheres and Their Application in Tissue Engineering

Xu-Dong Shi[a, b], Pei-Jian Sun[b], and Zhi-Hua Gan[b, c*]

[a] *Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China*
[b] *The CAS Key Laboratory of Engineering Plastics, Institute of Chemistry, Chinese Academy of Sciences (CAS), Beijing 100190, China*
[c] *State Key Laboratory of Organic-inorganic Composites, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China*

Abstract In this study, porous polylactide (PLA) microspheres with different structures were prepared through the multiple emulsion solvent evaporation method. By changing organic solvents (ethyl acetate and chloroform) and adding effervescent salt NH_4HCO_3 in the inner water phase, microspheres with porous capsular, matrix, microcapsular and multivesicular structures were prepared. The protein encapsulation and release, and the cell growth behavior of porous microspheres were further explored. Under the same inner water phase, microspheres prepared with chloroform had higher protein encapsulation efficiency and less protein release rate as compared with those prepared with ethyl acetate. Cell experiments showed that the relatively rough surface of microspheres prepared with chloroform was more favorable for the cell growth in comparison with the smooth surface of microspheres prepared with ethyl acetate. This study shows a simple and effective method to control the protein release and cell growth behaviors of polymer microspheres by tuning their porous structure.

Keywords Polylactide; Porous microspheres; Drug release; Cell; Tissue engineering

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INTRODUCTION

Microspheres fabricated from biodegradable polymers have been widely used in the research of tissue engineering because they are biocompatible, degradable and have large surface area. In this field, microspheres usually act as microcarriers or scaffolds to help the growth of cells and recover the defective tissues[1–7]. As an ideal carrier, microspheres should provide a controlled microenvironment to heal specific diseases or to guide the growth of cells and new tissues, which need to expose appropriate agents at the desired site with sufficient dose within the required time[8–10]. Therefore, the controlled release of therapeutic agents is also important for the real application of biodegradable polymer microspheres[11, 12].

It was reported that the porous structure of microspheres not only affected the release behavior of agents but also benefited the growth of cells. It can facilitate the diffusion of agent, cell attachment, migration and proliferation, and even new tissue ingrowth through the high porosity and enlarged surface area of microspheres[13–17]. Hence, it is meaningful to prepare the microspheres with different porous structures, and investigate their drug release and cell response behaviors. Among various preparation methods, the multiple emulsion solvent evaporation method is a convenient way to fabricate porous polymer microspheres[15, 18–21].

We previously prepared porous polylactide (PLA) microspheres by the modified emulsion solvent evaporation method, and altered the porous structure of microspheres through the changing of preparation parameters. Cell experiments showed that the pore size of porous PLA microspheres obviously effected the cells growth[22–24]. However, the protein encapsulation and release behaviors of porous PLA microspheres and its effect on cell growth still remain unclear.

Poly(lactic acid) (PLA) and its related polymers have been widely utilized to prepare microspheres, tissue engineering scaffolds and to encapsulate drugs[15, 25–32]. In this study, the amorphous poly(DL-lactide) (PDLLA) was used to prepare porous PLA microspheres by the modified emulsion solvent evaporation method. PLA microspheres with different porous structures were prepared by changing the organic solvents or adding the effervescent salt NH_4HCO_3 in the inner water phase. Bovine serum albumin (BSA) as the model protein was encapsulated into porous microspheres for exploring their protein encapsulation and release behaviors. Finally, osteoblast like cells MG-63 were cultured on porous PLA microspheres to assess their potential for cell cultivation.

* Corresponding author: E-mail zhgan@mail.buct.edu.cn
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EXPERIMENTAL

Materials

Poly(DL-lactide) (PDLLA) (M_w 7.5 × 10^4–1.2 × 10^5) and poly(vinyl alcohol) (PVA) (87% ~ 89% hydrolyzed, M_w 8.5 × 10^4–1.46 × 10^5) were purchased from Aldrich. Ammonium bicarbonate (NH_4HCO_3) with analytical grade was bought from Shanghai Sihewei Chemical Co. Ltd. Ethyl acetate, chloroform and dimethyl sulphoxide (DMSO) used in this work were of analytical grade and purchased from Beijing Chemical Co. Ltd. Bovine serum albumin (BSA) protein was biological grade and purchased from Beijing Xinjingke Biotechnology Co. Ltd. All above chemicals were used as received.

