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Fabrication of hollow porous PLGA microspheres for controlled protein release and promotion of cell compatibility

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ABSTRACT

This letter reports on the fabrication of hollow, porous and non-porous poly(D,L-lactide-co-glycolide) (PLGA) microspheres (MSs) for the controlled release of protein and promotion of cell compatibility of tough hydrogels. PLGA MSs with different structures were prepared with modified double emulsion methods, using bovine serum albumin (BSA) as a porogen during emulsification. The release of the residual BSA from PLGA MSs was investigated as a function of the MS structure. The hollow PLGA MSs show a faster protein release than the porous MSs, while the non-porous MSs have the slowest protein release. Compositing the PLGA MSs with poly(vinyl alcohol) (PVA) hydrogels promoted chondrocyte adhesion and proliferation on the hydrogels.

1. Introduction

The delivery and controlled release of bioactive materials such as proteins and growth factors are critical for the treatment of many diseases and tissue engineering with programmed local delivery [1,2]. Polymer microspheres are among the most effective candidates due to the flexibility in the design and manipulation of their structures and capacity [3–6]. Moreover, the encapsulation of biofunctional material in the MSs [7] has been demonstrated to be effective in maintaining the bioactivity of the material of interest, which is important for the subsequent local release [8]. PLGA is an FDA-approved biomaterial [9] with excellent biocompatibility and biodegradability, and frequently used to prepare protein carriers [10] for the regulation of cell behavior [11] in three-dimension scaffolds [12].

The polymer MS structures strongly influence the protein release kinetics [13]. The protein loaded in polymer MSs can travel through the pores and channels formed during the formation of protein-microsphere composites. For example, a highly porous poly(D,L-lactic acid)-poly(ethylene glycol) (PDLLA-PEG) MSs release a drug at a considerably higher rate than their non-porous counterparts [14]. In addition, hollow non-porous polymer MSs show rapid release of plasmid DNA [15]. However, the effects of hollow porous, porous and non-porous structures of PLGA MSs on protein release rates have not been systematically compared.

In this Letter, PLGA microspheres with controlled structures, *i.e.*, non-porous, porous and hollow porous were systematically prepared, and the protein encapsulation efficiency and release behaviors were comparatively studied. The protein-loaded porous and hollow porous MSs were composited with PVA hydrogels, which are potential candidates for cartilage repair [16] with the cell compatibility yet to be improved [17], to remarkably enhance the chondrocyte adhesion and growth on the hydrogels [18,19]. The effects of MS structures and releasing behavior on the cell growth on the hydrogels are discussed.

2. Experimental

PLGA (Mw = 50,000; lactide:glycolide = 1:1 (mole/mole), Ji'nan Daigang Biology, China) microspheres were prepared by a double emulsion method [3]. Briefly, a 4 mL PLGA/methylene chloride (CH_2Cl_2) solution (2.26 wt%) and a 0.4 mL BSA (15 mg) aqueous solution were emulsified at room temperature, followed by solvent evaporation in a 25 mL 0.5 wt% aqueous PVA solution with mechanical stirring at 1000 rpm. The MSs were thoroughly washed with deionized water and lyophilized for use. Hollow porous PLGA MSs were obtained by increasing the volume ratio of the aqueous phase to the organic phase to 1:2 and the BSA/PLGA weight ratio to 1:3.

Porous PLGA MSs were prepared by a modified water-in-oil-in-water (w/o/w) double emulsion method [20]. Briefly, 3 mL

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$PLGA/CH_2Cl_2$ (5 wt%) and 0.6 mL BSA (BSA/PLGA = 0.6, w/w) aqueous solutions were emulsified in an ice bath, followed by a second emulsification with a 4 wt% aqueous PVA solution (15 mL) to form a w/o/w emulsion with mechanical stirring at 700 rpm. The organic solvent evaporated during stirring in a 300 mL 0.5% PVA solution at 700 rpm for 4 h. The precipitate MSs were washed with deionized water five times and lyophilized.

