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Chondrocytes Behaviors Within Type I Collagen Microspheres and Bulk hydrogels: An *in vitro* study

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Cells niche, which is considered to be critical to the proliferation and differentiation of cells, is one of the most important aspects for the design and development of ideal scaffold in tissue engineering. The mass transfer property of the scaffold affects the nutrients supply and exchange of the other substance. In this study, we prepared collagen hydrogels in the forms of microspheres (CHM) and bulks (CHB) to investigate the mass exchange differences and their influence on embedded chondrocytes. CHM were developed by the emulsion method, which was efficient to load cells. Bovine serum albumin (BSA) was used as a diffusion model in CHM and CHB to evaluate the transport property of the hydrogels and the release kinetics. During the 4-week *in vitro* culture process, the contraction of the hydrogels, cell viability and morphology, DNA and glycosaminohlycan (GAG) contents were monitored at different intervals. The results suggested that CHM showed obvious superiority on transfer property than CHB, leading to better maintenance of the chondrocyte phenotype in CHM at the early stage of *in vitro* culture. Histological analyses indicated that lots of lacunae and homogeneous positive GAG staining appeared in CHM from day 7. In contrast, only a few lacunae and obscure GAG staining was found in the outer area of CHB after day 21.Without enough nutrients, the chondrocytes in the inner area of CHB had few secreted matrix. Based on the presented CHM system, a further developed construct is suggested as a promising alternative toward clinical application of engineered cartilaginous tissue.

1. Introduction

Once damaged, cartilage can hardly be repaired spontaneously due to lack of cells and nutrition. [1] In recent years, autograft and microinvasive techniques are major available treatments, but the final effect is not greatly satisfied.[2] Tissue engineering is considered to be one of the most promising treatments for cartilage defect repair and regeneration.[3] It has been proved that chondrocytes can secrete cartilaginous matrix in the hydrogel scaffold.[4] Hydrogels not only supply the implanted cells with three-dimensional microenvironment, but also act as the artificial extracellular matrix (ECM) to maintain the phenotype and function of cells.[5,6]

Collagen is the main component of the ECM in cartilage. It is regarded as superior natural polymer for tissue engineering because of its excellent properties, such as biodegradability and biocompatibility.[4,7,8] Although type II collagen is mainly contained in cartilage, there is no significant differences between the biological effects of collagen type I and II was observed on chondrogenesis of chondrocytes. [9] Chondrocytes have a high proliferation rate in collagen hydrogel, but they might tend to lose their phenotype after long time culture.[10] These dedifferentiated chondrocytes cannot synthesize the specialized extracellular matrix that is significant for cartilage defect repair and regeneration. Therefore, it is important to maintain the phenotype of chondrocytes by supplying a suitable microenvironment, including the interaction of cell-cell and cell-matrix.[11,12]

The limited mass exchange of large bulk materials might be a main reason causing incomplete differentiation or dysfunction of chondrocytes due to the insufficient nutrient and accumulated metabolite, especially when cell density and crosslinking density in scaffold are high.[13,14] Small size scaffolds, such as microspheres can overcome the obstacles at some extent. Meanwhile, shape and size of the defect area on cartilage are usually irregular, so it may be wise to fit the defect by accumulating micron-sized materials than the traditional bulk ones.[4,15] As reported in literatures, microspheres are generally made from synthetic polymers and/or natural polymers in forms of hydrogels or sponge scaffolds, and mainly applied in drug delivery or served as cells carrier.[16,17] Cells can be seeded into hydrogel during fabrication, while it is impossible for sponge to have cells planted during the preparation process. A major difference between those two forms of materials is the different growing status of cells, which will affect the cellular behavior. Hydrogel offers three-dimensional (3D) microenvironment, but sponge is based on two dimensions. In the 3D system, chondrocytes will keep the natural spherical morphologies.[18] Hydrogel microspheres can be fabricated by three main methods: microfluidic, droplets on non-adhesive surface and the emulsion method. Hong et al. developed a microfluidic material-processing chip to produce collagen microspheres.[19] Hui et al. made microspheres with 3~100uL collagen solution of different concentration (0.5, 1, 2 or 3 mg/mL) by pipetting droplets into a Petri dish.[20] But the productivity of these methods is limited, especially when the viscosity of the materials is high.[13,14]