Preparation of Microspheres

The detailed preparation method of PLA microspheres was described in our previous paper[22]. Briefly, 1.25 mL of 0.05 g/mL NH_4HCO_3 or 0.04 g/mL BSA aqueous solution was added into 4 mL of 0.03125 g/mL PDLLA/organic solvent, and the mixture was emulsified by a homogenizer (T10, IKA, Germany) in an ice bath. This primary emulsion was then poured immediately into 150 mL of 0.001 g/mL PVA aqueous solution, and stirred to produce a double emulsion. After the organic solvents were evaporated, porous PLA microspheres formed and were washed for the further usage.

Characterization of Microspheres

PLA microspheres and their slices cut by a blade were freeze dried and mounted on metal stubs. The surface and cross-section of microspheres were observed on a scanning electron microscope (SEM) (JEOL JSM-6700F, Japan) after coated with platinum. The size of microspheres was determined using an optical microscope (Leica DMLP, Germany) or SEM, through which 150 microspheres were counted to calculate their average diameter and size distribution.

Protein Encapsulation Efficiency

The BSA encapsulation efficiency was calculated from the amount of non-entrapped protein in the supernatant recovered after preparation and washing of the PLA microspheres[33]. The BSA concentration was determined *via* a standard curve by measuring the absorbance at 280 nm using a spectrophotometer (Shimadzu UV-1601, Japan). Each sample was repeated in triplicate and the average values of BSA encapsulation efficiency were determined.

In Vitro Protein Release

30 mg of lyophilized microspheres with encapsulated BSA was suspended in 5 mL of phosphate buffer (0.1 mol/L, pH = 7.4) and incubated at 37 °C. At pre-determined time intervals (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13 and 15 days), 3 mL of the supernate was withdrawn and replaced with 3 mL of fresh phosphate buffer. The supernate was filtered and assayed for protein concentration. The concentration of BSA in the supernate was determined by measuring the absorbance at 280 nm in the spectrophotometer. Experiments were repeated in triplicate and the average values of BSA concentration were determined.

Cell Experiments

MG-63 cells were cultured in Dulbecco's modified eagle medium (DMEM, low glucose, HyClone) supplemented with 10% fetal calf serum (FBS, HyClone), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco). Cells were maintained in cell culture flasks (25 cm^2) at 37 °C in a humidified incubator in the presence of 5% CO_2. The culture medium was replaced every other day. At confluence, cells were harvested with 0.25% trypsin-EDTA (HyClone) and resuspended in above culture medium for next use.

Cell cytotoxicity

The effect of PLA microspheres on cell cytotoxicity was illustrated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. Firstly, MG-63 cells were seeded on 96-well plates at a density of 5 × 10^4 cells/mL in 200 μL of culture medium per well. After 2 days, the cell confluence was reached. Then, PLA microspheres were placed into each well and cultured. The culture plates without microspheres were used as the control. After 2 days of cell culture, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well. After 4 h (37 °C) incubation, the above culture medium was removed and the precipitate of each well was dissolved in 200 μL of DMSO. The optical density (OD) of produced formazan in the solution was read at 570 nm in a plate reader (Thermo Multiskan MK3, USA). The percent cell viability of the control (none microspheres) cells was taken as 100%.

Cell cultivation on microspheres

PLA microspheres were first sterilized by ultraviolet irradiation at room temperature for 2 h. A certain amount of sterilized PLA microspheres were put into 24-well plate, and then MG-63 cells in culture medium with the concentration of 3 × 10^4 cells/mL were added to the wells. After co-cultured for 7 days, the cultured cells were stained by a dye solution (concentration of fluorescein diacetate (FDA): 1 mg/mL), and cell morphologies were observed through a fluorescence microscope (Olympus IX71, Japan)[34].

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA). Data were considered to be statistically significant at a level of $P < 0.05$.

RESULTS AND DISCUSSION

Preparation of Porous PLA Microspheres

In the emulsion solvent evaporation method, the removal rate of organic solvent was reported to play an important role in deciding the morphology of microspheres. In this study, ethyl acetate and chloroform were chosen to prepare microspheres with different porous structures due to their different solvent removal rates[23]. In addition, NH_4HCO_3 was also used in the inner aqueous phase to change the porous structure of PLA microspheres. Table 1 shows the preparation parameters of PLA microspheres with different porous structures.