The above-prepared PLGA microspheres were added into a 10 wt% aqueous solutions of PVA (degree of polymerization: 1750 ± 50, Sinopharm Chemical Reagent Co., Ltd.) with 20 wt% PLGA with respect to PVA, and gelled by six freeze-thawing cycles.

Primary human chondrocytes at passage 6 (CHON-001, American Type Culture Collection (ATCC)) were seeded (1×10^5 cell/mL) on sterilized hydrogels and cultured in complete medium of McCoy's 5A medium (Gibco®, Life Technologies Corportation, USA) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Jinuo Company, Hangzhou, China) under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was replaced every other day. After 1 and 3-day culture, the samples were gently rinsed with PBS, followed by treatment with 2.5 wt% glutaraldehyde overnight. The samples were washed and stained with a 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI, Beyotime Company, Shanghai, China) solution, incubated in dark at 37 °C for 5 min, washed with PBS, and imaged with an inverted fluorescent phase contrast microscopy (OLYMPUS IX51, Japan).

The PLGA particle size was determined with a S3500-special Microtrac Particle Size Analyzer (Microtrac, USA). The PLGA particles, hydrogels, and cell morphology, after carbon sputtering, were imaged with a scanning electron microscope (Hitachi™-1000, Tokyo, Japan) at 15 kV. The average pore size was determined by analyzing the SEM images using an Image-Pro Plus Software (Media Cybernetics). Ultrasound sonication was used to fracture the hollow microspheres for SEM imaging.

The encapsulated protein (BSA) in the MSs was determined by subtracting the protein feed before emulsification ($M_{\text{protein, feed}}$) by the residual protein in solution after emulsification ($M_{\text{protein, residual}}$),

$$M_{\text{protein, encaps}} = M_{\text{protein, feed}} - M_{\text{protein, residual}} \quad (1)$$

where the $M_{\text{protein, feed}}$ and $M_{\text{protein, residual}}$ values in the solutions were determined by using a BCA (bicinchoninic acid) protein assay (Zoman Biotechnology Co., Ltd., Beijing, China).

The protein encapsulation efficiency (%) was calculated as:

$$\text{protein encapsulation efficiency}\,(\%) = \frac{M_{\text{protein, encaps}}}{M_{\text{protein, feed}}} \times 100\% \quad (2)$$

The BSA load in microspheres (%) was calculated as:

$$\text{BSA load in microspheres}\,(\%) = \frac{M_{\text{protein, encaps}}}{M_{\text{microspheres}}} \times 100\% \quad (3)$$

The BSA release was conducted by incubating a suspension of the BSA-containing PLGA MSs in PBS at 37 °C for up to 30 days. At a predetermined time, the suspensions were centrifuged and the PBS solution was collected and replaced with fresh PBS. The 562 nm absorbance of the collected PBS solution was measured by a Spectra Max 190 microplate reader (Molecular Devices, USA). At least three specimens ($n \geq 3$) were tested for each sample.

Quantitative analysis of the cell adhesion and growth on the hydrogels was conducted with a CCK-8 assay. After incubation for 1, 3 or 5 days, the cell-seeded hydrogels were gently rinsed and moved to a new 24-well plate with fresh medium. The chondrocyte-seeded and cultured hydrogels in medium (1 mL) and a 100 μL CCK-8 solution were set as the experimental group. The hydrogels, CCK-8 (100 μL) and medium without cell (1 mL) was set as the control group. The two groups were both incubated for 4 h and then a 200 μL solution of each well was sampled to determine the absorbance at 450 nm by the microplate reader. The absorbance difference of the experimental group and the control group was regarded proportional to the cell numbers on the hydrogels and thus used as a measure of the cell adhesion and proliferation on the hydrogels.

