Chitosan modified poly(l-lactide) microspheres as cell microcarriers for cartilage tissue engineering

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ABSTRACT

The surfaces of poly(l-lactide) (PLLA) microspheres were modified by chitosan via a method of hydrolysis and grafting-coating to improve their compatibility to chondrocytes. The PLLA microspheres with a diameter of 74–150 μm were fabricated by an oil/water emulsion solvent evaporation method, followed by hydrolysis in alkaline solution to produce a larger number of carboxyl groups. Using water-soluble carbodiimide as a coupling reagent, chitosan was covalently grafted onto the microspheres. Due to the physical entanglement and insolubility at neutral pH, unbonded chitosan molecules were stably remained to yield a large amount of coated chitosan. Biological performance of the control PLLA and the chitosan-coated PLLA microspheres were assessed in vitro culture of rabbit auricular chondrocytes. After 24 h and 7 d culture, the chitosan-coated PLLA microspheres, especially the ones with larger chitosan amount, exhibited stronger ability to promote cell attachment and proliferation, and maintain the secretion function of the chondrocytes. Therefore, the chitosan-coated PLLA microspheres can be potentially used as the injectable cell microcarriers for chondrogenesis in cartilage tissue engineering.

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1. Introduction

Natural articular cartilage itself lacks a blood supply to support spontaneous self-repair. Tissue engineering and regenerative medicine have demonstrated their great success in regeneration of cartilage with normal function and bioactivity. In this process, a construct of chondrocytes/three-dimensional (3D) scaffold is often implanted into a defect site [1–3]. Therefore, the scaffolds play an important role in cartilage regeneration. There are various technologies for fabricating 3D porous scaffolds [4], including thermally induced phase separation (TIPS), freeze-drying, particle leaching, and 3D printing. More recently, injectable scaffolds have gained much attention due to their advantages of maintaining cell differentiated phenotype and minimal incision during the transplantation [5–7]. Biodegradable microspheres are a type of injectable materials, which can be used as cell microcarriers [5,8–10], delivery system for drugs [11] and growth factors [12] as well as further assembly into porous scaffolds to form thick construct for cell infiltration and clinical applications [13]. The microspheres could serve as the cell microcarriers in tissue regeneration because of their unique properties for scaling up 3D cell culture and large surfaces for promoting cell expansion and maintaining cell phenotype [5,8–10]. More importantly, microcarriers carrying cells can be directly injected into tissue defect site without undergoing an open surgery process.

Various types of microcarriers have been developed so far [8], of which synthetic polysters including polylactide (PLA), polyglycolide (PGA) and poly(lactide-co-glycolide) (PLGA) are particularly attractive due to their good mechanical properties, processibility, biocompatibility and biodegradability [9,10]. Acting as the cell carriers, however, surface modification of the polysters such as poly(l-lactide) (PLLA) is still required to improve their bioreponsivity for cells. One of the appealing and effective strategies is to enrich their surfaces with bioactive components such as fibronectin (Fn) and RGD (Arg-Gly-Asp) peptide sequence [14], forming a biomimetic interface.

Chitosan is another frequently applied biomaterial. It is composed of glucosamine and N-acetylglucosamine, which is structurally similar to glycosaminoglycan (GAG) produced by chondrocytes and other analogs such as chondroitin 4-sulphate, chondroitin 6-sulfate and keratin sulfate [14–16]. GAG is one of the principle components of the normal cartilage-specific extracellular matrix (ECM) and can stimulate chondrogenesis [17]. Therefore, similar bioactivity of chitosan to GAG can be expected. Previous results show that the chitosan-based constructs including 3D porous scaffolds [18], hydrogels [19] and microcarriers [20] are beneficial of wound healing. We also found that a chitosan-g-
lactose/heparin film could accelerate chondrocyte growth with preserved phenotype [21]. Moreover, the large number of amino groups on the chitosan molecules makes further modification possible by methods of covalent bonding [22], layer-by-layer assembly [23], plasma treatment [15] and grafting-coating [9,24].