Figure 1 shows the SEM images of PLA microspheres with different porous structures. For the microspheres whose inner aqueous phase is NH_4HCO_3, a lot of tiny pores with diameters about 1 μm on the smooth surface of PLA-E-N microspheres were observed, while many big pores with diameters around

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Table 1 Porous PLA microspheres prepared with different organic solvents and inner aqueous phase

Microspheres	Organic solvent	Inner aqueous phase
PLA-E-N	Ethyl acetate	5% NH_4HCO_3
PLA-C-N	Chloroform	5% NH_4HCO_3
PLA-E-H	Ethyl acetate	H_2O
PLA-C-H	Chloroform	H_2O

10 μm on the surface of PLA-C-N microspheres were found. PLA-E-N microspheres have the structure of hollow core and porous shell, while PLA-C-N microspheres have a homogeneously porous structure. For the PLA microspheres whose inner aqueous phase is water, both kinds of microspheres have smooth surface while the inner structure of microspheres is quite different. PLA-E-H microspheres are hollow with thin shell, while PLA-C-H microspheres have several holes inside. According to the classification of microspheres with different types of structure in Florence's work[35], microspheres shown in Fig. 1 have the structure of porous capsular (Fig. 1a), matrix (Fig. 1b), microcapsular (Fig. 1c) and multivesicular (Fig. 1d) type, respectively.

In the emulsion solvent evaporation method, the complete removal of organic solvents results in the formation of polymer microspheres. Figure 2 shows the formation process of PLA microspheres with different porous structures. During the preparation process of PLA microspheres, the removal rate of ethyl acetate is faster than that of chloroform, which results in the high osmotic between the microdroplets and the outer aqueous phase. This high osmotic can drive the unstable inner contents of microdroplets out, leads to the coalescence of some inner aqueous droplets, and finally results in the formation of hollow microspheres. Chloroform has a lower removal rate, which produces a concentrated and viscous microenvironment in the microdroplets. During the slow removal process of chloroform, the sites of inner aqueous droplets can develop into holes within microspheres, which finally lead to porous microspheres.

Comparing the microspheres structure in Figs. 1(a) and 1(b)

