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Collagen nanofiber-covered porous biodegradable carboxymethyl chitosan microcarriers for tissue engineering cartilage

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ABSTRACT

Nanofibrous collagen-coated porous carboxymethyl chitosan microcarriers (CMC-MCs) were successfully fabricated for use as injectable cell microcarriers. A modified phase separation method combined with temperature controlled freeze-extraction was used for formulating the CMC-MCs. Collagen nanofibers were immobilized onto the surfaces of the CMC-MCs via covalently anchoring some collagen molecules first and more molecules self-assembling into nano-scale fibrous networks afterward. Scanning electron microscopy and hydroxyproline colorimetry analysis revealed that more collagen was immobilized on the CMC-MCs with collagen molecules anchored initially. *In vitro* cell culture revealed that chondrocytes could adhere, proliferate, and remain differentiated on the nanofiber-coated CMC-MCs. Optical microscopy and confocal laser scanning microscopy showed that chondrocytes grew to confluence on the CMC-MCs within 3 days post-seeding. Subsequently, several confluent CMC-MCs attached to each other, forming tissue-like aggregates after 7 days culture. The mRNA expression of type II collagen was much stronger in chondrocytes cultured on the nanofiber-coated CMC-MCs for 7 days than those cultured in 24-well plates or on CMC-MCs without initial treatment. These porous CMC-MCs could be utilized for cultivating cells and for application in cartilage tissue engineering as injectable scaffolds for cell delivery.

1. Introduction

A wide range of tissue engineering systems using cells from the recipient is currently being investigated for the regeneration of cartilage [1–4]. The microcarrier (MC) system recently has gained much attention in cartilage tissue engineering [5,6]. An important advantage of this system is that cell-seeded MCs can be delivered directly to the place that needs repair; MCs and expanded cells can be injected or administered directly, thus eliminating the need for re-seeding the retrieved cells into a scaffold delivery system [7]. Nevertheless, major challenges still need to be addressed to make this system a clinical success [8].

When used *in vivo*, biodegradable MCs are especially appropriate since conventional cell MCs, such as Cytodex-3 and the Cultispher series, are less or even non resorbable by the host body [9–12]. Based on this consideration, porous chitosan MCs have been used to culture chondrocytes isolated from articulate cartilage of newborn rabbits [13]. Chitosan is a natural-origin polymer extracted from crustaceans [14]. However, the degradation rate of chitosan is relatively slow and uncontrollable [15]. Consequently, to accelerate and control the degradation rate of chitosan, we previously developed a technique to induce carboxyl groups into chitosan chains via a carboxymethylation reaction followed with EDC cross-linking. The degradation rate of the new chitosan derivative, carboxymethyl chitosan (CMC), was significantly accelerated compared to chitosan and could be regulated through different

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cross-linking extents by EDC, while retaining excellent mechanical properties [16].

Cross-linked CMC has many advantages such as non-toxicity, processibility, and controllable biodegradability; however, this novel polymer system is biologically inert and lacks cell binding sites [17]. Therefore, surface treatment is required to improve its biocompatibility. Many natural biomacromolecules, such as collagen, have domains in their molecules recognized as ligands that can specifically bind with integrin on cell membranes, and thus can effectively enhance cell attachment and spread [18]. Particularly, it has been supposed that nano-scale collagen fibers could provide the most favorably spaced binding sites and geometrical signals because their structure is similar to the natural collagen supramolecular arrangement in the extracellular matrix (ECM) [19–21].

In this context, for the first time we report here the nanofibrous collagen-coated CMC microcarriers (CMC-MCs) for chondrocyte culture, which can be expected to find important application as injectable scaffolds for cartilage tissue engineering. For this purpose, a modified phase separation method combined with temperature controlled freeze-extraction was used for formulating the CMC-MCs. Collagen nanofibers were immobilized onto the surfaces of the CMC-MCs via a convenient method. Scanning electron microscopy (SEM) and hydroxyproline colorimetry analysis (HCA) were used to qualitatively and quantitatively detect the amount of collagen immobilized on the CMC-MCs respectively. *In vitro* study revealed that chondrocytes could adhere, proliferate, and remain differentiated on these CMC-MCs. Optical microscopy and confocal laser scanning microscopy (CLSM) were employed to characterize the morphology of chondrocytes growing on the CMC-MCs. Chondrocyte growth was also evaluated at the protein synthesis and gene transcription levels.

2. Materials and methods

2.1. Materials

Chitosan was obtained from Qingdao Haisheng Co., Ltd (Qingdao, China). The degree of deacetylation was estimated to be 93.5% using the ^{1}H NMR method and the viscosity–average relative molecular weight was 1.8×10^6 Da. Lysozyme, Hank's Balanced Salt Solution (HBSS), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and monochloroacetic acid were purchased from Sigma. Dulbecco's minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone. All other inorganic reagents were purchased from Beijing Chemical Engineering Factory (Beijing, China) and were of analytical grade.

2.2. Preparation of porous CMC-MCs

CMC was synthesized according to a previous work [16]. Briefly, chitosan (10 g) was immersed into 100 ml of 50 wt% NaOH solution to swell and alkalize for 24 h in a flask. Monochloroacetic acid (15 g) dissolved in isopropanol (20 ml) was added into the reaction mixture dropwise for 30 min and stirred for 4 h at 50 °C. Then the reaction mixture was filtered to remove the solvent. The filtrate was dissolved in 100 ml of deionized water. After centrifugation and removal of the insoluble precipitate, ethanol (400 ml) was slowly added and the product was precipitated. The solid was filtered and then suspended in 80% ethanol (100 ml). The mixture was stirred for 30 min after 10 ml HCl (37%) had been added. The solid was filtered and rinsed in 80% ethanol until neutral, and then air-dried. The products were the H-form of CMC.