Herein PLLA microspheres are modified to obtain cell carriers, which can be used as cell delivery vehicle and an injectable scaffold. Surface hydrolysis of the microspheres produces abundant carboxyl groups, by which chitosan is immobilized by a grafting-coating technique (Scheme 1). In vitro culture of rabbit auricular chondrocytes is finally conducted to assess their biological performance.

2. Materials and methods

2.1. Materials

Poly(1-lactide) (PLLA; \(M_n = 200\) kDa and \(M_w = 400\) kDa) was purchased from China Textile Academy. Chitosan (deacetylation degree 85%, \(M_n = 6.2 \times 10^5\)) was obtained from Haidebei Bio-engineering Co. (Qingdao, China). Its density was measured as 1.42 g/cm\(^3\) by a bottle method. Poly(vinyl alcohol) 124 (PVA 124, average \(M_w = 85–124\) kDa and 98–99% hydrolyzed) was supplied by Shanghai Medicine and Chemical Company, China. \(p\)-Dimethylaminobenzaldehyde was supplied by Shanghai San’ai Chemical Company, China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), chondroitin sulfate, trypsin, toluidine blue O (TBO), fluorescein isothiocyanate (FITC) and fluorescein diacetate (FDA) were purchased from Sigma–Aldrich. Fluorescein isothiocyanate (FITC)-labeled chitosan (FITC-chitosan) was prepared as follows: 0.5% (w/v) chitosan/3% acetic acid solution was reacted with 0.5 mg/ml FITC at 4°C for 48 h and then dialyzed with 0.05 M acetic acid for 4 weeks [25]. All other chemicals and bioreagents were used as received.

2.2. Preparation of PLLA microspheres

PLLA microspheres were prepared by an oil/water emulsion solvent evaporation technique [26]. Briefly, 1 g PLLA was dissolved in 20 ml methylene chloride (CH\(_2\)Cl\(_2\)) to obtain 5% transparent PLLA/CH\(_2\)Cl\(_2\) solution. The solution was then poured into 100 ml deionized water containing 0.5% (w/v) PVA under agitation with a rate of 500 rpm by a mechanical stirrer. The agitation was lasted for 24 h at room temperature to evaporate the organic solvent. The microspheres were collected after centrifugated at 6000 rpm for 5 min, washed five times with triple-distilled water to remove PVA and then freeze-dried for 24 h. The PLLA microspheres with a diameter of 74–150 \(\mu\)m were separated by standard sieves and used for all the following experiments.

2.3. Surface hydrolysis of the PLLA microspheres

The PLLA microspheres were immersed in an aqueous solution of 2.5% NaOH at 60°C for a given period, and then rinsed successively with 0.1 M HCl and a large amount of deionized water. After lyophilized, surface hydrolyzed PLLA microspheres (PLLA-COOH microspheres) were obtained.

The amount of the carboxyl groups introduced onto the PLLA microspheres was determined by a method of TBO analysis [23]. Briefly, 20 mg dried PLLA-COOH microspheres were placed into a
glass tube containing 4 ml 0.5 mM TBO solution with a pH value of 7.2. Formation of an ionic complex between the –COO\(^-\) groups and the cationic dye was allowed to proceed for 12 h at room temperature. After discarding the residue TBO solution, the microspheres were further rinsed with 0.1 mM NaOH to remove the unbonded TBO molecules. The bonded TBO on the PLLA microspheres was then desorbed by incubation in 4 ml 50% acetic acid solution for 10 min. Absorbance at 633 nm was measured on a UV–vis spectrophotometer (UV-Probe 2550, Shimadzu). A calibration curve was obtained with TBO/50% acetic acid solution recorded at the same conditions. The calculation is based on the assumption that 1 mol TBO complexes exactly with 1 mol carboxyl groups [23].

