RGD Modified PLGA/Gelatin Microspheres as Microcarriers for Chondrocyte Delivery

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Abstract: Poly(lactide-co-glycolide) (PLGA)/gelatin composite microspheres were prepared by an emulsion solvent evaporation technique. RGDS peptides were further immobilized under the catalyzation of water soluble carboximidide (EDAC). Confocal laser scanning microscopy and transmission electron microscopy revealed that the gelatin was entrapped in the PLGA/gelatin microspheres with a manner of separated domains. The contents of the entrapped gelatin and immobilized RGDS peptides were quantified as 0.9 mg/20 mg and 2.1 µg/20 mg microspheres by hydroxyproline analysis and bicinchoninic acid protein assay, respectively. Moreover, difference in morphology of PLGA, PLGA/gelatin and RGDS modified PLGA/gelatin (PLGA/gelatin-RGDS) microspheres was observed by scanning electron microscopy. The PLGA/gelatin and PLGA/gelatin-RGDS microspheres lost their weight rapidly in PBS, but slowly in DMEM/fetal bovine serum. Rabbit auricular chondrocytes were seeded onto the microspheres in vitro to assess their biological performance and applicability as cell carriers. Results show that amongst the PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres, the latter ones have the best performance in terms of chondrocyte attachment, proliferation, viability and sulfated glycosaminoglycans secretion.


Keywords: poly(lactide-co-glycolide); microspheres; gelatin; RGD; chondrocytes

INTRODUCTION

Cartilage regeneration in tissue engineering relies greatly on the scaffolds of various shapes and microstructures.¹,² As a conventional pathway, the chondrocytes are cultured in a porous or hydrogel scaffold made of biological or synthetic polymers.³,⁴ However, in many cases, construction of the cartilage with larger dimensions is hard to achieve because of the poor exchange of nutrients and metabolic wastes.⁵–⁷ Recently, more attention has been paid to injectable scaffolds because of the minimal incision during transplantation,⁸–¹² among which the microspheres have been used as the initial supports in tissue engineering. Moreover, the microspheres can function as cell microcarriers,⁵–⁹ delivery vehicles for drugs¹³–¹⁹ and growth factors,²⁰–²⁵ and injectable scaffolds as well.²⁶,²⁷

The large surface area of the microspheres enables sufficient exchange of nutrients and metabolic wastes and allows rapid cell expansion.⁵–⁷ Compared to the porous counterparts, cell culture on the microspheres may produce a larger number of cells within a relatively short period. Moreover, the routine trypsinization procedure, which may be harmful to the cells, can be avoided as cell expansion can be achieved through simple addition of new microspheres. The cell-seeded microcarriers can be directly injected into the defect, which can then assemble into a three-dimensional scaffold in vivo to induce cell infiltration and tissue regeneration. The transplanted cells can proliferate and differentiate in situ in a native histological condition as well, which is beneficial to maintaining their phenotype.

Poly(ε-lactide-co-glycolide) (PLGA) has been widely used in the fields of tissue engineering and drug delivery. In addition to forming porous scaffolds, this material can also be easily formulated into microspheres which have been frequently functioned as drug delivery vehicles.¹⁴–¹⁹ More recently, efforts have been made to use the micro-
spheres as injectable scaffolds for cartilage tissue engineering. To enhance the positive biological response, however, surface modification of the PLGA scaffolds and microspheres is usually required. Currently, an appealing and effective strategy is to incorporate bioactive species such as cell growth factors, peptides and proteins into the material, resulting in the biomimetic scaffolds and microspheres with bioactive functions. So far we have developed several facile approaches including covalent bonding, grafting and coating and layer-by-layer assembly to anchor the bioactive species onto polymeric membranes and scaffolds.

Many natural biomacromolecules such as gelatin contain biologically active domains such as RGD sequences, which are recognized as ligands that can specifically bind with integrin on the cell membrane. Thus, these molecules can effectively promote cell adhesion, migration, differentiation and proliferation. Furthermore, the use of synthetic RGD and RGD derived peptides for surface modification is advantageous to the use of proteins, because the short sequences may undergo less conformational changes. Moreover, their concentration and spacing on the substrate can be modulated to enable the effective attachment and morphology maintenance of the cells.