Porous CMC-MCs were prepared using a phase separation technique according to a previous work [13]. Briefly, CMC aqueous solution (6%, w/v) was obtained, filtered and degassed. This solution (70 ml) was then poured into a 250 ml Wolff bottle containing 30 ml carbon tetrachloride, 105 ml toluene, and 5 ml span-80, and the mixture was stirred at 3000 rpm by a mechanical stirrer. The agitation was continued for 30 min at 25 °C to produce uniform sized beads. Then the beads were immediately poured into 1000 ml liquid nitrogen (−196 °C). The MCs were frozen overnight, dehydrated in pre-cooled absolute ethanol (−20 °C) for 48 h [22], and then washed extensively with absolute ethanol and deionized water at room temperature and air-dried.

Subsequently, two different CMC-MCs were fabricated. CMC-MCs comprised solely of CMC were termed CMC-0-MCs. Those cross-linked in the mixture comprised of 0.25 mg/ml collagen were termed CMC-25-MCs, and those in the 0.5 mg/ml collagen mixture were designated as CMC-50-MCs. The process, referred to as initial collagen treatment, can be summarized as follows. The CMC-MCs were cross-linked by immersion in 50 mM MES (pH 5.5) containing 30 mM EDC and 6 mM NHS in a mixture with certain amounts of type I collagen. After reaction for 4 h, the spheres were washed twice in 0.1 M Na_2HPO_4 (pH 9.1) for 1 h. Finally, the spheres were washed with 1 M NaCl for 2 h and with 2 M NaCl for 1 day, followed by washing with distilled water and air drying [16].

2.3. Swelling index measurements

Dried CMC-MCs were first wetted by immersion in 70% ethanol, washed three times with cold distilled water, and incubated in phosphate buffered saline (PBS, pH 7.2) for 12 h. Water contents were measured by weighing wet CMC-MCs after the excess water had been wiped from the surface. The swelling index (SI) was calculated from the following equation:

$$\mathrm{SI} = (W_{\mathrm{wet}} - W_{\mathrm{dry}})/W_{\mathrm{dry}} \times 100\%,$$

where W_{wet} and W_{dry} are the wet weight and dry weight of the CMC-MCs, respectively.

2.4. Fabrication of nanofibrous collagen coated CMC-MCs

Solutions of type I collagen were prepared from rat tail tendons using a modified method as previously reported [23]. In this method, tendons from the tails of Wistar rats (200–300 g) were extracted under 70% ethanol. The tendons were washed with absolute ethanol and placed in

sterilized 0.02 M acetic acid (approximately 100 ml solution/g wet tendon). Collagen from the tendons was allowed to solubilize in acetic acid for 72 h at 4 °C. The mixture was then centrifuged at 14,000 rpm for 45 min, and the supernatant was collected and centrifuged at 20,000 rpm for 30 min. The clear, collagen-containing supernatant was collected and stored at 4 °C. The concentration of collagen was determined after lyophilization by BCA protein assay (Sigma).

Subsequently, CMC-MCs were coated with collagen nanofibers through automatic self-assembly. For this purpose, the pH and osmolarity of the liquid collagen solution had to be raised to physiological levels (pH 7.2 and osmolarity 300 mOsm) by thoroughly mixing the following in a 1.5 ml centrifuge tube quickly at 4 °C:

a. Seven microliters of 5% (w/v) sodium bicarbonate.
b. One hundred microliters of HBSS (10× concentrated).
c. A predetermined volume of collagen solution to obtain the desired final concentration in a final volume of 1 ml.
d. A predetermined amount of CMC-MCs.
e. A predetermined amount of purified water to bring the final solution volume to 1 ml.
f. A predetermined amount of 0.1 M NaOH to obtain a final solution pH of 7.2.

The mixtures were thoroughly mixed by a vibrator at 4 °C and incubated at 37 °C for 15 min to allow collagen molecules to assemble into fibrous networks on the surface of the CMC-MCs.

2.5. Characterization of CMC-MCs

To observe the MC morphology by SEM (KYKY-2800, China), samples were mounted on a metal stub and coated with gold. Pore diameter estimates were obtained by analyzing the digital SEM images from sectioned samples. HCA was employed to quantitatively detect the amount of collagen on the CMC-MCs. The CMC-MCs were placed in a glass tube containing 3 ml of 12 M HCl solution. After the tube was sealed at reduced pressure, it was incubated at 110 °C for 24 h to completely degrade the collagen and CMC. After evaporation of HCl, the residues were dissolved in 2 ml water, and 0.5 ml of 50 mM chloramine-T solution was added. The sample was incubated at 25 °C for 25 min, followed by the addition of 1 ml 3.15 M perchloric acid solution. Five minutes later, the mixture was treated with 1 ml of 10% 4-dimethylaminobenzaldehyde/ethylene glycol monomethyl ether solution at 65 °C for 20 min. The absorbance at 560 nm was measured under a microplate reader (Bio-Rad 550). The collagen content was quantified by referring to a calibration curve obtained with pure rat tail type I collagen (Sigma) under the same conditions.

Table 1
Primers used for RT-PCR analysis and expected sizes of PCR products

Genes	Direction	Primer sequences	Size (bp)
β-actin	Sense	5′-TGGACTTCGAGCAAGAGA-3′	450
	Antisense	5′-TACACCTAGTCGTTCGTC-3′	
Pro-collagen II	Sense	5′-GCACCCATGGACATTGGAGG-3′	323
	Antisense	5′-AGCCCCGCACGGTCTTGCTT-3′	

2.6. Chondrocyte culture

Chondrocytes were isolated from the articulate cartilage of a newborn rabbit (New Zealand white). Briefly, cartilage tissue was incised into small pieces. Chondrocytes were isolated by incubating the cartilage segments in DMEM with a high glucose concentration containing 0.2% type II collagenase (Gibco) at 37 °C for 4 h. The isolated chondrocytes were centrifuged and then re-suspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was then seeded in a 100-mm polystyrene cell culture dish (Corning®, USA, seeding density 2×10^4 cells/cm^2) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After a confluent cell layer was formed (after 2–3 days), the cells were detached using 0.25% trypsin in PBS and resuspended in the supplemented culture medium as described above for use in the following experiments.