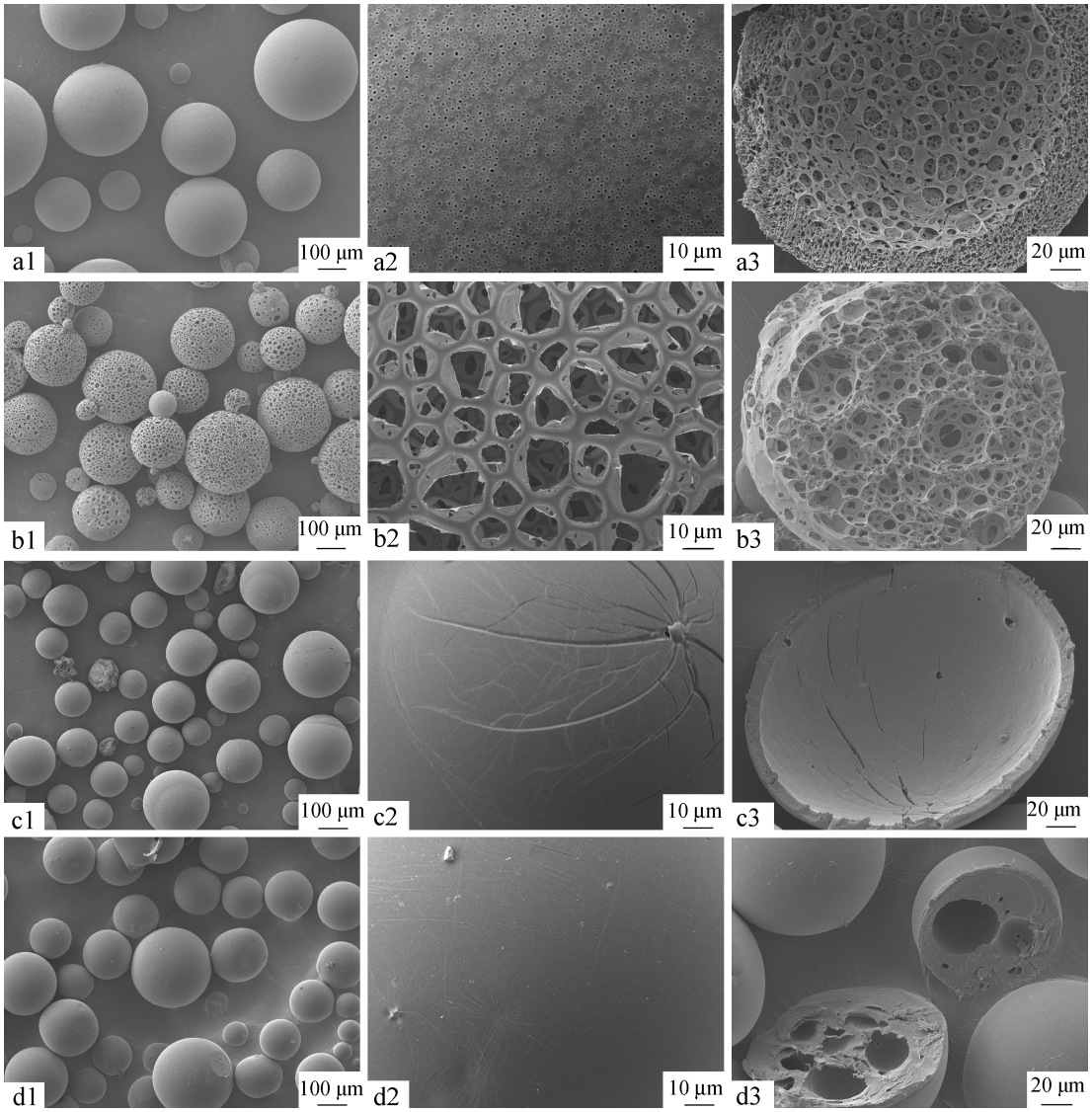


Fig. 1 SEM images of porous PLA microspheres, their enlarged surface and cross-section: PLA-E-N (a1–a3), PLA-C-N (b1–b3), PLA-E-H (c1–c3), PLA-C-H (d1–d3)

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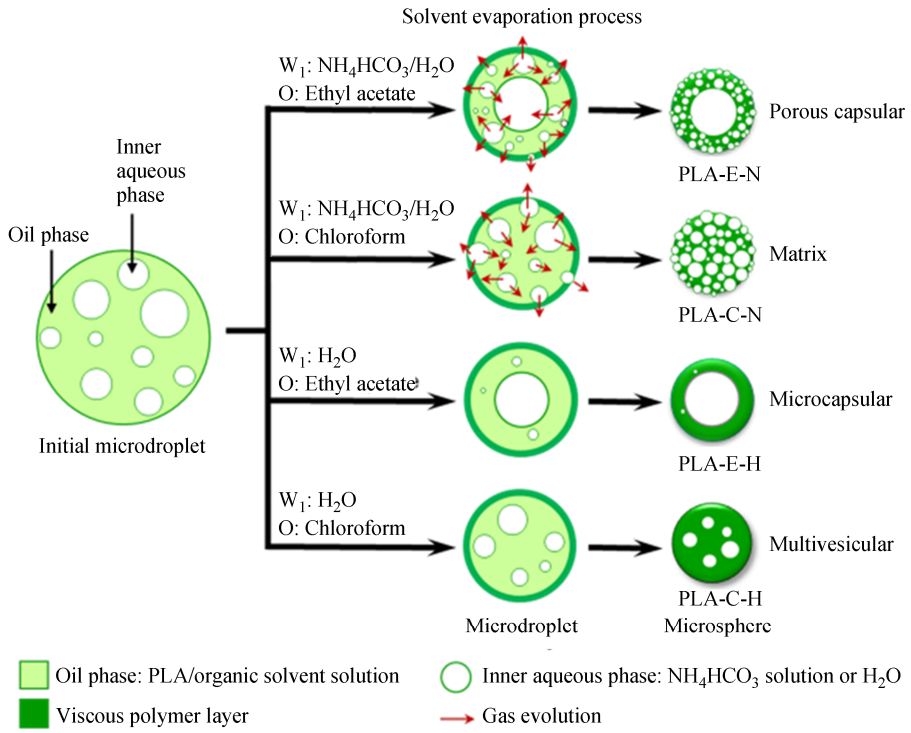