In this study, we prepared collagen hydrogel microspheres (CHM) with chondrocytes implanted by water-in-oil emulsion method. The preparation process was designed to be mild, and parameters of this method were optimized. Collagen hydrogel bulk (CHB) was prepared to serve as controls. Transfer property of CHM and CHB was carefully investigated and the influence on implanted cells was compared.

2. Experimental

2.1 Chondrocytes isolation and culture

Chondrocytes were isolated from new-born New Zealand white rabbits. Cartilages were collected and treated by 0.25 mg/mL of trypsin for 30 minutes and 2 mg/mL of collagenase type II for 6 hours at 37°C. After being filtered by 100 μm strainer and centrifugation, chondrocytes were re-suspended in alpha-modified Eagle' s medium (α-MEM, Hyclone, Beijing) containing 10% fetal bovine serum (FBS, Gibco, USA), 1% vitamin C and antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL) and cultured at 5% CO_2 at 37°C.

2.2 Fabrication of CHM and CHB

Collagen type I was extracted from calf skin with pepsin in acetic acid. Collagen hydrogel microspheres (CHM) were made by water-in-oil emulsion method. Briefly, collagen solution that was neutralized by 1M NaOH in ice-bath and had a final concentration of 6.5 mg/mL. The neutralized collagen solution was injected into precooled polydimethylsiloxane (PDMS) and stirred at 500 rpm by magnetic stirrer in an ice bath. After 10 min of emulsification, the system was moved into a water bath at 37°C and kept on stirring for 30 min. The CHM were produced during this stirring process. Then, CHM were collected by gentle centrifugation and washed with PBS for three times. To prepare cells-loaded CHM, cells were evenly suspended in neutralized collagen solution and then followed the same method described above. To investigate the effect of cell density on the size of CHM and cell-loading efficiency, the final cell densities were set to 1.0×10^6 cells/mL, 5.0×10^6 cells/mL and 1.0×10^7 cells/mL, respectively. Collagen hydrogel bulk (CHB) and cell-loaded CHB were prepared by gelation of 100 μL neutralized collagen solution in cylindrical mold (diameter 6.4 mm, depth 3.1 mm) with the concentration of 6.5 mg/mL at 37°C for 30 min. Only CHM and CHB with cell density of 5.0×10^6 cells/mL were chosen for 4-week *in vitro* culture.

2.3 Size and size distribution of CHM

The collected CHM were distributed in PBS buffer and imaged under a light microscope. Images were taken at random area (more than 10) in the dish and the sizes were measured with software (NanoMeasurer 1.2). Accordingly, the size distribution was calculated by OriginPro 9.0 (OriginLab Corp.).

2.4 Morphology of hydrogels

The hydrogels were washed with PBS and fixed in 0.25% glutaraldehyde at 4°C overnight. Then the hydrogels were dehydrated in a series of graded ethanol and dried in a critical point dryer after using isoamyl acetate to replace ethanol. The morphology of hydrogels was observed by scanning electron microscopy (SEM, Hitachi S-4800, Tokyo, Japan) after coating with gold in an ion sputter.