The weight loss ratio (%) of the microspheres after hydrolysis is defined as 
\[
\text{Weight loss} \% = \frac{(W_1 - W_2)}{W_1} 
\]
where \(W_1\) and \(W_2\) represent the weights of the PLLA microspheres before and after surface hydrolysis, respectively.

2.4. Grafting-coating of chitosan on the hydrolyzed PLLA microspheres

After surface hydrolysis, the PLLA microspheres were incubated in 8 mM EDAC/PBS at 37 °C for 2 h, followed by rinsing with a large amount of deionized water to remove the physically adsorbed EDAC [27]. The microspheres were then incubated in 1% chitosan/3% acetic acid solution at 37 °C for 2 h with occasional shaking. After centrifugated, the microspheres were sequentially rinsed with fresh PBS and water and then lyophilized. Since chitosan used here is not soluble in water, besides the chemically bonded, the physically adsorbed or entangled chitosan was also largely retained on the microsphere surfaces [24]. Thus, comparatively larger amount of chitosan can be introduced by this grafting-coating approach.

Chitosan amount on the microspheres was quantitatively detected by UV–vis spectroscopy after the polymers were hydrolyzed in HCl solution [28]. Briefly, 15 mg microspheres were hydrolyzed in 2 ml 6 M HCl solution in a sealed glass tube at 100 °C for 12 h to obtain the degradation products. After removal of HCl at 70 °C, the residues were dissolved in 2 ml water, into which 1.5 ml 3.5% (v/v) acetyl acetone/0.33 M sodium phosphate tribasic dodecylzyl-2,5-dimethyl tetrazolium bromide (MTT) solution was added into each tube, respectively. The chondrocytes were cultured on the control PLLA microspheres and chitosan modified PLLA microspheres for 24 h and 7 d, 40 μl 5 mg/ml MTT solution was added into each tube, respectively. The chondrocytes were continually cultured for another 4 h. During this period, viable cells could reduce the MTT to formazan pigment, which was then dissolved by 400 μl dimethyl sulfoxide (DMSO) after removal of the culture medium. 150 μl of the DMSO solution was added into a well of a 96-well tissue culture polystyrene plate, whose absorbance at 590 nm was recorded by a microplate reader (Bio-Rad 550). All data were averaged from three parallel experiments.

2.5. Chondrocyte culture

Chondrocytes were isolated from cartilage tissue of rabbit ears (New Zealand white) under the institutional guideline and were routinely cultured [9]. Briefly, the cartilage tissue was cut into small pieces. The chondrocytes were isolated by incubating the cartilage pieces in Dulbecco’s minimum essential medium (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% fetal bovine serum (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% CO\(_2\) 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 the PLLA microspheres (control sample) and the chitosan-coated microspheres were sterilized by 75% ethanol solution and UV irradiation. After washed by PBS (pH 7.4), they were placed into plastic tubes (20 mg/tube), which can prohibit cell adhesion. Into each tube, 20 × 10⁴ chondrocytes were seeded. The final concentration of the microspheres was controlled at 20 mg/ml. In order to accelerate cell adhesion onto the microspheres, the tubes were shaken every 15 min in the first 1 h and then cultured as described above.

2.6. Cell viability

Cell viability was measured by MTT (3-(4,5-dimethyl)thiazol-2-yl-2,5-dimethyl tetrazolium bromide) assay [9]. After the chondrocytes were cultured on the control PLLA microspheres and chitosan modified PLLA microspheres for 24 h and 7 d, 40 μl 5 mg/ml MTT solution was added into each tube, respectively. The chondrocytes were continually cultured for another 4 h. During this period, viable cells could reduce the MTT to formazan pigment, which was then dissolved by 400 μl dimethyl sulfoxide (DMSO) after removal of the culture medium. 150 μl of the DMSO solution was added into a well of a 96-well tissue culture polystyrene plate, whose absorbance at 590 nm was recorded by a microplate reader (Bio-Rad 550). All data were averaged from three parallel experiments.