In this study, we intend to develop a PLGA based cell carrier with enhanced bioactivity for chondrocyte delivery, which may be used as an injectable scaffold for cartilage repair. The PLGA/gelatin composite microspheres are fabricated by an emulsion solvent evaporation technique. RGDS peptides are then immobilized by bonding with the gelatin molecules under the catalyzation of water soluble carbodiimide (EDAC). Finally, the microspheres are used to culture chondrocytes in vitro to assess their biological performance and applicability as the cell carriers.

MATERIALS AND METHODS

Materials

PLGA (85/15 lactide/glycolide ratio, $M_n = 108,000$, $M_w = 203,000$) was purchased from China Textile Academy, China. Gelatin and RGDS (arginine-glycine-aspartic-serine, Arg-Gly-Asp-Ser) were obtained from Shanghai Chemical Industries and Shanghai Xibao Biotech, China, respectively. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), rhodamine B isothiocyanate, 1,9-dimethylmethylene blue and chondroitin sulfate were purchased from Sigma-Aldrich. Enhanced bicinechinonic acid (BCA) protein assay kit was purchased from Beyotime Institute of Biotechnology, China. Chloramine T and poly(vinyl alcohol) 124 (PVA 124, Mw 85,000-124,000, 98-99% hydrolyzed) were supplied by Shanghai Medicine and Chemical Company, China. Ninhydrin and p-dimethylaminobenzaldehyde were supplied by Shanghai San’aisi Chemical Company, China. Rhodamine labeled gelatin (Rd-gelatin) was prepared by mixing 10 mg rhodamine B isothiocyanate with 50 mL 0.5% (w/v) gelatin solution at 4°C for 48 h. The free dye was dialyzed off in triple-distilled water for 4 weeks. All other chemicals and reagents were used as received.

Preparation of PLGA/Gelatin Composite Microspheres

PLGA/gelatin microspheres were fabricated using a double emulsion solvent evaporation technique (W/O/W). Briefly, 1 mL 20 mg/mL gelatin solution was emulsified in 5 mL 50 mg/mL PLGA/methylene chloride solution containing 1 mL emulsifier (Tween 80/Span 80: 5/1, v/v) under sonication for 15 min. The water-in-oil emulsion was added dropwise to a stirred 70 mL 3% w/v PVA and 3% v/v emulsifier aqueous solution. After sonication for 15 min, a multiple water-in-oil-in-water (W/O/W) emulsion was formed. The multiple emulsion was stirred for 24 h (600 rpm) at room temperature to evaporate the organic solvent, followed by treatment in a desiccator under vacuum for 12 h to remove the residual methylene chloride. The microspheres were centrifuged at 3,000 rpm for 5 min, washed 5 times with triple-distilled water to get rid off PVA, emulsifiers and free gelatin, and lyophilized for 24 h. The PLGA/gelatin microspheres were then separated by standard sieves and stored in a desiccator at 4°C. Pure PLGA microspheres were prepared and sieved at the same conditions except that gelatin was not added.

To determine the gelatin distribution by confocal laser scanning microscopy (CLSM, Bio-Rad Radiance 2100), the normal gelatin was substituted by Rd-gelatin to prepare the PLGA/Rd-gelatin microspheres. The excitation wavelength was set at 543 nm. The sections of gelatin entrapped microspheres were investigated by transmission electron microscopy (TEM, PHILIPS TECNAI-10). The microspheres were encapsulated in epoxy resin for 3 day and cut into sections of about 100 nm in thickness, which were mounted on copper grids and examined after uranyl acetate staining for 5 min.

Immobilization of RGDS Peptides onto the PLGA/Gelatin Microspheres

EDAC was used to covalently immobilize RGDS peptides onto the PLGA/gelatin microspheres. The protocol was similar to that used for immobilization of peptides on an amino-enriched substrate. Briefly, the RGDS peptides (0.25 mg/mL in PBS) were activated by 8 mM EDAC at 37°C for 15 min under agitation. The PLGA/gelatin microspheres were immersed into the activated RGDS peptide solution at room temperature for 2 h. After rinsed with fresh PBS (pH 7.4), the PLGA/gelatin microspheres were lyophilized. The PLGA and PLGA/gelatin microspheres treated by EDAC solution without RGDS were used as controls. All the microsphere samples were stored at 4°C before use.