Fig. 2 Schematic illustration of the formation process of PLA microspheres with different porous structures

with that in Figs. 1(c) and 1(d), the addition of NH_4HCO_3 produces more and interconnected pores in PLA microspheres. NH_4HCO_3 is an effervescent agent that is mainly responsible for the well-developed porous structure of the resultant microspheres in the modified emulsion evaporation method. The decomposed products of NH_4HCO_3—gas bubbles of carbon dioxide and ammonia—not only stabilize the primary emulsion, but also create and connect pores within PLA microspheres, which results in higher porosity of microspheres. In addition, the average diameters of PLA-E-N, PLA-C-N, PLA-E-H and PLA-C-H microspheres are (225 ± 61), (191 ± 57), (135 ± 49) and (104 ± 33) μm correspondingly. The porous structure resulted from NH_4HCO_3 obviously increased the diameters of microspheres.

BSA Encapsulation and Release Behaviors of Porous PLA Microspheres

Recent approaches of scaffold design in tissue engineering have been pursued to integrate bioactive factors into scaffold for promoting the growth of cell and new tissue. The encapsulation and release behavior of bioactive factors will affect cell growth behavior. Bovine serum albumin (BSA) is a serum albumin protein which is used as a nutrient in cell culture and plays an important role in regulating cell attachment to biomaterials[36]. To figure out the protein encapsulation and release behaviors of porous microspheres, BSA was used as the model protein and encapsulated in the four types of porous PLA microspheres (shown in Fig. 1).

The calculated data shown in Table 2 demonstrate that the BSA encapsulation efficiency and the production ratio of microspheres made by two organic solvents are different. It was reported that organic solvents could play an important role in the protein entrapment efficiency and protein bioactivity of PLA microspheres[37]. In this work, it was also observed that microspheres prepared with chloroform had higher BSA encapsulation efficiency and production ratio than those prepared with ethyl acetate, while no matter whether NH_4HCO_3 was added or not. Comparing microspheres prepared with the same solvent, the addition of NH_4HCO_3 did not obviously affect the BSA encapsulation efficiency (Table 2). In this study, the removal rate of ethyl acetate was so fast that some PLA easily precipitated from their emulsion and even wrapped on the stir bar, which led to the lower encapsulation efficiency and production ratio of microspheres.

Figure 3 shows the SEM images of BSA loaded PLA microspheres, their enlarged surface and cross-section. The cross-section results of the PLA microspheres shown in Figs. 3(a3)–3(d3) indicate that there are a lot of floccules of BSA assembled inside the hollow microspheres prepared with ethyl acetate, while for the microspheres prepared with chloroform, fibrous BSA homogeneously distributes in the small inner holes. These four kinds of microspheres (Fig. 3) have similar average sizes. Their average diameters are (244 ± 53), (200 ± 36), (221 ± 49) and (194 ± 34) μm, respectively.

Table 2 BSA encapsulation efficiency and production ratio of PLA microspheres prepared with different organic solvents and inner aqueous phase

Microsphere	Encapsulation efficiency (%)	Production ratio (%)
PLA-E-N	60.60	82.40
PLA-C-N	86.74	92.63
PLA-E-H	59.44	67.66
PLA-C-H	91.42	96.46