2.7. Cell morphology and distribution

Chondrocytes on the microspheres were observed under SEM after cultured for 7 d. After fixed by 2.5% glutaraldehyde at 4 °C for 24 h, the cells were sequentially dehydrated in a series of ethanol solution (30%, 50%, 75%, 95% and 100%). The microspheres were further treated by acetic and isoamylacetate successively, each for 15 min at room temperature. Finally, critical point drying was performed, and the cells were observed under SEM (Stereoscan 260, Cambridge) after coated with a thin gold layer.

After the chondrocytes were cultured for 7 d, cell distribution on the microspheres was also observed by fluorescence microscopy (Bio-Rad Radiance 2100). To visualize the viable cells, the cells were incubated in 5 μg/ml FDA/PBS for 10 min. In this process, FDA (no fluorescence) could penetrate through the cell membranes and was hydrolyzed into fluorescein by the viable cells.

2.8. GAG secretion assay

The total content of sulfated GAGs secreted by the chondrocytes was determined quantitatively using a 1,9-dimethylmethylene blue method with chondroitin sulfate as a standard [16,29]. Briefly, the microcarriers were rinsed by PBS for three times after the chondrocytes were cultured for 14 d, and then were digested with 150 μg/ml papain in a buffer of 50 mM KH\(_2\)PO\(_4\), 5 mM EDTA, and 5 mM cysteine-HCl at pH 6.8 and 60 °C for 6 h. The dye solution was prepared by dissolving 16 mg of 1,9-dimethylmethylene blue in 1000 ml distilled water containing 3.04 g glycine, 2.37 g NaCl and 95 ml 0.1 M HCl. 2.5 ml 1,9-dimethylmethylene blue solution was added to 100 μl papain digested solution. After 5 min, absorbance at 520 nm was measured by UV–vis spectroscopy.

2.9. Statistical analysis

Experimental data were analyzed using ANOVA. The significant level was set as \(p < 0.05\). Results are reported as mean ± standard deviation.
3. Results and discussion

3.1. Surface hydrolysis of PLLA microspheres

To overcome the disadvantage of poor cell–material interaction, the PLLA microspheres were stably coated with a chitosan layer by a grafting-coating technique. The surface hydrolysis breaks some of the ester groups, yielding carboxyl (–COOH) and hydroxyl (–OH) groups on the PLLA chain termini, by which immobilization of bioactive components such as proteins, enzymes, growth factors, polysaccharides and peptides becomes possible (Scheme 1). Fig. 1 shows that amount of the carboxyl groups (–COOH) on the PLLA microspheres increased rapidly at the first 2 min, then reached almost a constant value of $1.2 \times 10^{-9}$ mol/mg microspheres. At time 0 the measured value belongs to the original carboxyl groups at PLLA chain termini. The constant platform of COOH amount implies that the dissolution or dissociation rate of the oligomeric fragments equilibrates with that of the hydrolysis.

Hydrolysis in principle is a partial degradation of the polymer chains. Therefore, weight loss unavoidably occurred with the reaction time, as shown in Fig. 2. Along with prolongation of the hydrolysis, the weight loss ratio increased rapidly. For instance, a weight loss of 18% was achieved at 10 min, and even over 40% at 50 min. SEM observation showed that the original PLLA microspheres were spherical with hill-like surface morphology (Fig. 3a), which should be a result of inhomogeneous shrinkage during solvent evaporation. 2 min hydrolysis did not bring notable alteration (Fig. 3b), whereas 10 min hydrolysis created needle-like cracks with a length of several micrometers on the microspheres (Fig. 3c). The cracks and grooves were further developed at 30 min (Fig. 3d). When the time was further prolonged to 50 min (Fig. 3e), the entire microspheres were severely eroded, resulting in surface breakage, volume shrinkage and a hollow structure. This observation is in accordance with the weight loss results (Fig. 2). Based on these results, the microspheres hydrolyzed for 2 and 10 min were chosen for further studies.