2.7. In vitro studies

The mixture of chondrocytes and sterilized CMC-MCs were inoculated into a 24-well plate (Corning®, USA, density 1×10^5 cells/mg of CMC-MCs) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The number of attached cells was determined after the sample had been seeded for 24 h. When the CMC-MCs were suspended uniformly in the medium via pipette

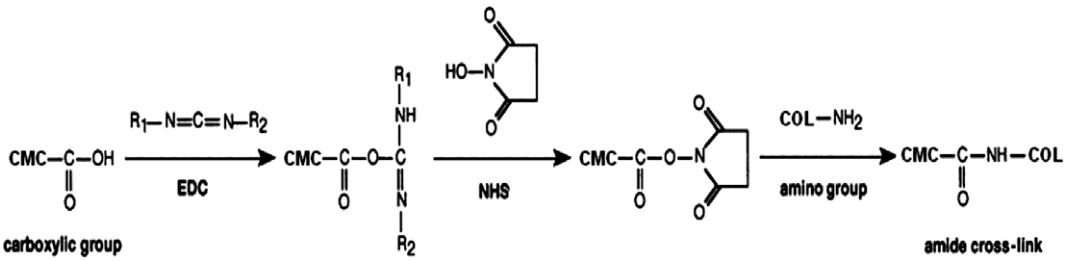

Fig. 1. Mechanism of covalent anchoring collagen (COL) on CMC-MCs using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). $R_1 = –CH_2–CH_3$; $R_2 = –(CH_2)_3–NH^+–(CH_3)_2\ Cl^-$.

blowing, 150 μl of solution was taken out with a pipette and placed into a 96-well plate. Before the attached cells were harvested by trypsin, two washings with PBS were performed. The detached cells were then counted using a haemocytometer. Cell proliferation was similarly determined and all data are averaged from three parallel experiments. Cell viability was tested by conventional MTT assay [16].

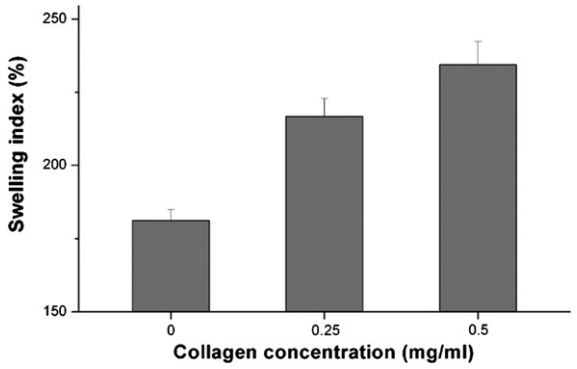

Fig. 2. Swelling index of porous CMC-MCs prepared by EDC cross-linking method containing different amounts of collagen. Data are presented as means ± SD ($n = 6$).

2.8. Cell distribution and morphologies

The aggregates of cells/MCs were fixed with 2.5% glutaraldehyde in PBS at 4 °C for 4 h, and then cryosections (10 μm thick) were cut. To visualize the viable cells under confocal laser scanning microscopy (CLSM) (Olympus, Japan), the samples were incubated in Rhodamine-Phalloidin (to stain F-actin) for 15 min in the dark and then rinsed with PBS. Rhodamine-Phalloidin is a triphenylmethane-derived dye commonly used in cell morphology examination [24,25]. The emission wavelength used for this purpose was 488 nm.

2.9. Type II collagen immunofluorescence

After 3 days culture, the cells/MCs aggregates were fixed with 2.5% glutaraldehyde in PBS at 4 °C for 4 h. Subsequently, they were washed three times with PBS and inoculated for 1.5 h at 25 °C with a mouse monoclonal antibody against rabbit type II collagen (Neomarkers, USA). After extensive washing with PBS, the second immunoreaction was performed for 20 min at 25 °C with