The amount of unreacted peptides was determined using the bicinchoninic acid protein assay. Briefly, after the immobilization reaction, 20 μL supernatant of the RGDS
containing solution was reacted with 200 μL BCA working solution at 70°C for 60 min. The absorbance at 560 nm was recorded by a microplate reader (Bio-Rad 550). The RGDS peptide content was quantified by referring to a calibration curve obtained with a known RGDS concentration exposed to the same conditions. The immobilized RGDS on the PLGA/gelatin microspheres was obtained from the difference between the feeding and the detecting amount in the supernatant. In this study, at a constant feeding ratio of RGDS/microspheres (1 mg/g), about 10.4% of the peptides were immobilized (~104 μg/g or ~2.1 μg/20 mg RGDS/microspheres).

The morphology of the PLGA, PLGA/gelatin and RGDS modified PLGA/gelatin (PLGA/gelatin-RGDS) microspheres was observed under scanning electron microscopy (SIRION 100, FEI) after coating with a thin gold layer.

Measurement of the Gelatin Content
Hydroxyproline (Hyp) analysis was employed to quantitatively detect the amount of gelatin entrapped in the PLGA/gelatin microspheres. Briefly, the microspheres were placed in a glass tube containing 2 mL 6 M HCl solution. After being sealed at reduced pressure, the tube was heated at 120°C for 24 h to degrade the gelatin and the PLGA completely. After evaporation of HCl at 70°C, the residues were dissolved in 2 mL water. One milliliter of 0.05 M chloramine-T solution was added and reacted with this solution at 25°C for 20 min, followed by addition of 1 mL 3.15 M perchloric acid solution. After 5 min, the mixture was treated with 1 mL 10% dimethylaminobenzaldehyde/ethylene glycol monomethyl ether solution at 60°C for 20 min. Absorbance at 560 nm of the final solution was measured on a UV-vis spectrophotometer (UV-Probe 2550, Shimadzu). The gelatin content was quantified by referring to a calibration curve obtained with pure gelatin suffered from the same treatments.

Weight Loss of the Microspheres
Weight change of the microspheres was monitored as a function of incubation time in PBS and Dulbecco’s minimum essential medium (DMEM)/10% fetal bovine serum (FBS) at 37°C, respectively. The microspheres were taken from the mediums at a predetermined time interval, washed with distilled water, lyophilized and weighed. The weight loss ratio was defined as (W0 − Wt)/W0 × 100%, where W0 and Wt represent the initial weight and the weight at the detecting time, respectively. The amount of the gelatin released from the microspheres during the PBS incubation was also quantified by the hydroxyproline (Hyp) analysis as described above.

Chondrocyte Seeding, Adhesion and Proliferation
Chondrocytes were isolated from cartilage tissue of rabbit ears (New Zealand white) under the institutional guideline and were routinely cultured.8 Briefly, the cartilage tissue was cut into small pieces. The chondrocytes were isolated by incubating the cartilage pieces in DMEM containing 0.2% collagenase type II (Sigma) at 37°C for 5 h under agitation. The isolated chondrocytes were centrifuged, resuspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were then seeded in an 11 cm plastic tissue culture dish (Falcon) and were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. After a confluent cell layer was formed, the cells were detached using 0.25% trypsin in PBS and were resuspended in PBS, and used for the experiments.

A known weight of PLGA (control sample), PLGA/gelatin and PLGA/gelatin-RGDS microspheres were sterilized by 75% ethanol solution and UV irradiation.36–38 After washed by PBS and cell culture medium, they were placed into plastic tubes (20 mg/tube) which can prohibit cell adhesion. Into each tube 10 × 106 chondrocytes were seeded. The final concentration of the microspheres for all the samples was controlled as 20 mg/mL. To promote the cell adhesion on the microspheres, the tubes were shaken every 15 min in the first 1 h. The culture conditions were same as described above. The attached cell number was determined after the cells were seeded for 24 h. Before harvesting the attached cells by trypsinization, two-gentle washings with PBS were performed. The detached cells were then counted under a haemocytometer. All data were averaged from three parallel experiments.

Chondrocyte Viability Detected by MTT Assay
The cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl-2,5-dimethyl tetrazolium bromide) assay during the cell culture. Briefly, into each culture tube 100 μL MTT solution (5 mg/mL) was added. The cells were continually cultured for another 5 h. During this period, viable cells could reduce the MTT to formazan pigment, which was dissolved by 300 μL dimethyl sulphoxide (DMSO) after removal of the culture medium. The absorbance at 490 nm was recorded by a microplate reader (Bio-Rad 550).