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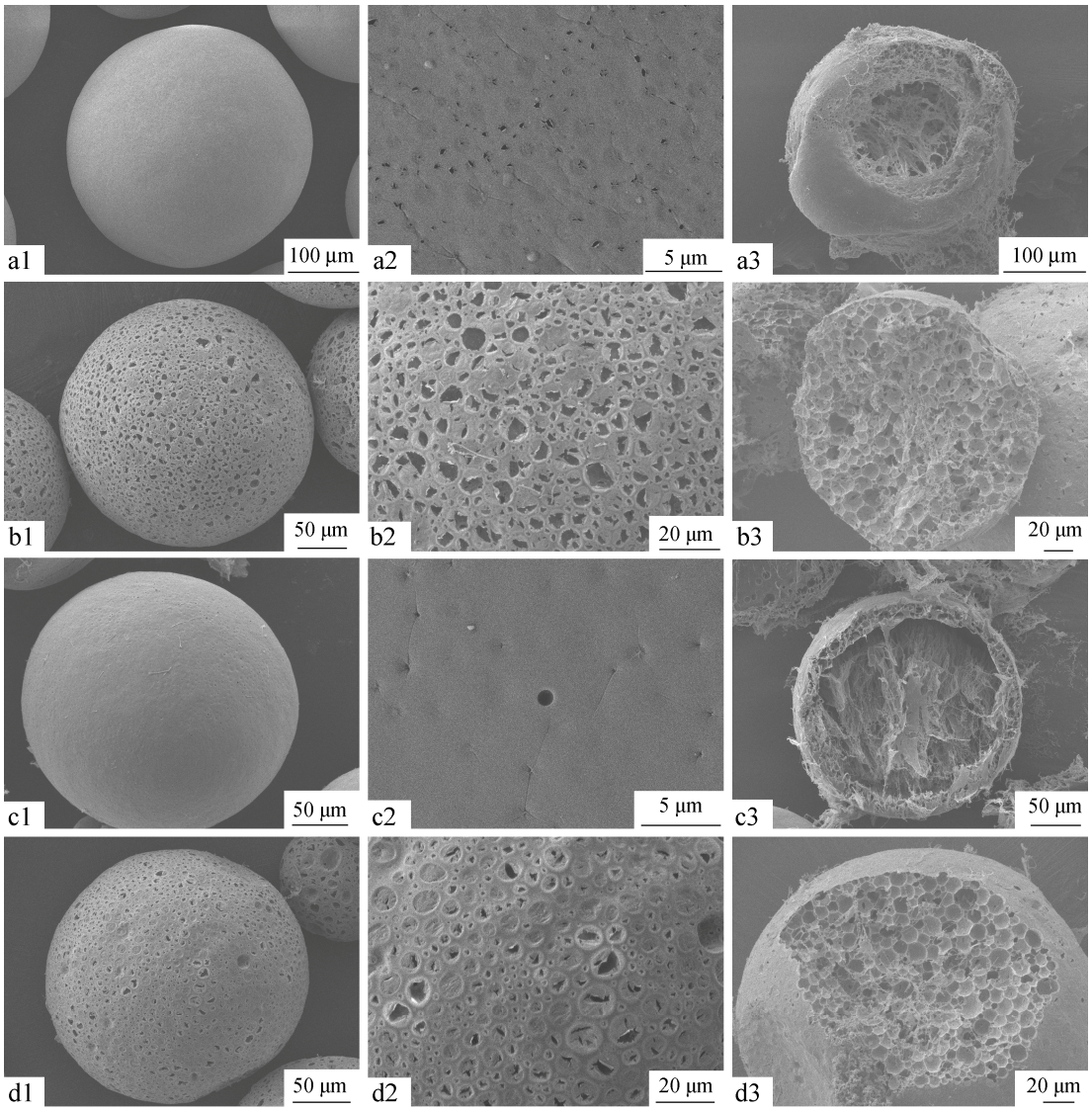


Fig. 3 BSA loaded porous PLA microspheres, their enlarged surface and cross-section as a function of organic solvents and the NH_4HCO_3 in the inner aqueous phase: PLA-E-N (a1–a3), PLA-C-N (b1–b3), PLA-E-H (c1–c3), PLA-C-H (d1–d3)

Comparing Fig. 3 with Fig. 1, the addition of BSA in inner aqueous phase generates more pores in PLA microspheres. It is because BSA can act as a surfactant to stabilize the primary emulsion during the formation of microspheres in the modified emulsion solvent evaporation method[38], which leads to the more porous structure of microspheres as shown in Fig. 3.

The release profiles of different PLA microspheres were monitored for 15 days. The results are shown in Fig. 4. The BSA releases of all microspheres have a similar trend, while the extent of protein release changes with the microspheres structure. After an initial burst by 7 days, these plots show remarkable decrease in the BSA release rate. The final released quantities of BSA for all samples are less than 45% of the total protein encapsulated in microspheres. The addition of NH_4HCO_3 in the inner aqueous phase is found to have an influence on the BSA release profiles of microspheres. Comparing plot a with c (or comparing plot b with d), it is observed that microspheres prepared with