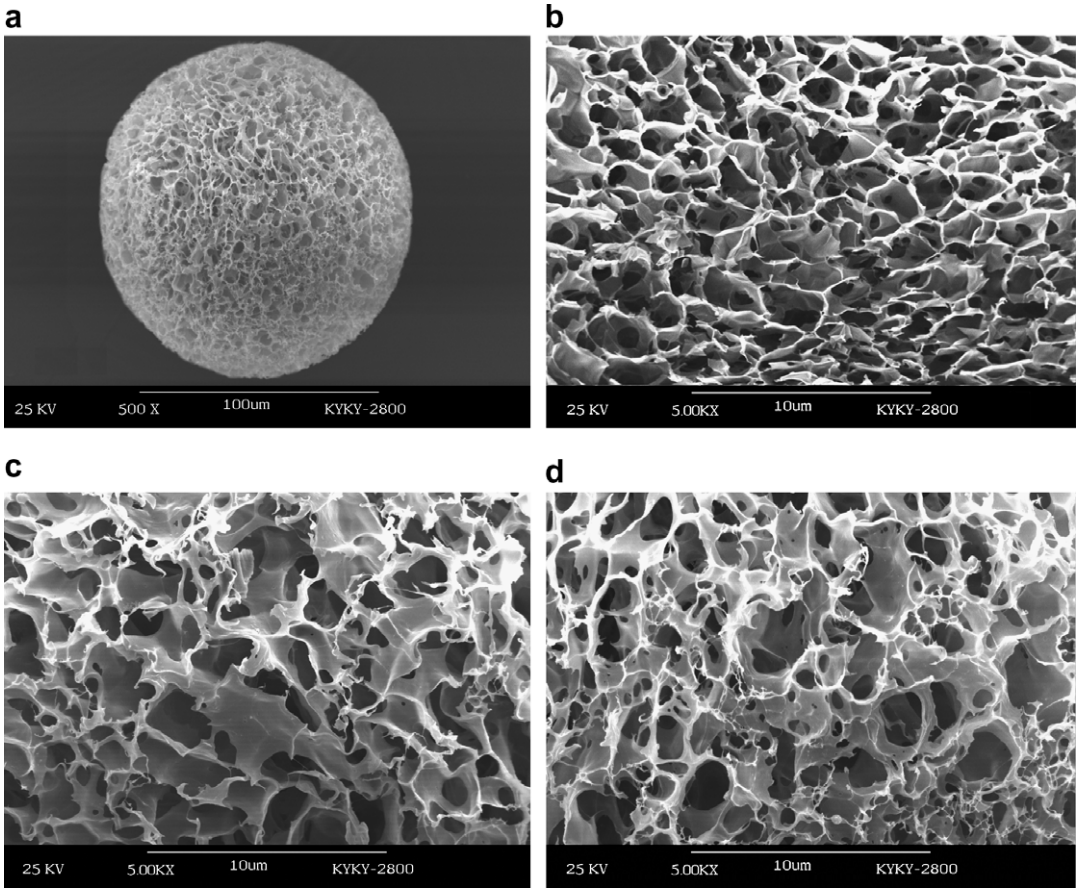

Fig. 3. SEM images showing surface morphologies of porous CMC-MCs (a) prepared by EDC cross-linking method. (b) CMC-0-MCs, (c) CMC-25-MCs, and (d) CMC-50-MCs.

fluorescein isothiocyanate (FITC)-conjugated antibodies raised in goat against mouse IgG (Dake, USA). After three washes in PBS, specimens were observed under CLSM (Olympus, Japan).

2.10. Type II collagen mRNA expression analysis

After 3 and 7 days inoculation, chondrocytes were harvested and total RNA was extracted as described [26]. Reverse transcription was carried out using a commercially available kit (Promega, USA). In this process, 1 mg of extracted RNA was reverse transcribed to generate full-length first strand cDNA for use as a template for PCR amplification. After the reverse transcription reaction, 3 ml product was amplified using a PCR kit (Promega, USA). The reactions were carried out for both the target gene pro alpha1 type II collagen and β-actin as the housekeeping gene. Amplified PCR products were analyzed by ethidium bromide staining after gel electrophoresis. The primers for the rabbit cDNAs are indicated in Table 1.

2.11. Statistical analysis

Data are presented as means ± SD. Statistical comparisons were performed using Students *t*-test, and $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Characterization of MCs

To firmly immobilize collagen nanofibers onto the surfaces of CMC-MCs, pre-treatment of the polymer matrix is necessary because of the absence of active sites in their molecules. We have developed a convenient and effective method to covalently introduce free collagen molecules onto the CMC matrix. The method makes use of the reaction between the carboxyl groups (–COOH) of CMC and the amino groups ($–NH_2$) on collagen chains (Fig. 1). The addition of NHS increases the rate and degree of cross-linking [27]. Without additional procedures, free collagen molecules were successfully grafted onto the polymer to form amide bonds (–CONH–) when EDC and NHS were used to prepare amide bond cross-linked CMC.

We took the CMC-MCs with and without initial collagen treatment for further studies. Fig. 2 shows the SI of CMC-MCs as a function of collagen concentration in the cross-linking reaction process. The SI, a value calculated by dividing the weight increase of wet MCs by the dry weight of MCs, increased with the concentration of collagen added. This indicates that CMC-MCs prepared using greater amounts of collagen had less extensive cross-linking and higher surface hydrophilicity.

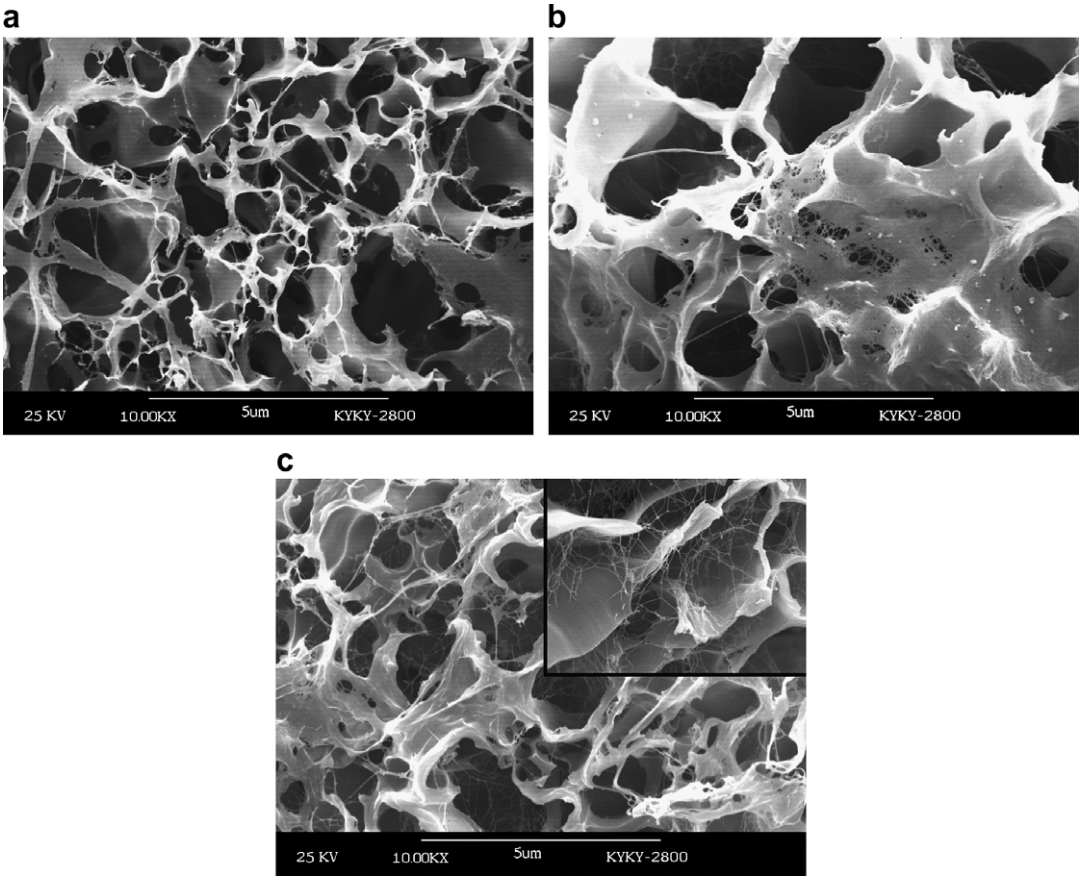

Fig. 4. SEM images showing the collagen nanofiber-coated CMC-MCs prepared by EDC cross-linking method. (a) CMC-0-MCs, (b) CMC-25-MCs, and (c) CMC-50-MCs. The image in (c) is shown in higher magnification; this image was chosen to qualitatively identify the existence of the collagen nanofibers.