Chondrocyte Distribution and Morphology
The chondrocytes on the microspheres were observed under SEM after cultured for 7 day. Before measurement, the cells were sequentially dehydrated in a series of ethanol solutions. The microspheres were further treated by isopropanolacetate for 15 min at room temperature. Finally, critical point drying was performed, and the cells were observed under SEM (Stereoscan 260, Cambridge) after coating with a thin gold layer.

Glycosaminoglycans (GAGs) Secretion Assay
The total content of sulfated glycosaminoglycans (GAGs) secreted by the chondrocytes was quantified using a 1,9-dimethylmethylene blue method with chondroitin sulfate as
Briefly, the microspheres were collected and freeze-dried after the chondrocytes were cultured for 7 days. The freeze-dried microspheres were digested with papain in a buffer of 0.1 M KH₂PO₄, 5 mM Na₂EDTA (disodium ethylenediaminetetraacetic acid), and 5 mM cysteine·HCl at pH 6.0 and 60°C for 6 h. The dye solution was prepared by dissolving 16 mg of 1,9-dimethylmethylene blue in 1 L distilled water containing 3.04 g glycine, 2.37 g NaCl and 95 mL 0.1 M HCl. Two milliliters of 1,9-dimethylmethylene blue solution were added to 200 µL papain digested solution. After 5 min, the absorbance was measured at 525 nm by UV-vis spectroscopy (UV-Probe 2550, Shimadzu).

Statistical Analysis

The experimental data were reported as mean ± standard deviation. Statistical analysis was performed using the two population Student’s t-test. The significance level was set as p < 0.05.

RESULTS

Fabrication of PLGA/Gelatin Composite Microspheres

In this study, the optimal gelatin and PLGA concentrations were set as 20 mg/mL and 50 mg/mL, respectively. Under these conditions, the yield of the microspheres could be up to ~80% with a gelatin entrapment ratio of 45.5% and a gelatin content of 0.9 mg/20 mg PLGA microspheres. A representative particle size distribution for the microspheres is shown in Figure 1. The majority of the microspheres are below 100 µm in diameter. Therefore, the microspheres fabricated under these conditions and with a size smaller than 100 µm shall be used for all the following experiments.

To figure out the gelatin distribution within the microspheres, Rd-gelatin was entrapped under the same conditions. An overall homogeneous fluorescence emission from the microsphere was recorded by confocal microscopy [Figure 2(a)], implying that the gelatin exists within the entire microsphere with no sign of a core-shell structure. Yet, uneven distribution of the emission from the microsphere can be observed, as more clearly demonstrated by the line profile [Figure 2(b)]. This would mean that there exist microscopically detectable phase domains, with the brighter regions of Rd-gelatin and the darker regions of PLGA. This is reasonable since intrinsically these two polymers are not thermodynamically miscible.

Distribution of the gelatin domains in the microspheres was further confirmed by TEM [Figure 2(c)]. In this experiment, the microspheres were embedded in epoxy resin, and the sliced thin films were suspended in water. Since gelatin is dissolvable in water, in this process the gelatin domains were removed from the thin slices, leading to vacancies which were separated by the continuous PLGA phase, as indicated by the arrow heads in Figure 2(c). Therefore, one can conclude that gelatin is entrapped in the PLGA microspheres in a manner of separated domains.

Figure 2. (a) CLSM image of a PLGA microsphere loaded with Rd-gelatin, and (b) a line profile of the fluorescence intensity derived from (a) to show the distribution of gelatin. (c) TEM image of part of a PLGA/gelatin microsphere. The dark background in (c) is epoxy resin.
Surface Morphology of the Microspheres

Although all the microspheres have a spherical macroscopic structure (Figure 3), compared to the smooth surface of the PLGA microspheres [Figure 3(a)] folds and creases were observed on the surfaces of the PLGA/gelatin [Figure 3(b)] and the PLGA/gelatin-RGDS microspheres [Figure 3(c)]. Obviously the surface change is attributed to the gelatin incorporation.