3. Results and discussion

Fig. 1 shows the SEM images of the non-porous, porous, and hollow porous MSs. The microsphere size is in the range of 25–65 μm. The non-porous MSs are solid and smooth on the surface (Fig. 1a). The porous MSs have an average pore diameter of (1.31 ± 0.84) μm (Fig. 1b). The co-emulsion with BSA at specific formulations yielded hollow MSs, the internal structures and the porous shells of which were exposed by fracturing the spheres with sonication (Fig. 1d–f). The shell thickness varies from about 2 μm to 10 μm, containing pores with an average diameter of about (1.0 ± 0.36) μm (Fig. 1d–f).

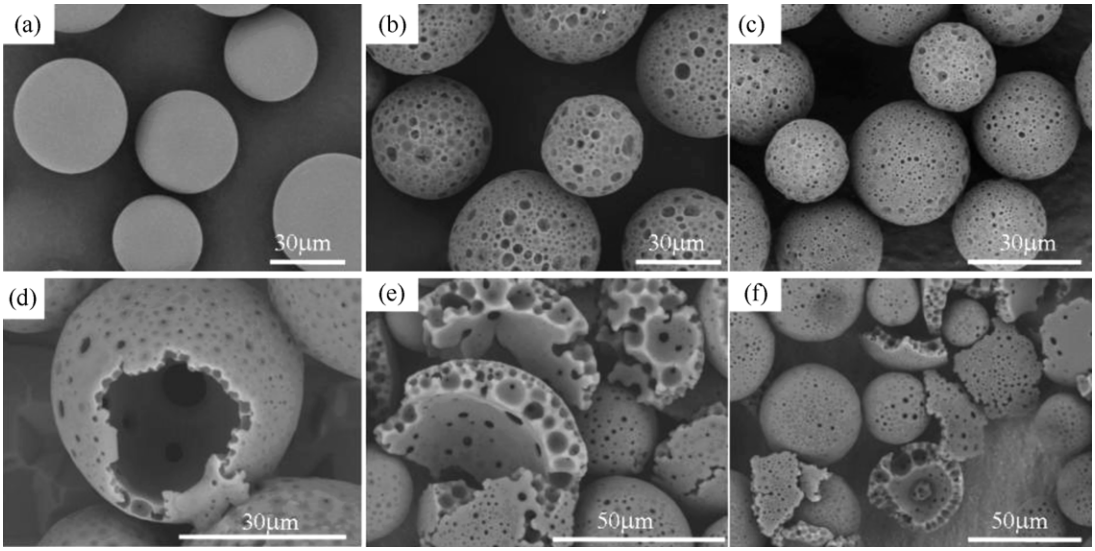

Fig. 1. SEM images of the PLGA microspheres: (a) non-porous MSs, (b) porous MSs, (c) hollow porous MSs, and (d–f) broken hollow porous MS.

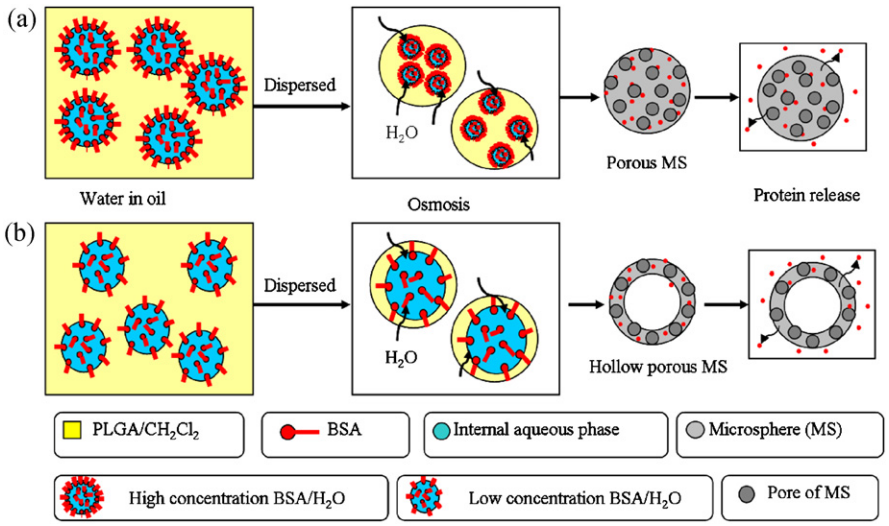

Fig. 2. The schematic illustration of the formation of porous microspheres (a) and hollow porous microspheres (b) and the protein release.