Their surface morphologies were observed using SEM as shown in Fig. 3. A porous structure with abundant interconnective open pores in the range of 1–3 μ could be clearly seen on all CMC-MCs. Whether subjected to initial collagen treatment or not, all CMC-MCs had smooth pore walls. This observation is in accordance with the cross-linking process, which was performed after the formulation of open porous structure. Different morphological characteristics could be found for the surfaces of CMC-MCs produced with collagen added (Fig. 3b–d). Fiber-like edges with lengths less than one micron emerged on the surfaces of the CMC-25-MCs and CMC-50-MCs. Together with the results of SI, one can conclude that collagen molecules had been immobilized onto the sphere surfaces for the next step: collagen self-assembly.

3.2. Nanofibrous collagen assembly on the modified CMC-MCs

SEM observation qualitatively revealed that there indeed were nano-scale collagen fibers on the surfaces of the CMC-25-MCs and CMC-50-MCs (Fig. 4). The smooth walls of the open pores were clearly covered with the nanofibers. There was a larger amount of collagen on the CMC-50-MCs according to these micrographs; moreover, the amount of collagen was quantified by HCA (Fig. 5). Hydroxyproline detection revealed that the initial collagen treatment did significantly increase the amount of collagen in CMC-25-MCs (6.2 ± 0.7 μg/mg, collagen/MCs) compared with untreated CMC-0-MCs (1.4 ± 0.3 μg/mg). On the other hand, the amount of collagen in CMC-50-MCs (7.3 ± 0.4 μg/mg) was just slightly increased compared with that of CMC-25-MCs (6.2 ± 0.7 μg/mg). Since higher initial collagen concentration during the cross-linking reaction causes more collagen molecules to be anchored, it is understandable that the grafted collagen was increased. The larger amount of surface anchored collagen provides more sites for free collagen molecules to assemble with; however, not all anchored collagen molecules are flexible enough to initiate nano-scale self-assembly [21].

After initial collagen treatment, collagen molecules were anchored onto the CMC matrix. Upon incubation of these CMC-MCs in the process of collagen self-assembly into nanofibrous networks, interactions would take place between MC-anchored collagen molecules and free collagen molecules at physiological conditions. During this process, free collagen molecules would automatically intertwine and assemble with the anchored collagen molecules. Consequently, physically assembled collagen fibers could be firmly immobilized on the CMC-25-MCs and CMC-50-MCs after rinsing with water or PBS, because collagen is insoluble in solution of physiological pH. The as-cast collagen layer was relatively thick and stable due to the existence of these anchored collagen molecules. In contrast, physically adsorbed collagen molecules on untreated CMC-0-MCs could be easily rinsed off with PBS solution of neutral pH.

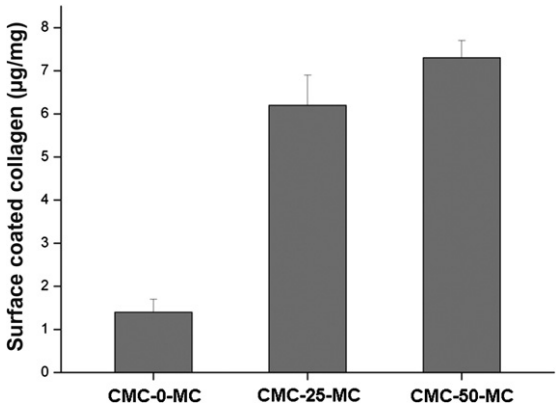

Fig. 5. HCA detection of the amount of collagen on various CMC-MCs. Data represent as means ± SD ($n = 6$).

3.3. Chondrocyte growth on the CMC-MCs

Nanofibrous collagen provides the CMC-MCs with improved compatibility to cells, as demonstrated by the results of *in vitro* chondrocyte culture (Fig. 6). MTT assay

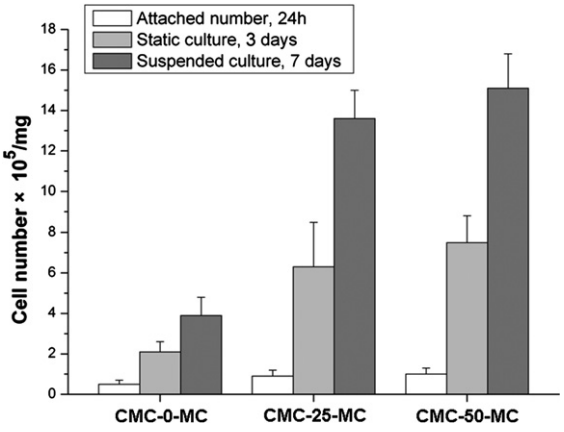

Fig. 6. Chondrocyte numbers on various CMC-MCs at culture times of 24 h, 3 days and 7 days. Cell seeding density 1×10^5 cells/mg of MCs.