Weight Loss of the Microspheres

Weight loss of the microspheres was monitored as a function of time in PBS and DMEM/FBS at 37°C, as shown in Figure 4. The release amount of gelatin from both PLGA/gelatin and PLGA/gelatin-RGDS microspheres in PBS increased along with the incubation time, with a faster rate at the initial stage but slower at the later stage, that is

![Figure 3. SEM images to show the surface morphology of (a) PLGA, (b) PLGA/gelatin, and (c) PLGA/gelatin-RGDS microspheres.](image)

![Figure 4. (a) Release percentages of gelatin from the PLGA/gelatin and PLGA/gelatin-RGDS microspheres in PBS at 37°C as a function of incubation time. No significant difference (p > 0.05) was found between any time point. (b) Weight loss of PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres in PBS and DMEM/FBS at 37°C as a function of incubation time, as well as the release of PLGA from the PLGA/gelatin and PLGA/gelatin-RGDS microspheres in PBS.](image)
following parabola regimes [Figure 4(a)]. At day 7, the released gelatin percentages from the PLGA/gelatin and PLGA/gelatin-RGDS microspheres were 57% and 59%, which increased to 67% (0.6 mg) and 73% (0.66 mg) at day 14, respectively. No significant difference ($p > 0.05$) for these two samples was found at each time interval. Compared to the faster release of gelatin in PBS, the weight loss of the control PLGA microspheres was rather slow regardless of the type of mediums (e.g. PBS or DMEM/FBS), although it follows a parabola regime too [Figure 4(b)]. The total weight loss at day 14 in PBS and in DMEM/FBS was 1.6% and 1.3%, respectively. By contrast, the weight loss of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres in PBS was very fast, and significantly faster than that of the control PLGA microspheres ($p < 0.05$). At day 14, the weight loss percentages of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres reached to 7.9% and 8.3%, respectively. However, in DMEM/FBS the weight loss rate of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres was significantly slower ($p < 0.05$). For example, at day 14, the weight loss percentages of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres in DMEM/FBS were only 3.1% and 2.7%, respectively. The release of PLGA from the PLGA/gelatin-RGDS and PLGA/gelatin microspheres in PBS was specially monitored to compare with the weight loss of the control PLGA microspheres. As shown in Figure 4(b), the release of PLGA from the PLGA/gelatin-RGDS and PLGA/gelatin microspheres was significantly faster than that from the control PLGA microspheres after 14 days in PBS ($p < 0.05$).

**Figure 5.** SEM images to show the morphology of (a,d) PLGA, (b,e) PLGA/gelatin, and (c,f) PLGA/gelatin-RGDS microspheres after they were incubated in (a–c) PBS and (d–f) DMEM/FBS at 37°C for 7 day.

Compared to the identical surface morphology of the PLGA microspheres before [Figure 3(a)] and after PBS [Figure 5(a)] or DMEM [Figure 5(d)] incubation, many micron pores emerged on the PLGA/gelatin [Figure 5(b)] and PLGA/gelatin-RGDS [Figure 5(c)] microspheres after they were incubated in PBS for 7 day. Moreover, after incubation some of the composite microspheres lost their

**Figure 6.** Chondrocyte numbers on PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres after the cells were cultured for 24 h and 7 day. After 7 days, the chondrocyte number on the PLGA/gelatin-RGDS microspheres was significantly higher than that on the PLGA microspheres ($p < 0.05$). Cell seeding density $10^5$ cells/mL; microsphere density 20 mg/mL.
spherical shape too. On the contrary, after incubation in DMEM/PBS for 7 day, no obvious micron pores were observed on the PLGA/gelatin [Figure 5(e)] and PLGA/gelatin-RGDS [Figure 5(f)] microspheres. These results coincide very well with the weight loss experiments [Figure 4(b)].

Chondrocyte Growth on the Microspheres

Generally, the chondrocyte number counted before 24 h is regarded as the attached cells. As shown in Figure 6, the order of attached cell number on the microspheres was: PLGA < PLGA/gelatin < PLGA/gelatin-RGDS, but no significant difference was found. After culturing for 7 day, the cells on all the microspheres were expanded, and their numbers were found to be $2.1 \times 10^4$, $4.4 \times 10^4$, and $5.4 \times 10^4/20$ mg PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres, respectively. However, a significant difference ($p < 0.05$) was found between the cell number on the PLGA/gelatin-RGDS and PLGA microspheres.