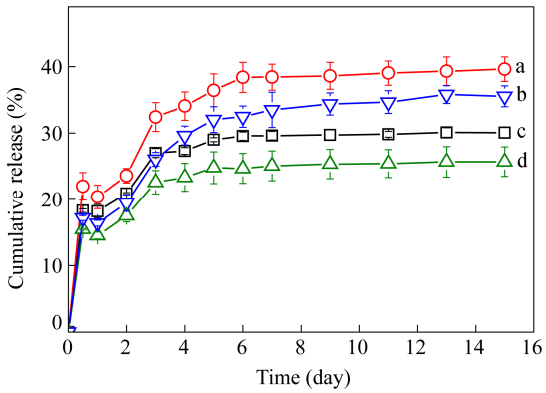


Fig. 4 Comparison of *in vitro* BSA release profiles from PLA microspheres with different porous structures: PLA-E-N (a), PLA-C-N (b), PLA-E-H (c), PLA-C-H (d)
Each group was separately prepared and tested (n = 3, each group) for up to 15 days. Each data point was recorded in the average and standard deviation (SD).

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NH_4HCO_3 have a faster BSA release rate than those without NH_4HCO_3. The organic solvents also affect the release behavior of microspheres through the change of microspheres porous structure. With the same inner aqueous phase, PLA microspheres prepared with ethyl acetate release more BSA than those prepared with chloroform. It is apparent that the porous structure of microspheres has an influence on the BSA release profiles of microspheres. Hence, the release behavior of microspheres can be controlled by changing the organic solvents and adding of NH_4HCO_3 in the inner aqueous phase.

Usually, the rate of BSA released from PLA microspheres is thought to mainly depend on their diffusion through the microspheres and the rate of polymer degradation[13, 39, 40]. Compared with Fig. 3, the SEM images of PLA microspheres (Fig. 5) reveal that all BSA loaded microspheres do not obviously degrade after immersion in phosphate buffer for 31 days. Due to the good adhesion between BSA and PLA matrix, some BSA remained in microspheres and would be released with the degradation of PLA microspheres[41]. In this study, the porous structure of microspheres is mainly responsible for the different BSA release behaviors considering the slow degradation rate of PLA matrix.

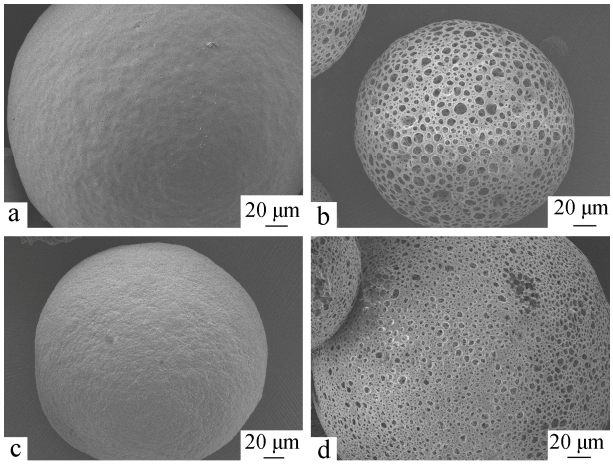


Fig. 5 SEM images of BSA loaded PLA microspheres after immersing in PBS solution for 31 days: PLA-E-N (a), PLA-C-N (b), PLA-E-H (c), PLA-C-H (d)

The rapid initial burst release of BSA can be ascribed to the porous structure of all microspheres samples. The addition of NH_4HCO_3 broke the PLA skin layers covering on pores and microspheres, which was convenient for BSA to release. Moreover, the hollow structure of microspheres prepared with ethyl acetate was more favorable than the tortuous path in microspheres prepared with chloroform for the diffusion of BSA. The larger BSA concentration gradient between the microspheres prepared with ethyl acetate and the outer water phase drove a more rapid release rate of BSA[42]. The protein release results of PLA microspheres suggest that the porous structure of microspheres displayed a great influence on the agents release behavior of microspheres.

Cell Experiments

In vitro cell culture study is an effective method for the biocompatibility test of materials due to its well-controlled variables and higher sensitivity than *in vivo* studies[33]. For eventual application *in vivo*, the cytotoxicity of porous PLA microspheres and microspheres with BSA should be tested at first. Microspheres with two representative porous structures —hollow and homogeneous porous—were selected to culture cells. They are PLA-E-N, PLA-C-N, BSA loaded PLA-E-N and BSA loaded PLA-C-N. Figure 6 shows the results of MTT assay, which present the cell viability of MG-63 cultured with microspheres on the second day of cell culture. Although there was a slight decrease in the viability after the cells contacted with PLA microspheres, the cell viability of all microspheres was still higher than 85%, indicating a good biocompatibility of the microspheres samples[43]. This slight decrease of cell viability may be ascribed to the minim residual organic solvent in the microspheres[36, 44–46].