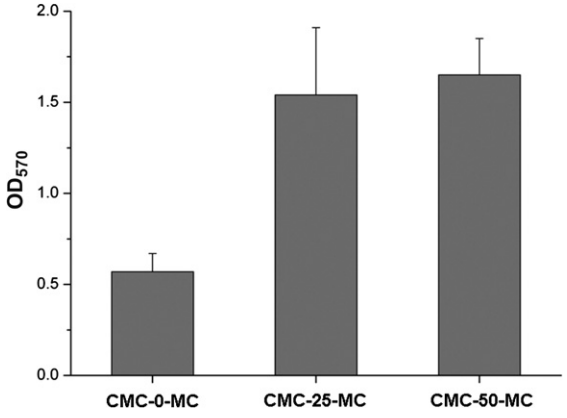

Fig. 7. MTT assay of chondrocytes grown on various CMC-MCs after 7 days culture. The data indicate no significant difference ($p > 0.05$) between CMC-25-MCs and CMC-50-MCs. Cell seeding density 1×10^5 cells/mg of MCs.

recorded a similar tendency of cytoviability on these MCs (Fig. 7). Compared to the CMC-0-MCs, both the cell attachment number and the cell growth rate were improved for the CMC-25-MCs and CMC-50-MCs. However, less difference was observed between the CMC-25-MCs and CMC-50-MCs, which is consistent with the HCA results.

Observations under CLSM and optical microscopy confirmed the existence of viable chondrocytes on the surfaces of CMC-MCs (Fig. 8). The cell morphology was observed with CLSM after staining chondrocytes with Rhodamine-Phalloidin (Fig. 8a–c). Note that the red regions represent viable chondrocytes. The results showed that chondrocytes could adhere and grow on all the CMC-MCs, but the cell densities were quite different. Cells grew to confluence on the CMC-25-MCs within 3 days post-seeding (Fig. 8a and c), while fewer chondrocytes were found on the CMC-0-MCs with collagen just physically adsorbed (Fig. 8b). Moreover, no significant difference could be observed between the CMC-25-MCs and CMC-50-MCs (data not shown). This is consistent with the cell proliferation and viability results (Figs. 6 and 7). Fig. 8d shows that several confluent CMC-25-MCs attached to each other forming tissue-like clusters after 7 days culture. Clustering of MCs within incubation suggests that cells were probably secret-

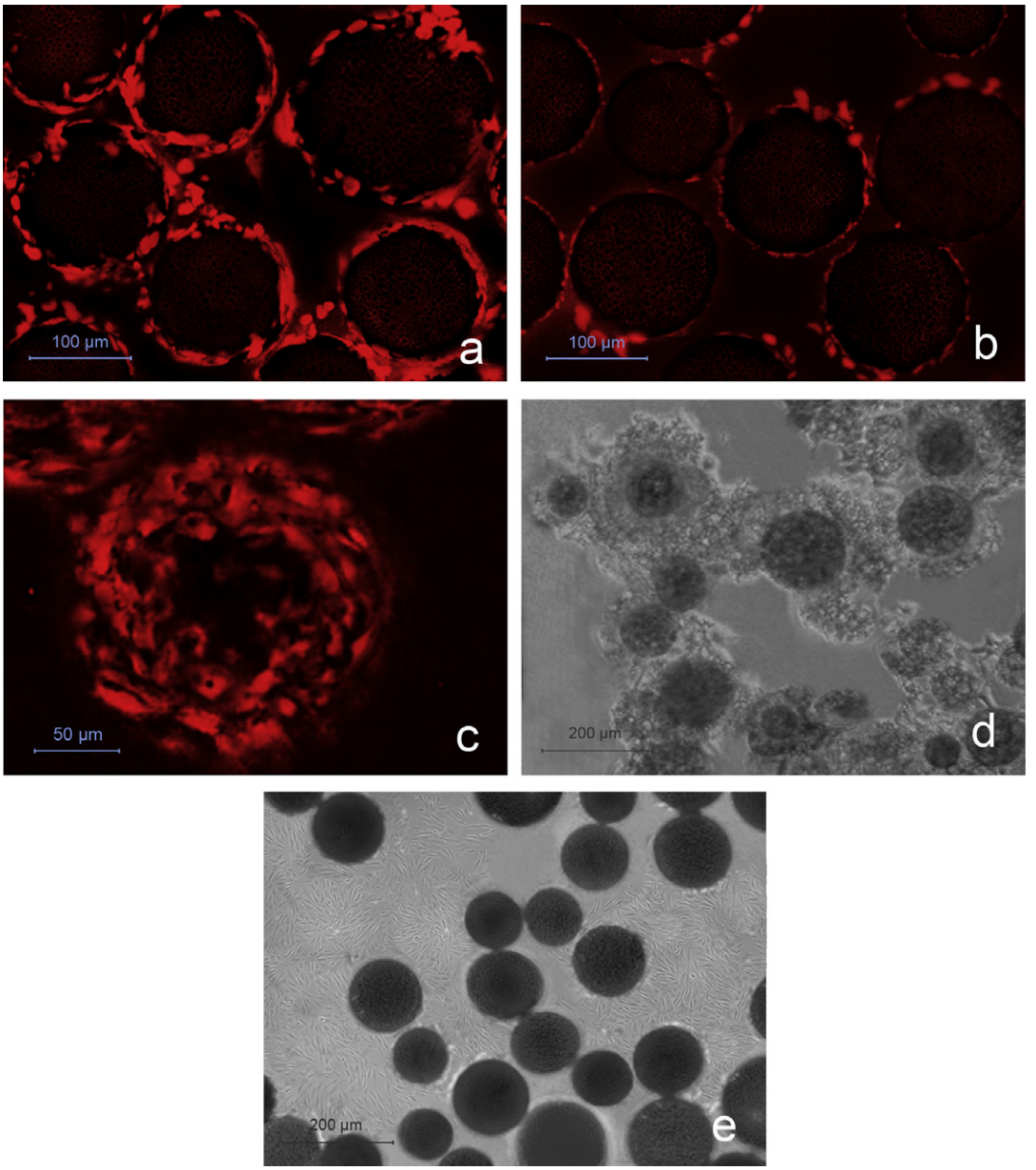

Fig. 8. Micrographs showing morphology on the CMC-MCs at different culture times. CLSM images of cells on (a) CMC-25-MCs and (b) CMC-0-MCs after 3 days culture, and (c) representing higher magnification of (a); microphotographs of cells on (d) CMC-25-MCs and (e) CMC-0-MCs after 7 days culture. Cell seeding density 1×10^5 cells/mg of MCs.

ing ECM components. On the other hand, the microphotographs of CMC-0-MCs in wells demonstrated that these MCs were floating in culture medium and much fewer cells could be seen on the MCs (Fig. 8e). Together with the cell proliferation and viability results, one can conclude that the CMC-MCs coated with nanofibrous collagen are more favorable for chondrocyte attachment and growth.