2.5 Mass transfer property of hydrogels

To better understand the transfer property of CHM and CHB, bovine serum albumin (BSA, 66.43kDa, on the order of growth factor) was used as a release model in the hydrogels. Neutralized collagen solution was mixed with BSA solution. The concentration of collagen was 6.5 mg/mL and the final concentration of BSA in the hydrogel was 1600 μg/mL. BSA-loaded CHB and CHM were prepared by the same way as cells-loaded hydrogels. Three CHB samples (300 μL) and 3.7 mL PBS were added into 10mL Eppendorf tube. To attain the same release condition with CHB, CHM made by 300 μL collagen solution were suspended in PBS with a final volume of 4 mL and were pipetted into 10mL Eppendorf tube. All tubes were put in the incubator at 37°C. 500 μL of the incubated solution were sampled for measurement at intervals of 10, 20, 30, 40, 50 min, 1, 2, 3, 4, 5, 6, 7, 8, 9, 18, 24 and 48 h. 500 μL fresh PBS was added into the tubes to balance the volume at each interval. BSA concentrations in sampled 500 μL liquid were measured by Coomassie brilliant blue method with ultraviolet spectrophotometer. The release ratio of BSA is calculated as following: $R=C/C_{max}*100\%$, where R is the release ratio, C is the cumulative concentration of BSA in the buffer, and C_{max} is the total concentration of BSA in the testing system. Based on the acquired data, the releasing kinetics were studied by the first order exponential decay equation (ExpDec1, $y=y_0+A*\exp(-x/t)$).

2.6 Contraction of hydrogels

During the in vitro culture process, the sizes of CHM and CHB were recorded by digital camera under light microscope on day 0, 1, 2, 3, 4 and 5. The hydrogel size was measured with software (NanoMeasurer 1.2) based on the microphotographs. The diameters of the hydrogels were calculated and the contraction ratios were investigated.

2.7 Cell viability and cell morphology

The viability of cell in the hydrogel was assessed by fluorescence staining after culturing for 1, 3, 7 and 14 days in vitro. CHM and CHB samples were attained and washed three times with PBS for 5 min and then immersed in PBS containing fluorescein diacetate (FDA, 1 μg/mL, Topbio Science, Beijing, China) and propidium iodide (PI, 1 μg/mL, Solarbio, Beijing, China) for 10 min. The samples were washed again by PBS and imaged by confocal laser scanning microscopy (CLSM, TCS SP 5, Leica).

2.8 Biochemical analysis

To understand the proliferation of cells in hydrogels at the early stage of the *in vitro* culture (day 0, 0.5, 1, 3), both CHM and CHB were sampled to investigate the DNA contents. Both DNA and glycosaminohlycan (GAG) contents were detected in CHM and CHB samples with longer culture period (day 7, 14, 21 and 28). All hydrogel samples were washed with PBS for three times, and then freezing dried at -60°C. Lyophilized samples were carefully weighed by Mettler Toledo XP205 (with a weigh resolution of 0.01 mg) and then digested in 0.1% papain solution at 60°C for 12 hours. Clear supernatant were collected after centrifugation at 4000 rpm and then divided into two parts for the test of DNA and GAG content. DNA content was measured by Hoechst 33258 (B1302, Sigma). Briefly, 10 μL of supernatant was added into 2mL of Hoechst 33258 solution and measured by fluorometry. Blyscan sGAG assay kit (B100, Biocolor) was used to measure GAG content. Briefly, 20 μL of supernatant was added into 1 mL of Blyscan dye reagent and mixed by a shaker for 30 minutes. Then unbound dye was removed by centrifugation. After dissolved in dissociation reagent, the dye content was measured by using Varioskan™ Flash Multimode Reader (Thermo Scientific, USA).

2.9 Histological analysis

After culture for 1, 4, 7, 14, 21 and 28 days *in vitro*, hydrogel samples were harvested and fixed in 4% paraformaldehyde for 1h at

4°C. The samples were washed three times with PBS to remove paraformaldehyde and then frozen sectioned with a thickness of 20 μm. Since the inner and outer areas of CHB show different histological conditions, the sections attained by inner or outer area of CHB were identified and marked as CHBi and CHBo, respectively. The sections of CHM, CHBi and CHBo were stained by hematoxylin-eosin (HE) and toluidine blue (TB) to investigate the cell morphology and extracellular matrix (ECM).