Fig. 2 schematically illustrates the formation of the porous and hollow porous spheres *via* a double emulsion route. Herein, the protein, BSA, acts as the surfactant and porogen during the emulsification process due to its unique amphiphilic nature and water solubility. The BSA molecules preferentially distribute in water and the water-oil interface, forming a gel-like film around oil droplets *via* noncovalent interactions [21,22]. Therefore, a relatively higher BSA content (*e.g.*, 199 mg/mL) may allow for the stabilization of smaller droplets with large specific surface area (Fig. 2a), whereas droplet coalescence may occur in the emulsion with a relatively low BSA content (Fig. 2b). As an extreme example with 20 mg/mL of BSA, during the second emulsification, the internal water phase may form a large droplet encapsulated by the PLGA/CH_2Cl_2 phase (Fig. 2b), whereas small droplets at high BSA content may be encapsulated by PLGA/CH_2Cl_2 droplets (Fig. 2a). After solvent evaporation, with 199 mg/mL of BSA in the internal water phase, porous MSs were obtained. With 20 mg/mL of BSA in the internal water phase, hollow porous MSs were obtained.

The porogen BSA content had a great influence on the pore size. During the formation of double emulsions, the osmosis pressure drives water penetration from the external water phase into internal water phase, leading to the formation of interconnected pores and finally porous PLGA spheres after solvent evaporation (Fig. 2) [20]. It is reasonable to expect smaller pores to have a lower osmosis pressure, which corresponds to a lower BSA concentration in the internal water phase. For example, the BSA used for porous MSs was about 199 mg/mL and 20 mg/mL for hollow porous MSs, respectively. The BSA concentration in the internal aqueous phase of porous MSs is higher than that in the hollow porous MSs. The pore size increased with the BSA concentration (Fig. 1b and c).

During emulsification, most of the BSA in the aqueous phase was encapsulated in the PLGA spheres. The penetration of water into the PLGA spheres will dissolve the encapsulated proteins. According to the formation mechanism (Fig. 2), the large pores in the hollow spheres allow for the dissolution of BSA into water [23], while the complicated micro-channels in the porous MSs can retain more proteins. Thus, the BSA encapsulation efficiency was (64.1 ± 2.4)% for the non-porous MSs, (21.0 ± 4.0)% for the porous MSs, and (4.6 ± 0.5)% for the hollow porous MSs, respectively (Table 1). The BSA loads (Table 1) were (8.0 ± 0.3)% for the non-porous MSs, while the data for the porous MSs and the hollow porous MSs were (12.6 ± 2.4)% and (1.5 ± 0.2)%, respectively.

The diffusion through pores and interconnecting channels is a predominant release mechanism for BSA-loaded PLGA MSs [24]. The structures of these PLGA MSs showed remarkable influence on the protein release behaviors. Fig. 3a shows the BSA release profiles

Table 1
The BSA encapsulation efficiency and the BSA load in PLGA microspheres.