MTT assay was further used to detect the chondrocyte viability as a function of culture time (Figure 7). The viability on the control PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres increased 2, 2.3, and 2.4 times compared to 1 day after the cells were cultured for 7 day, respectively. Compared to that of the control PLGA microspheres, a significantly higher optical density ($p < 0.05$), which is proportional to the cell viability, was recorded on the PLGA/gelatin and PLGA/gelatin-RGDS microspheres after the cells were cultured for 3 day. Figure 7 shows that the PLGA/gelatin-RGDS microspheres always have the highest cell viability throughout the culture period.

After the cells were cultured for 7 day, their morphology was observed by SEM (Figure 8). Compared to a few cells

![Figure 7. Optical density (Abs, analyzed by a MTT assay method) of chondrocytes on PLGA, PLGA/gelatin and PLGA/gelatin-RGDS microspheres as a function of culture time. The cell viability of the PLGA/gelatin-RGDS microspheres was significantly higher ($p < 0.05$) than that of the PLGA and PLGA/gelatin microspheres after 7 days. Cell seeding density $10^3$ cells/mL.](image)

![Figure 8. SEM images to show cell morphology on (a) PLGA microspheres, (b) PLGA/gelatin microspheres, and (c) PLGA/gelatin-RGDS microspheres after the chondrocytes were cultured for 7 days.](image)
on the PLGA microspheres [Figure 8(a)], a relatively larger number of cells were observed on the PLGA/gelatin microspheres [Figure 8(b)], especially on the PLGA/gelatin-RGDS microspheres [Figure 8(c)]. This observation is consistent with the cell viability results (Figure 7). While the chondrocytes on the PLGA microspheres were polygonal, those on the PLGA/gelatin and PLGA/gelatin-RGDS microspheres were more round-shaped with abundant cytoplasm. As the chondrocytes in their natural ECM show a round or elliptical shape, one can conclude that the composite microspheres are more biomimetic to support the chondrocyte adhesion and growth.

The amount of secreted GAGs by the chondrocytes after culturing for 7 day on the microspheres was further quantified and compared in Figure 9. It shows that the total GAGs amount on the PLGA/gelatin and PLGA/gelatin-RGDS microspheres are $\times 3$ and $\times 4$ times higher than that on the PLGA microspheres, respectively [Figure 9(a)]. The amount of GAGs secreted by the chondrocytes grown on the PLGA/gelatin-RGDS microspheres was significantly higher ($p < 0.05$) than that on the PLGA microspheres, but no significant difference was found between the PLGA/gelatin and PLGA/gelatin-RGDS microspheres after 7 days. Cell seeding density $10^4 \times 10^5$ cells/mL.

**DISCUSSION**

Although suitable for clinical uses, the biological performance of the synthetic polyesters such as PLGA is not good enough in terms of cell adhesion, proliferation and phenotype preservation. To obtain injectable cell carriers with better cell responsiveness, gelatin and RGDS peptides are incorporated into the PLGA microspheres, following the procedures illustrated in Scheme 1. In this process, the gelatin molecules are physically entrapped into the hydrophobic PLGA matrix during the W/O/W emulsion. Under the catalyzation of EDAC, the RGDS peptides are further immobilized by amidization with the gelatin molecules. It has to note that during this process the gelatin molecules are more or less crosslinked too, which accounts for the stability of the entrapped gelatin.

After optimizing the fabrication conditions, we found that in a system of 3% PVA and Tween80/Span80 (5/1, v/v), the obtained microspheres have narrower size distribution with minimum debris by-products. We also compared the influence of concentrations of gelatin (10 mg/mL and 20 mg/mL) and PLGA (10, 30, and 50 mg/mL) on the microsphere yields and the gelatin entrapment ratios. Results show that at a given PLGA concentration, a higher gelatin concentration yielded microspheres with a higher gelatin content. While at a larger PLGA concentration, both the gelatin entrapment ratio and the microspheres yield were improved.

The incorporated gelatin molecules provide the necessary anchoring sites for further immobilization of the cell specific moieties such as RGD peptides. Here we coupled RGDS by the convenient EDAC chemistry. The experiment showed that at a constant feeding ratio of RGDS/microspheres (1 mg/g), the grafting efficiency of RGDS increased steadily along with the reaction time, and reached to a platform after 2 h. Since the primary purpose of this work is to demonstrate the possibility of the protocol, here the reaction time was fixed at 2 h at which the maximum
amount of immobilized RGDS could be expected at the present experimental conditions.