2.10 Statistical analysis

GAG, DNA content and diameter contraction were analyzed with an one-way ANOVA followed by Bonferroni post-hoc correction (SPSS statistics 20.0, IBM Inc.). All data are reported as mean ± standard deviation with the significance level set at $p < 0.05$.

3. Results and discussion

3.1 Morphology analysis

The SEM pictures (Fig.1) showed that the morphology and structure of CHM and CHB were equivalent. The collagen fibers in CHM and CHB were uniform in diameter, and some close assemble fibers can be found in CHM as shown in the picture. Fibers in both samples entwined to form three dimension networks that showed similar pore size and porosity. Via the morphology study by SEM, the identity of fiber size and network structure between CHM and CHB meant that the collagen in CHM experienced no destruction during the fabrication.

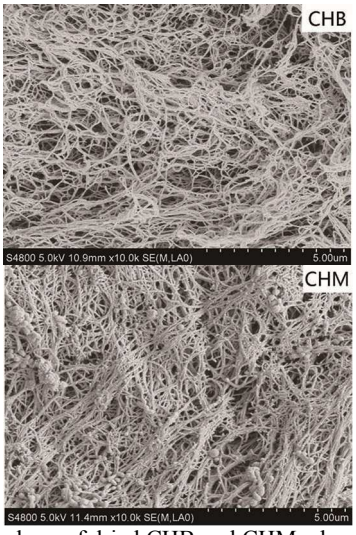

Figure 1. Morphology of dried CHB and CHM, observed by SEM.

3.2 Mass transfer analysis

The release of BSA from hydrogels reflected the diffusion situation of molecules such as nutrient, growth factors and metabolites, which were necessary for chondrocytes to proliferate and secrete matrix. Fig.2 showed the release ratios (R) of BSA in CHM and CHB. The first sampling time was 10 minutes, while the R value in the CHM and CHB was about 80.67% and 2.31%, respectively. The ExpDec1 equations fitted the release process of CHM and CHB very well with the adj. R-squares 0.9724 and 0.9950, respectively. According to the fitting equation, the release ratio at equilibrium (R_e) of CHM would reach at 95.90% and the time for 90% and 95% of R_e were 0.66 and 1.46 hours. By comparison, the R_e of CHB was 68.55% and the time for 90% and 95% of R_e were 8.17 and 10.63 hours. It was obvious that the BSA in CHB needed longer time to migrate to the soaking solution. BSA release tests were carried out in the study of Chia et al., and similar result was observed.[21] They found that 90% of BSA was released from collagen microspheres within 10 min. Distinctly different results of BSA release behaviors of CHM and CHB reflect the different mass exchange property of these two hydrogels. It is reasonable to consider that BSA in the central area of CHB could not easily diffuse to the surface because of the longer distance. The different mass transfer property between CHM and CHB might be one of the main reasons leading to the significantly different results in cell culture.

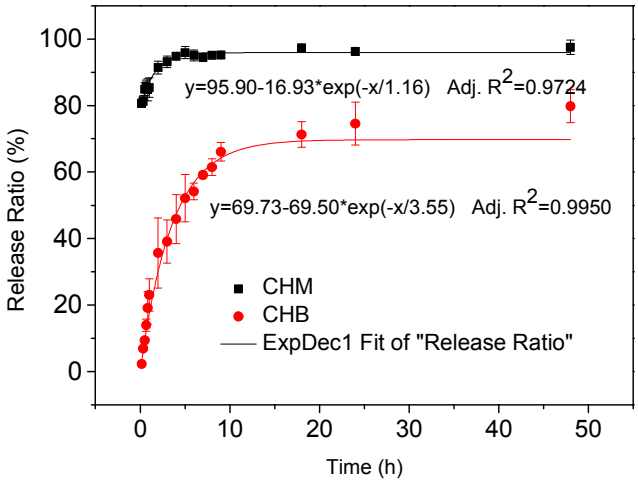

Figure 2. Release ratio of BSA and their ExpDec1 fit lines.