In order to reveal type II collagen produced by chondrocytes seeded on the CMC-25-MCs, the aggregates of cells/MCs cultured for 3 days were studied by immunofluorescence analysis under CLSM (Fig. 9). Red fluorescence indicated cells labeled with Rhodamine (Fig. 9a). Green fluorescence appeared in the same field of vision, which indicated positive collagen II staining for cells and ECM (Fig. 9b). Type II collagen-expressing cells also displayed red fluorescence (Fig. 9c). The immunofluorescence results indicated that chondrocytes seeded on the CMC-25-MCs remained differentiated and secreted cartilage ECM components.

The mRNA expression of type II collagen in chondrocytes cultured on CMC-MCs (or 24-well plates) for 3 days and 7 days, respectively, was analyzed by RT-PCR with β-actin as the housekeeping gene (Fig. 10). Chondrocytes from each group exhibited a similar level of type II collagen gene expression on day 3 (Fig. 10a). However, much stronger gene expression for type II collagen was observed on the CMC-25-MCs and CMC-50-MCs after 7 days of cultivation (Fig. 10b), while on 24-well plates the expression level was the lowest, even much lower than the expression on day 3.

It has been reported that regeneration of cartilage could be achieved when the involved biodegradable scaffold could accommodate production of a cartilage-specific ECM, together with stimulation of cell proliferation [28–

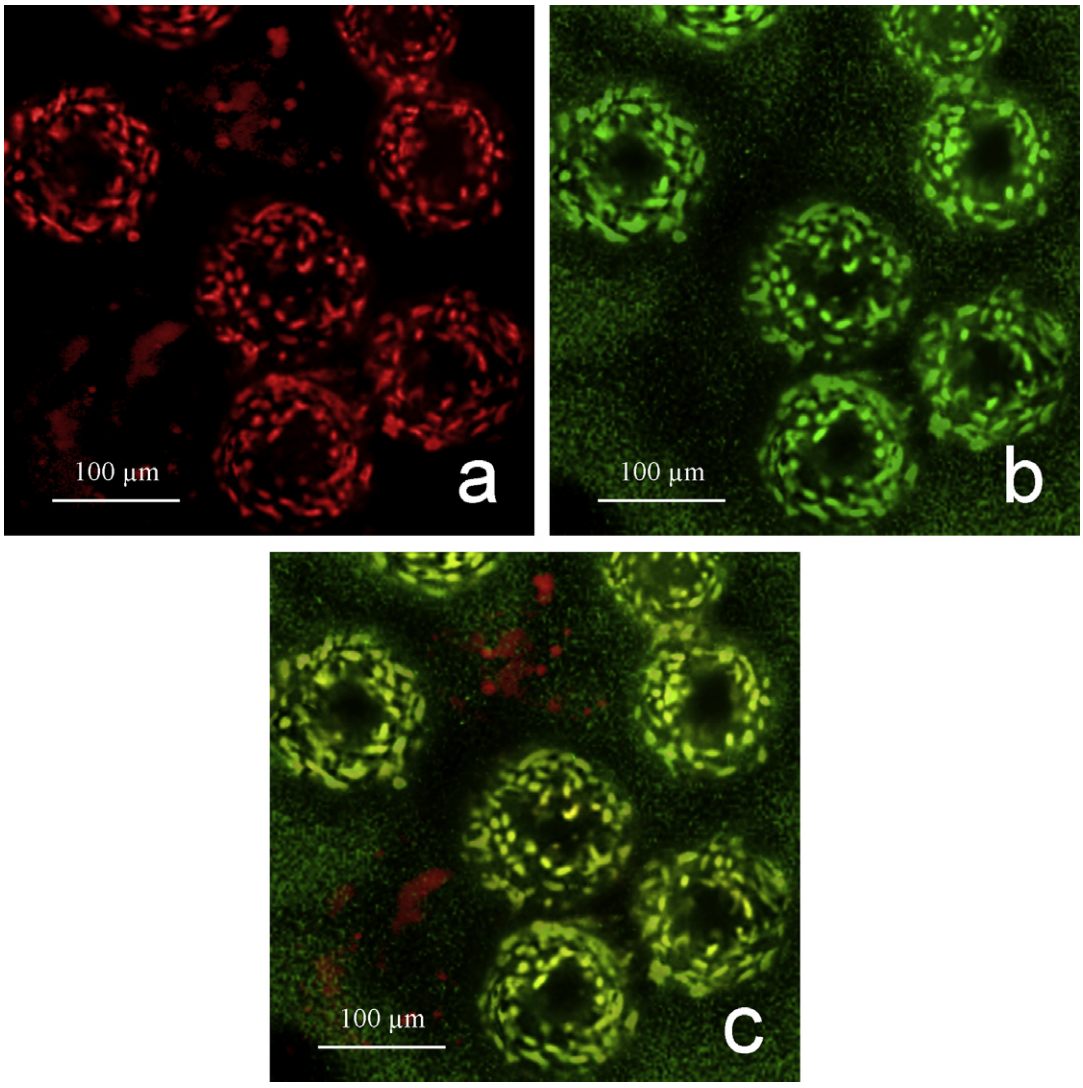

Fig. 9. CLSM images of chondrocytes on CMC-25-MCs double-labeled with rhodamine and collagen II (FITC-conjugated). (a) Cells labeled with rhodamine, (b) cells positive for type II collagen, (c) overlap of (a), and (b); double-labeled cells displayed yellow staining. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