![Fig. 1. COOH density on the PLLA microspheres as a function of hydrolysis time. The hydrolysis was conducted in 2.5% NaOH solution at 60 °C.](image1)

![Fig. 2. Weight loss ratio of the PLLA microspheres as a function of hydrolysis time. The hydrolysis was conducted in 2.5% NaOH solution at 60 °C.](image2)

![Fig. 3. SEM images of (a) the original PLLA microspheres and the PLLA microspheres hydrolyzed in 2.5% NaOH solution at 60 °C for (b) 2 min, (c) 10 min, (d) 30 min and (e) 50 min.](image3)
3.2. Grafting-coating of chitosan on the hydrolyzed PLLA microspheres

Now that the carboxyl groups have been introduced, the PLLA microspheres can be now modified further. The grafting-coating produced covalently grafted chitosan on the microsphere surface by forming amide bonds, while some un-grafted chitosan molecules were physically entangled and intertwined with the covalently grafted chitosan layer since the chitosan is only acidic soluble. Therefore, by contrast to a thin layer produced by most simple grafting methods, a comparative large amount of chitosan can be obtained [9].

Fig. 4 shows that the immobilized chitosan amount was as large as $8.7 \pm 2.1 \mu g/mg$ on the PLLA-chit2 microspheres (the chitosan-coated PLLA microspheres with an initial hydrolysis time of 2 min), and significantly increased to $14.2 \pm 2.4 \mu g/mg$ on the PLLA-chit10 microspheres (the chitosan-coated PLLA microspheres with an initial hydrolysis time of 10 min) ($p < 0.05$). Taking into account the sphere diameter (74–150 μm), PLLA density (1.27 g/cm³) and chitosan density (1.42 g/cm³), a simple calculation gives that the surface chitosan density and the chitosan thickness are 0.21 μg/mm² and 0.34 μg/mm², and 145 nm and 237 nm, respectively. Since the surface –COOH groups are almost same, the higher chitosan amount of the PLLA-chit10 microspheres should be attributed to their rougher surfaces (Fig. 3c), which cause more physical adsorption of the chitosan. This is in good agreement with the SEM observation (Fig. 5a), in which smoother surfaces of the chitosan-coated microspheres were identified. Moreover, the initial needle-like cracks (Fig. 3c) disappeared, implying that they were covered by a chitosan film which was visible in some places. By substituting the normal chitosan with FITC-chitosan, confocal laser scanning microscopy (CLSM) observation reveals that the black PLLA microsphere was evenly covered by a bright skirt (Fig. 5b). All these results confirm that a chitosan layer has been successfully formed on the PLLA microspheres.

3.3. In vitro chondrocyte growth on the microspheres

After chitosan-coating, the microspheres are expected to have better biocompatibility with respect to cell attachment and growth. In vitro chondrocyte culture was thus conducted and the results are shown in Figs. 6–9. Viable cells were confirmed at 24 h on all the samples regardless of the chitosan immobilization (Fig. 6). After 7 d culture, the cytoviability was greatly improved too, indicating that the cells can proliferate. However, compared with the control PLLA microspheres, higher cell viability was measured on the chitosan-coated PLLA microspheres at 24 h and 7 d. At the same culture time, the cytoviability of the PLLA-chit10 microspheres was signif-

![Fig. 4. Chitosan contents on the PLLA microspheres which were initially hydrolyzed for (a) 2 min and (b) 10 min.](image)

![Fig. 5. (a) A SEM image of the chitosan-coated PLLA microsphere. (b) A CLSM image to show the distribution of FITC-labeled chitosan on the microsphere surface. The initial hydrolysis time was 10 min.](image)

![Fig. 6. Chondrocyte viability on PLLA microspheres before and after chitosan modification. Cell seeding density $20 \times 10^4$ cells/ml; microsphere density 20 mg/ml; culture time 24 h and 7 d.](image)
Fig. 7. SEM images to show cell morphology on the PLLA microspheres at a culture time of 7 d. (a) Control PLLA microspheres. (b) and (c) Chitosan-coated PLLA microspheres which were initially hydrolyzed for 2 min and 10 min, respectively. (d), (e) and (f) are higher magnification of (a), (b) and (c), respectively. Cell seeding density $20 \times 10^4$ cells/ml; microsphere density 20 mg/ml.

icantly higher ($p < 0.05$) than that of the PLLA-chit2 microspheres too.