3.3 Influence of cell density

The mean sizes of the fresh prepared CHM samples loaded with 1×10^6, 5×10^6 and 1×10^7 cells/mL were 222.7±82.4, 202.4±61.9 and 194.8±66.3 μm, respectively. Size distributions and normality tests of the samples were shown in Figure 3a. The results indicate that the average size of CHM slightly decreased when the cell density increased. The one way ANOVA overall analyses showed that the average sizes were significantly different at the 0.05 level. The means comparisons between 1×10^6 and 5×10^6, 1×10^6 and 1×10^7 were significantly different, but that difference between 5×10^6 and 1×10^7 was not significant. Corresponding to the size analysis, CLSM results of the fresh prepared CHM samples encapsulated cells of different densities were shown in the Figure 3b. The pictures indicated that CHM could efficiently encapsulate chondrocytes at different densities, and cells were uniformly distributed in the microspheres.

The CLSM pictures showed that the cell viability and cell morphology had no obvious difference between CHM and CHB when the cells were encapsulated at the early stage of *in vitro* culture. Combined the results of SEM and CLSM together, it indicated that the emulsion method did not change the structure and biocompatibility of collagen hydrogel or affect the viability of cells. Based on the acquired results, it is reasonable to consider that the different cell responses in CHM and CHB *in vitro* culture were mainly caused by the different mass exchange property of the hydrogels.

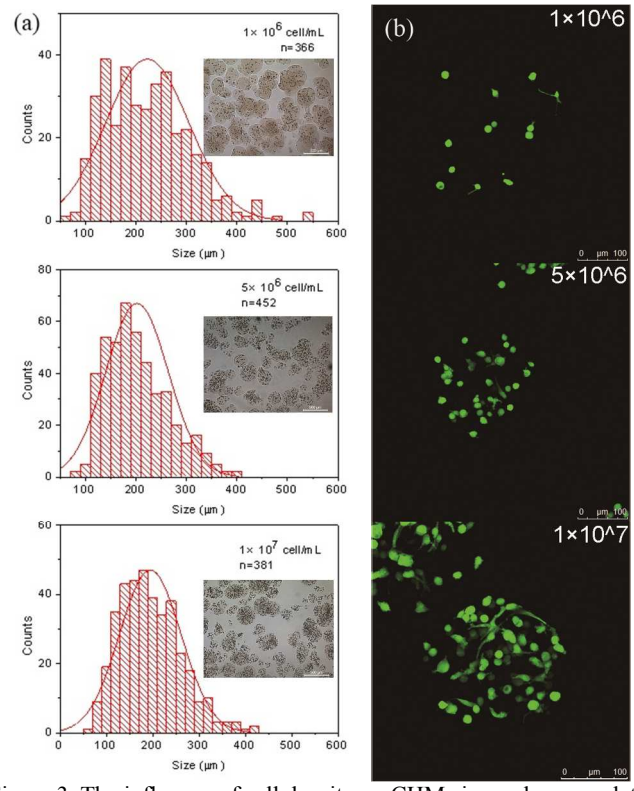

Figure 3. The influence of cell density on CHM size and encapsulate efficiency. (a) Size distributions of CHM loaded with 1×10^6, 5×10^6 and 1×10^7 cells/mL, , which were calculated based on the microshphere images under low magnification ligh microscope; (b) CLSM pictures of cell loaded CHM. Scale bars represent 100 μm.