Microspheres	BSA encapsulation efficiency (%)	BSA load in PLGA microspheres (%)
Nonporous MSs	64.1 ± 2.4	8.0 ± 0.3
Porous MSs	21.0 ± 4.0	12.6 ± 2.4
Hollow MSs	4.6 ± 0.5	1.5 ± 0.2

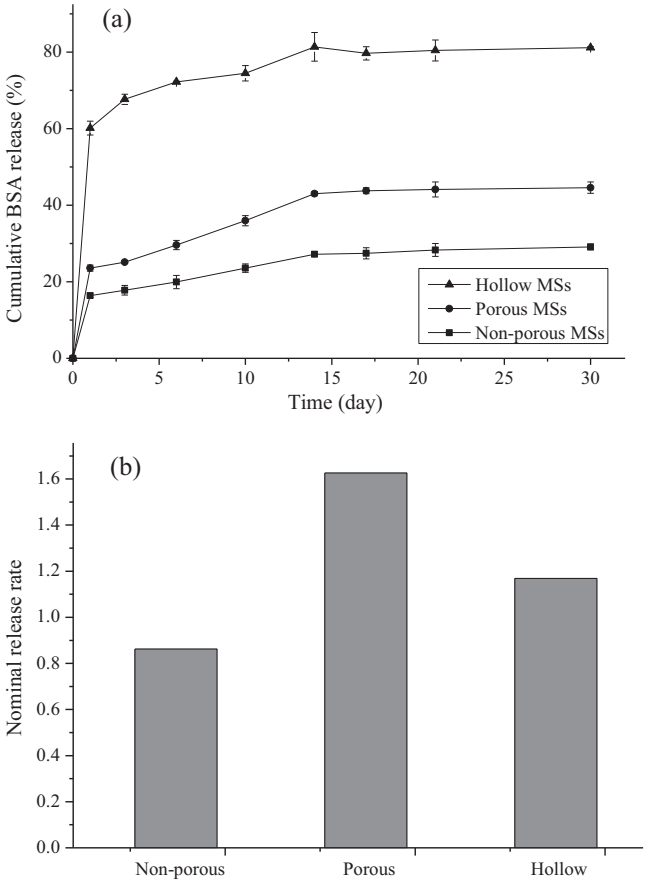

Fig. 3. (a) The BSA release profiles from hollow, porous and non-porous MSs. (b) The nominal release rate of the non-porous, porous and hollow porous MSs.

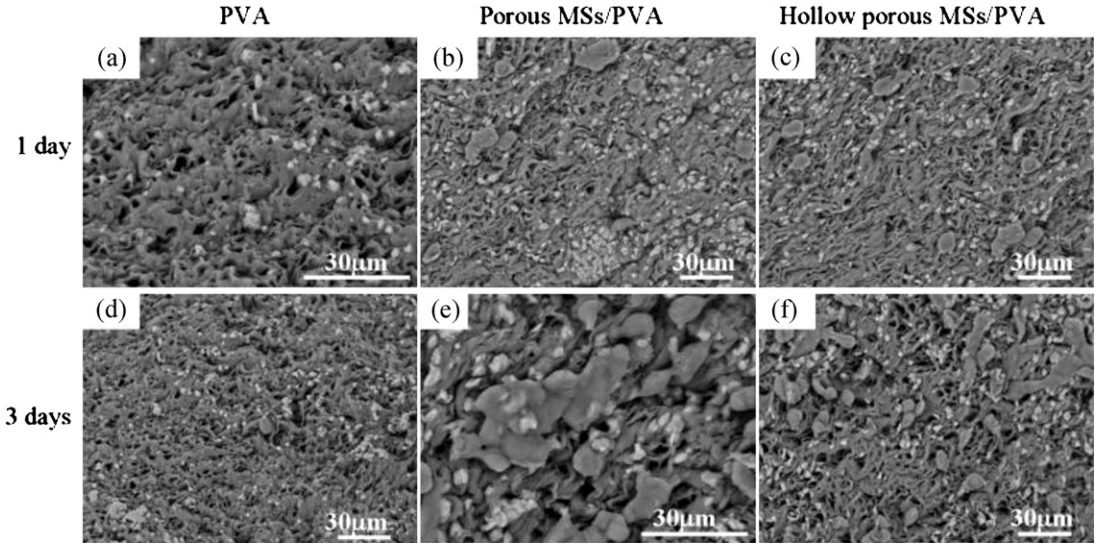

Fig. 4. Representative SEM images of different hydrogels seeded and cultured with chondrocytes for (a–c) 1 day and (d–f) 3 days.