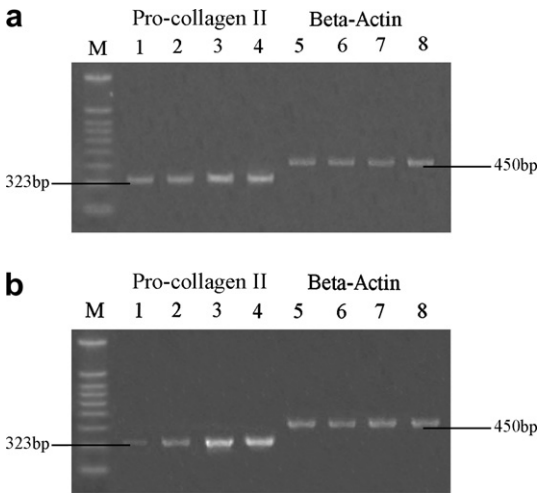

Fig. 10. Type II collagen mRNA expression detected by RT-PCR analysis in chondrocytes cultured for 3 days (a) and 7 days (b). (M) Marker, (1, 5) 24-well plate, (2, 6) CMC-0-MCs, (3, 7) CMC-25-MCs, and (4, 8) CMC-50-MCs. β-actin was used as the housekeeping gene.

30]. However, few of these materials presented an ability to synthesize type II collagen, which is a well documented marker of hyaline articular cartilage always found in freshly isolated chondrocytes, and which also accounts for 50–70% of the solid matrix in articular cartilage. The present results confirmed that collagen nanofiber-coated CMC-MCs were capable of activating a redifferentiation process allowing chondrocytes to express and produce type II collagen. This was further supported by an increase in type II collagen mRNA expression (Fig. 10), which was followed by the production of the secreted type II collagen, as detected by immunofluorescence analysis (Fig. 9). HCA and RT-PCR data (Figs. 5 and 10) indicated that increased collagen amounts resulted in increased mRNA levels of type II collagen in cells/MCs aggregates. By comparing the data on day 3 with the data on day 7, the stimulatory effect of CMC-25-MCs and CMC-50-MCs on type II collagen mRNA was clearly demonstrated.

Chitosan are commonly used natural polymers for tissue engineering applications [31] because of their biocompatibility as well as high mechanical strength. Since the degradability of chitosan is poor, we previously developed a technique to significantly accelerate its degradation rate via carboxymethylation and EDC cross-linking. However, the new produced polymer, cross-linked CMC, exhibited poor cell-material interactions because it lacked specific binding sites. Therefore, the polymer should be either coated or blended with other active molecules to improve cell affinity. CMC-MCs formulated with collagen anchored in the polymer matrix followed by collagen self-assembly on the surfaces demonstrated better cell adhesion, proliferation, and differentiation. This is probably because of the existence of cell specific domains in the collagen nanofibers, which enhanced their biocompatibility. Modification of the polymers' surface to mimic ECM has been widely investigated [20,32]. Cell adhesion to ECM is mediated by cell-surface receptors, one important class being the integrins. These receptors bind to short amino acid sequences on integrin binding proteins known as RGD sequences [33], which are abundant within ECM proteins such as collagen. Coating the surface with collagen had been demonstrated to improve cell adhesion to polymer matrixes [34].

The collagen microstructure on the polymer surface also plays an important role in cell adhesion and growth because natively folded proteins could provide binding sites (e.g., RGD sequences) distributed in the most appropriate concentration and spacing [35]. In the present study, free collagen molecules automatically assembled with MC-anchored collagen molecules into nano-scale networks. This structure could promote cell adhesion and the spread of the cells on the CMC-MCs. Chondrocytes were seen to attach to the CMC-25-MCs and CMC-50-MCs within 24 h of incubation, whereas the CMC-0-MCs required 48 h or more for the same degree of cell adhesion. Moreover, immobilizing self-assembled collagen nanofibers onto the polymer MCs seems to be a much better approach than simply blending or physical coating. Physically adsorbed molecules could desorb quickly, and therefore may not provide a long-term surface effect on cell adhesion and growth.

The aim of this study was to fabricate nanofiber-based biodegradable MCs as injectable scaffolds and to evaluate the influence of the collagen nanofibers on the biological activities of cultured chondrocytes. It is known that cells behave differently on various topographic substrates, and cells respond to the geometry of biomaterial surfaces [36,37]. Several research groups have reported that geometric variables of a biomaterial surface affect cell response [38,39]. However, few reports have focused on the cell response to micro-porous scaffolds with nano-fibrous surfaces. To our knowledge, there have been no studies of chondrocyte activities in cultures established in micro-porous and nano-fibrous structures. Previous reports have shown that osteoblasts showed improved attachment, growth, alkaline phosphatase synthesis, and deposition of calcium-containing mineral in a nanophase ceramic [40,41]. Based on our results and those from previous reports, we conclude that a scaffold composed of nano-topographic components is biologically favorable. Thus, a scaffold with nanometer structural components should be the preferred design for the fabrication of functional tissue-engineered scaffolds.

4. Conclusions

We reported a simple method of fabrication of nanofibrous collagen-coated CMC-MCs that can be used as injectable scaffolds through a modified phase separation method and thereafter collagen anchoring-assembling. Our results demonstrated that naturally structured collagen on the polymer MCs plays an important role in cell adhesion and cell growth. *In vitro* chondrocyte culture revealed better cell attachment, proliferation, and differentiation on the CMC-MCs immobilized with self-assembled collagen nanofibers. Cells were observed to grow into a tissue-like structure after 7 days of culture. Immunofluorescence and RT-PCR analysis confirmed that these MCs supported chondrocytes to express and produce cartilage ECM components. In summary, the CMC-MCs developed in the pres-

ent study can be used as injectable scaffolds for various biomedical applications.

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