3.4 Contraction of hydrogels

Contraction of hydrogels with cell embedded is a common phenomenon during the *in vitro* culture.[22,23,24] Figure 4 showed the diameter contraction of cell-loaded CHM and CHB during the initial 5-days culture *in vitro*. CHM contracted faster than CHB. Diameters of CHM shrank sharply on day 1 to around 60% of the original size, and kept relatively stable afterwards. The average diameter of CHB contracted to 85% of the original size on day 1, and continues to contract to around 45% of the original size on day 5. The contraction of hydrogel leads to a higher cell density and change of network structure in the hydrogels. In our study, according to the results of contraction ratio, cell density in CHM kept higher than in CHB from day 4, which could be ascribed to both the contraction and cell proliferation. High cell density was considered to be in favor of conservation of chondrocytes phenotype.[25] Normally, chondrocytes harvested from culture dish showed fibroblast-like morphology. After seeded in the hydrogel, these fibroblast-like chondrocytes will cause the fast contraction of hydrogel.[26] The change of morphology to round-shape not only implied recovery of cell function but also was the endpoint of contraction. Meanwhile, contractions could also enhance the density of collagen fiber and gel stiffness[12], which could further decrease the contraction. The results in Figure 4 indicated that the contraction of CHM terminated on day 1, suggesting earlier recovery of chondrocytes than CHB.

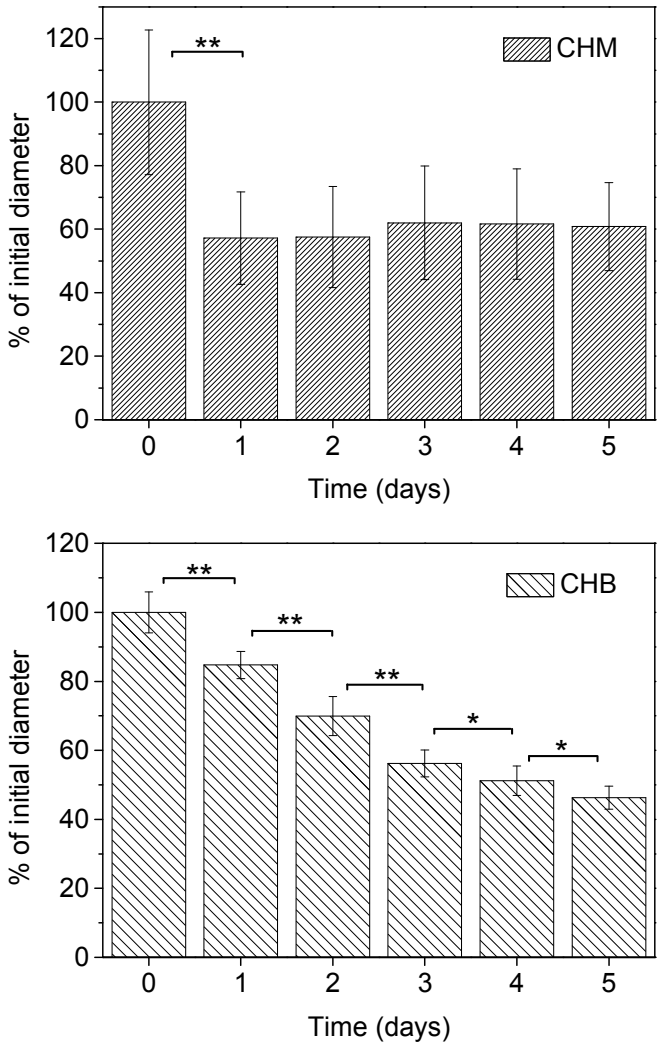

Figure 4. Diameter contraction of CHM (n =129~238) and CHB (n=10~11) in 5 days *in vitro* culture. (*p< 0.05, and **p< 0.01, same below)

3.5 Cell viability and cell morphology

If the scaffolds could balance the permeability and mechanical property, provide good protection, enough nutrient and quick removal of metabolite, the suitable micro-environment for chondrocytes might result in a good phenotype recovery and high matrix production.[27,28] The cells condition was evaluated from the DNA contents and CLSM pictures which were taken at different intervals. Figure 5a showed the DNA contents in CHM and CHB on day 0, 0.5, 1 and 3, which reflected the proliferation of loaded cells at the early stage of the *in vitro* culture. After about 12 hours, the chondrocytes proliferated so exuberantly in CHM that the DNA content doubled and the percentage of DNA to dry weight of CHM was about 2.5-fold to that of CHB. The proportion (DNA/dry weight) of CHB was unchanged until day 3, whereas that of CHM kept on growing, and showed 1.6-fold and 0.9-fold higher than that of CHB on day 1 and day 3. Also, the prolifieration of chondrocytes in both CHM and CHB indicates the good cytocompatibility of the scaffold. With the increase of culture time, more GAG would be secreted in the hydrogels, leading to the inaccuracy of the proportion (DNA/dry weight). Therefore, only the first three days of culture period were monitored.