FDA is a non-fluorescent molecule but can produce green fluorescence in viable cells. The distribution of viable cells on PLA microspheres could be clearly observed after the staining of FDA. Figure 7 shows the fluorescence microscopy results of MG-63 cells. The cells were cultured on different PLA microspheres for 7 days. It was obvious that more MG-63 cells distributed homogeneously on microspheres

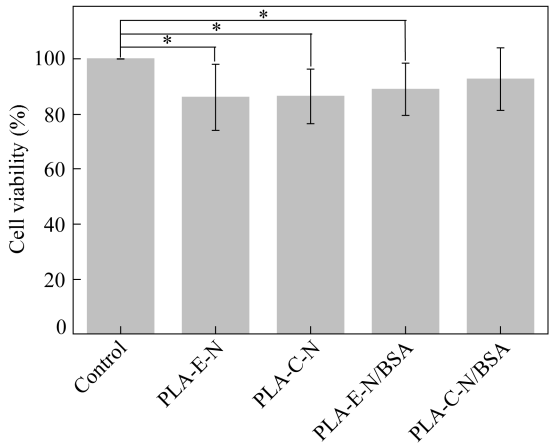


Fig. 6 The MTT assay of PLA microspheres with different porous structures and culture plate as control (* $P < 0.05$, $n = 5$)

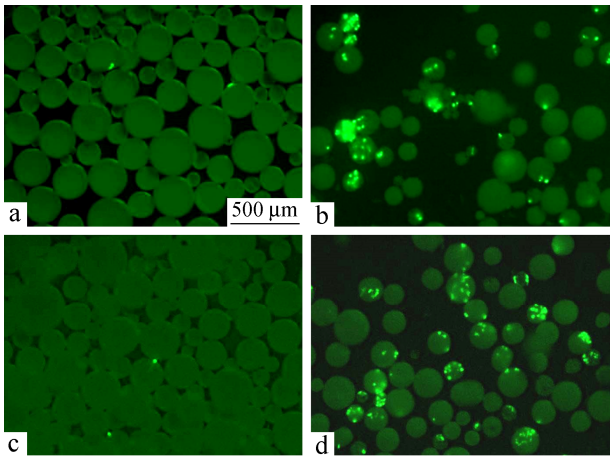


Fig. 7 Fluorescence microscopy photographs of MG-63 cells on PLA microspheres with different porous structures cultured for 7 days: PLA-E-N (a), PLA-C-N (b), PLA-E-N with BSA (c), PLA-C-N with BSA (d)
The scale bar in pictures represents 500 μm.

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prepared with chloroform than those prepared with ethyl acetate. The increased cell number could be ascribed to the relatively rough surfaces of microspheres prepared with chloroform than the smooth surface of microspheres prepared with ethyl acetate. Nevertheless, the addition of BSA in microspheres did not have an obvious effect on cells growth, which is contradictory to the previous study[36]. In our experiments, the topography of microspheres surface was thought to have more effects on cells attachment and growth than BSA.

CONCLUSIONS

PLA microspheres with different porous structures were prepared by the multiple emulsion solvent evaporation method through the changing of organic solvents and the adding of NH_4HCO_3 in the inner water phase. The organic solvent was found to play an important role to affect microsphere structure, and therefore the protein encapsulation and release behaviors. The addition of NH_4HCO_3 had a great effect on the porosity and protein release behaviors of microspheres. Four types of porous microspheres were obtained, which were porous capsular, matrix, microcapsular and multivesicular, respectively. Under the same inner aqueous phase, microspheres prepared with chloroform had higher BSA encapsulation efficiency and less BSA release rate than those prepared with ethyl acetate. The addition of NH_4HCO_3 in microspheres increased the BSA release rate of microspheres. Cell experiments showed that these PLA microspheres had good cell viability and different cell growth behaviors. It was concluded that the relatively rough surface of microspheres prepared with chloroform was more in favor of the growth of cells compared with those prepared with ethyl acetate. This study gives a simple and effective method to control the porous structure, bioactive factor release and cell response of biodegradable polymer microspheres, which will be beneficial for future study on the application of porous biodegradable polymer microspheres in tissue engineering.

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