After 7 d culture the chondrocytes could be found on all the samples (Fig. 7). Noticeably, there were a larger number of cells aggregating and anchoring tightly on the modified microspheres (Fig. 7b, c, e and f), in particular on the PLLA-chit10 microspheres (Fig. 7c and f). Some of the cells bridged between these microspheres to form agglomerates. By contrast, only a few separate cells were observed on the control (Fig. 7a and d), hardly to link the microspheres together. Observation by fluorescence microscopy (Fig. 8) is in good agreement with the above results. More chondrocytes (the bright green dots) aggregated and distributed densely on the chitosan-coated microspheres (Fig. 8b and c) to form cell clusters, particularly on the ones having higher amount of chitosan (Fig. 8c), whereas a fewer cells were found on the control microspheres (Fig. 8a).

Fig. 8. Fluorescence microscopic images to show cell distribution on the PLLA microspheres after 7 d culture and FDA staining. (a) Control PLLA microspheres. (b) and (c) Chitosan-coated PLLA microspheres which were initially hydrolyzed for 2 min and 10 min, respectively. Cell seeding density $20 \times 10^4$ cells/ml; microsphere density 20 mg/ml.
To further compare cell functions on different microspheres, secretion of GAG was quantified using a 1,9-dimethylmethylene blue method, as shown in Fig. 9. GAG is one of the main components of cartilage matrix secreted by chondrocytes. When culturing in vitro, secretion of GAG by chondrocytes can be regarded as a sign of maintenance of cell phenotype. Fig. 9 reveals that the chondrocytes on all the samples could normally secret GAG after cultured for 14 d. However, a higher level of GAG was measured on the chitosan-coated PLLA microspheres (Fig. 9b) and than that of the control PLLA microspheres (Fig. 9a). Significant difference (p < 0.05) and non-significant difference (p > 0.05) were found between the PLLA-chit10 microspheres and the PLLA-chit2 microspheres with the control, respectively. Altogether with the viability results and morphology observation, one can conclude that the chitosan-covered PLLA microspheres have stronger ability to support chondrocyte attachment, proliferation and phenotype preservation, thus have greater opportunity to be used as cell carriers for biomedical applications.

4. Conclusions

Chitosan-coated PLLA microspheres have been successfully prepared by a method of surface hydrolysis and grafting-coating. The hydrolysis produces more carboxyl groups and needle-like cracks on the microsphere surfaces. With still longer reaction time such as 50 min, severe erosion and weight loss takes place. The immobilized chitosan is 8.7 ± 2.1 μg/mg and 14.2 ± 2.4 μg/mg microspheres on the PLLA-chit2 microspheres and the PLLA-chit10 microspheres, respectively, which correspond to a surface chitosan density and a chitosan thickness of 0.21 μg/mm² and 0.34 μg/mm², respectively. In vitro cell culture shows that the chitosan-covered PLLA microspheres, especially the PLLA-chit10 microspheres, have stronger ability to support chondrocyte attachment, proliferation and phenotype preservation. Therefore, they have greater opportunity to be used as cell carriers for biomedical applications, in particular as an injectable scaffold for cartilage repair.

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References


Fig. 9. Secreted GAG amount on the microspheres after 14 d culture. (a) Control PLLA microspheres. (b) and (c) Chitosan-covered PLLA microspheres which were initially hydrolyzed for 2 min and 10 min, respectively. Cell seeding density 2 × 10⁴ cells/ml; microsphere density 20 mg/ml.