Unlike the PLGA, dehydration of the gelatin/water droplets resulted in the gelatin microspheres with fluctuant structures on their surfaces (image not shown). It is possible that during the water evaporation process, the gelatin droplet may form a skin layer first. After complete evaporation of the remained water, uneven shrinkage of the skin layer will eventually forms these fluctuant structures. The same process can of course occur during fabrication of the PLGA/gelatin microspheres. In the present case, the different shrinking ability of the PLGA and gelatin components may aggravate the surface textures [Figure 3(b)]. The more creases observed on the PLGA/gelatin-RGDS microsphere surfaces are caused by the rehydration and subsequent redehydration procedures [Figure 3(c)].

In DMEM/FBS, the weight loss rate of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres was significantly slower \((p < 0.05)\) [Figure 4(b)]. One possible reason could be that the existing proteins in the DMEM/FBS may retard the release of gelatin because of their structure similarity. It is worth mentioning that the EDAC treatment can also crosslink the gelatin molecules besides the RGDS immobilization. Nevertheless, the relatively slower weight loss rate in DMEM is beneficial for use of these microspheres in the cell culture.

Figure 4(b) shows also the weight loss of PLGA from both PLGA/gelatin and PLGA/gelatin-RGDS microspheres, which was calculated correspondingly by subtraction of gelatin weight loss from the total weight loss. It shows that the weight loss of PLGA from the composite microspheres was always larger than that from the pure PLGA microspheres. For example, at day 14 in PBS, the weight loss ratio of PLGA from both composite microspheres reached to \(\sim 5\%\). Since the PLGA degradation speed should be less influenced by incorporation of the gelatin, we expect that this extra weight loss should be attributed to the mechanical instability of some PLGA tiny particles in the composite microspheres, which can be partly evidenced by the TEM [Figure 2(c)] and SEM observation (Figure 5). This conveys a hint that the gelatin domains may be embedded with some tiny PLGA particles in the composite microspheres. Upon dissolution of the gelatin glue, these tiny particles shall be released, leading to formation of these micron pores and the larger weight loss as shown in Figure 4(b).

The composite microspheres thus obtained should have better performance on cell adhesion and growth. Chondrocytes were thus cultured to detect the applicability of the microspheres as the injectable cell carriers. It is known that the RGD peptide family can bind with the integrins on cell membrane, thus facilitating cell adhesion. Cell adhesion is the first event in which the anchorage-dependent cells come into contact with a substrate. Only after that event the cells can spread and proliferate. Since the gelatin contains also lots of bioactive domains including RGD peptides, our strategy then results in more cytocompatible microcarriers which are suitable to attract and deliver live cells as chondrocytes. The above results show that on the PLGA/gelatin-RGDS microspheres all the cell attachment, proliferation, viability and GAGs secretion are significantly improved, endowing these microspheres with applicability as chondrocyte microcarriers and an injectable scaffold for \textit{in vivo} chondrogenesis.

**CONCLUSIONS**

Biodegradable and cytocompatible PLGA/gelatin and PLGA/gelatin-RGDS microspheres have been facilely prepared by a water-in-oil-in-water emulsion solvent evaporation method (W/O/W). The gelatin was entrapped in the microspheres in a manner of separated domains. After gelatin incorporation, folds and creases appeared on the surfaces of the PLGA/gelatin and the PLGA/gelatin-RGDS microspheres, which are caused by the rehydration and subsequent redehydration procedures. The physical and biological performance of the PLGA/gelatin and PLGA/gelatin-RGDS microspheres was compared with the PLGA microspheres. The PLGA/gelatin and PLGA/gelatin-RGDS microspheres have a slower rate of weight loss in DMEM/FBS than that in PBS, implying that the existing proteins in the DMEM/FBS may retard the release of gelatin. \textit{In vitro} cell culture found that the PLGA/gelatin-RGDS microspheres can most effectively support the attachment, proliferation, viability and GAGs secretion of chondrocytes.
With their injectable feature, the PLGA/gelatin-RGDS microspheres are potentially applicable as cell carriers, which may be further piled into a three-dimensional scaffold for in vivo chondrogenesis.

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