of different MSs at room temperature. The initial burst release was found dependent on the microsphere structures: the burst release was (16.4 ± 0.5)% from non-porous MSs, (23.6 ± 0.8)% from the porous MSs, and (60.1 ± 1.8)% from hollow porous MSs, respectively. Subsequent *pseudo*-linear release kinetics was observed for all the systems up to day 14. The nominal release rates (the slope of the *pseudo*-linear release period) were 1.63 for porous MSs, 1.17 for hollow porous MSs, and 0.86 for non-porous MSs, respectively (Fig. 3b). The BSA release profiles showed a plateau after day 14. Up to day 30, the cumulative BSA release reached (29.1 ± 0.8)% for non-porous MSs, (44.6 ± 1.5)% for porous MSs, and (81.2 ± 0.3)% for hollow porous MSs, respectively. The non-porous MSs had the slowest BSA release rate because the protein inside had to travel through the inner channels of non-porous MSs. In contrast, it was much easier for the protein in the hollow porous MSs to diffuse into PBS.

Protein adhesion is critical for cell adhesion and growth on surfaces [25]. The protein-releasing PLGA spheres greatly promoted the cell adhesion and growth on PVA hydrogels (Fig. 4). As the BSA-loaded PLGA microspheres were composited with PVA hydrogels, which are usually poor for cell attachment ([14], Fig. 4a), the chondrocyte adhesion and growth were remarkably enhanced after *in vitro* seeding and culture for 3 days (Fig. 4 b, c, e and f). The chondrocytes adhered and spread well on the hydrogels, indicating their excellent affinity to the hydrogels.