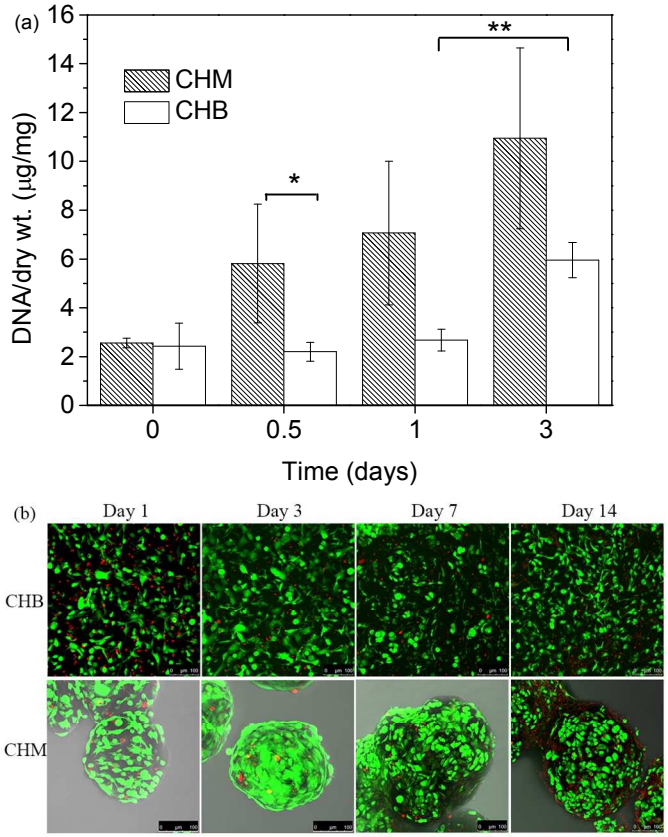

Figure 5. The cell viability and cell morphology in CHM and CHB. (a) The chondrocytes proliferation detected by DNA assay. (n=3) (b) Confocal laser scanning microscopy pictures of CHB and CHM loaded with 5×10^6 cells/mL. Scale bars represent 100 μm.

For different culture period, cells morphology in CHM and CHB was observed using CLSM and the images were shown in Figure 5b. According to the images, chondrocytes regained the chondrogenic phenotype and lots of cell clusters appeared in the CHM. On day 1, most of the cells in both hydrogels kept the spherical morphology, while a small amount of them tended to show fibroblast morphology. With the elongation of culture time, until day 7, there was almost no spreading out chondrocytes in the CHM. On the contrary, chondrocytes in the CHB spread out and became fibriform from day 3 onwards and the apparent cell density was lower than that in the microspheres.

3.6 Biochemical analysis

The GAG secreted by chondrocytes during the *in vitro* culture was abundant, especially when the cells were cultured longer than 7 days. Figure 6 showed the GAG contents and proportion of GAG to DNA in the hydrogels when cultured for 7, 14, 21 and 28 days. According to Figure 6a, the GAG content in CHM increased significantly on day 14 than that on day 7, and then gradually increased in the next 2 weeks. Meanwhile, the GAG content in CHB showed a slow increase at the first 2 week, but rose up to around 7.6-fold on day 21 compared with that on day 14, and then doubled the GAG content in the 4th week. Compared the GAG contents in CHM and CHB, it was obvious that the content in CHM was always higher than that in CHB. Especially, after cultured for 14 days, the GAG content in CHM was 10-fold higher than that in CHB (p=0.039). The difference of GAG content between these hydrogels becomes less pronounced when the culture period extends to 28 days.