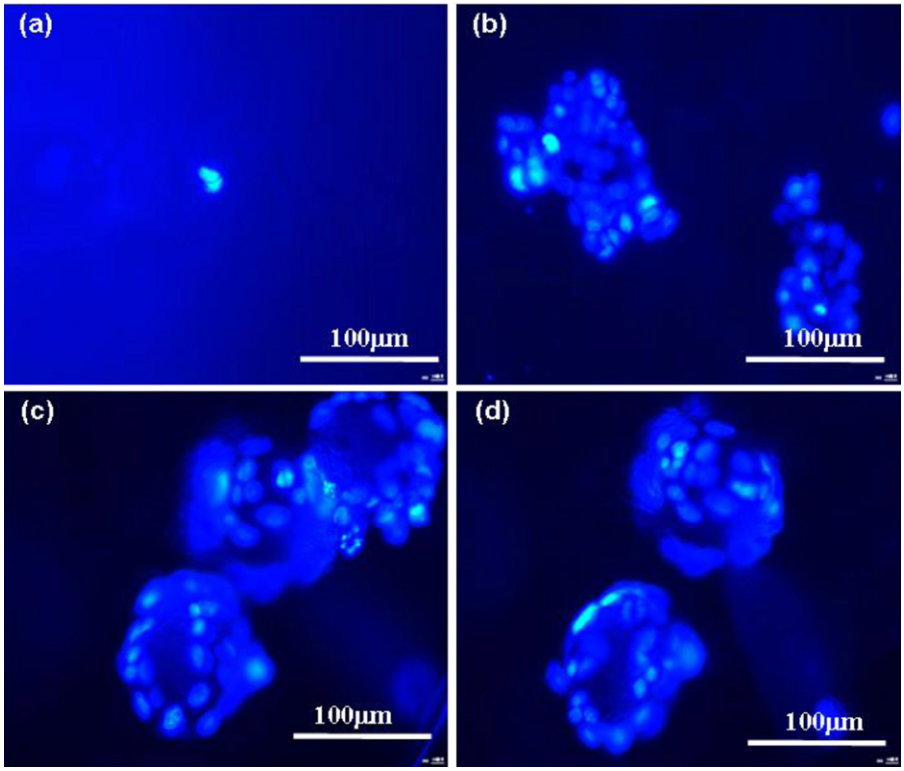

Fig. 5. The fluorescent microscopy images of chondrocytes cultured for 3 days *in vitro* on (a) PVA, (b) non-porous MSs/PVA, (c) porous MSs/PVA, and (d) hollow porous MSs/PVA hydrogels. The PLGA MS content was 20 wt%. DAPI staining was conducted to contrast the nuclei of the chondrocytes.

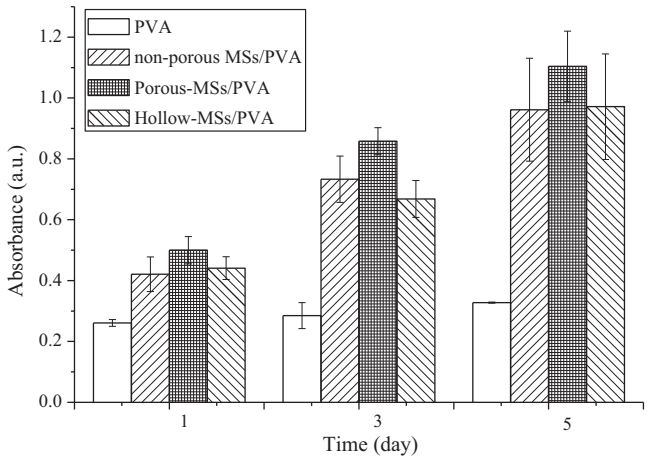

Fig. 6. The absorbance difference obtained by CCK-8 assay of chondrocytes cultured for 1, 3 and 5 days on PVA, non-porous MSs/PVA, porous-MSs/PVA and hollow-MSs/PVA composite hydrogels containing 20 wt% PLGA microspheres. The absorbance differences were calculated by subtracting the absorbance of experimental group to that of control group.

Fig. 5 compares the representative fluorescent images of chondrocytes on the PVA (Fig. 5a), non-porous MSs/PVA (Fig. 5b), porous MSs/PVA (Fig. 5c), and hollow MSs/PVA hydrogels (Fig. 5d) after 3-day culture. More cells were found on the composite hydrogels in comparison to PVA hydrogels, while the cell number on the porous MSs/PVA appeared more than that on the composite hydrogels. There were no significant differences in the cell number on the hydrogels with hollow porous MSs and non-porous MSs.

Quantitative assessment with a CCK-8 assay further revealed the effect of protein release on the cell growth on these composite hydrogels (Fig. 6). The reported differential absorbance values of the experimental group and the control group were presumably proportional to the cell number on the hydrogels. After 1, 3 and 5 days of culturing, the absorbance difference increased for each composite hydrogels except for the virgin PVA hydrogel. In contrast, low absorbance was observed for the PVA hydrogels. These results indicate that the protein-releasing composite hydrogels significantly promote cell adhesion and growth. Moreover, the porous MS/PVA hydrogels appeared more favorable for the cell growth compared to the hydrogels containing non-porous and hollow porous PLGA spheres, provided that the microsphere contents were the same. These results are in good agreement with the fluorescent microscopy observations.

4. Conclusion

Nonporous, porous, and hollow porous PLGA microspheres were prepared using a modified double emulsion method. The effect of the porous and hollow structures on the protein encapsulation and release behaviors has been systematically investigated. The hollow porous MSs had the highest BSA release rate while the non-porous MSs had the slowest protein release. In contrast, the porous MSs showed the maximum protein load and an intermediate release rate. These protein-loaded PLGA MSs, when composited with PVA hydrogels, promote the cell adhesion and proliferation. These results indicate the potential to use such microsphere composite hydrogels for programmed and controlled release of bioactive molecules for drug delivery or tissue engineering scaffolds.

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