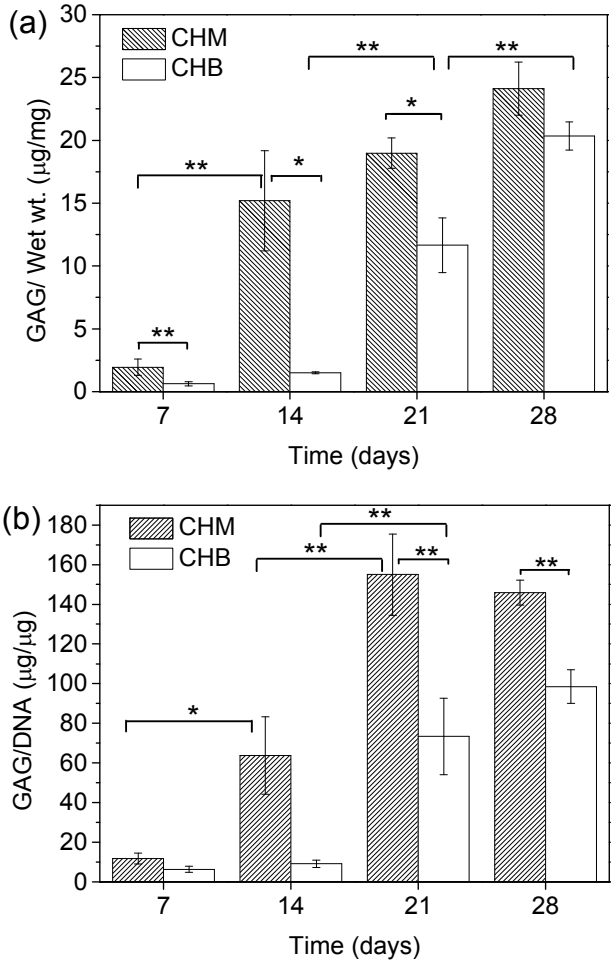

Figure 6. Biochemical analyses of secreted GAG in hydrogels. (a) The secreted GAG increasing in CHM and CHB; (b) The proportion of GAG to DNA in CHM and CHB. (Day 7, 14 n=2; Day 21, 28 n=3)

As a major component of extracellular matrix, the quantity of GAG secreted by chondrocytes can reflect the function expression of the cells. According to Figure 6b, it was found that the proportions of GAG to DNA in both CHM and CHB increased with the culture period. The chondrocytes in CHM maintained the proportion of GAG to DNA on day 28. Different from the situation in CHM, the chondrocytes in CHB secreted little GAG in the first 2 weeks. In comparison, the GAG/DNA values in CHM were significant higher than those in CHB, from 6.9-fold, 2.1-fold to 1.5-fold on day 14, 21 and 28, respectively. Quantitative and qualitative analyses of GAG indicated that the secreting of GAG in CHM is stronger and 1-2 weeks ahead than that in CHB.

3.7 Histological analysis

Figure 7 showed the HE staining results of CHM and CHB. In CHB, the encapsulated cells spread on day 1 and reached the highest cell density on day 4, following continuing decrease of cell density from day 7 to day 28. Furthermore, the cells maintained the shape and separated distribution, which meant the phenotype was not recovered during the process. Only a few lacunae could be found in the CHB samples from day 7 to day 28. On the contrary, it could be seen that the cell density in the CHM increased and the phenotype of cells was spherical. Large amount of cartilage lacunae was found in the CHM samples on day 7. Then the lacunae became more obvious

on day 14 and afterwards. Cells in the CHB maintained the fibroblast-like morphology with few clusters, which matched the studies made by Van Susante and Schuman.[10,11] Several clusters could be observed in the CHBo while the situation in CHBi is even worse. This might be explained by the mass exchange difference as mentioned in the BSA release test. Nutrient could only diffuse into the outer area of the gel and was consumed by cells in the outer area, leading to the insufficient nutrient supply and dysfunction of chondrocytes in the inner area of hydrogel.

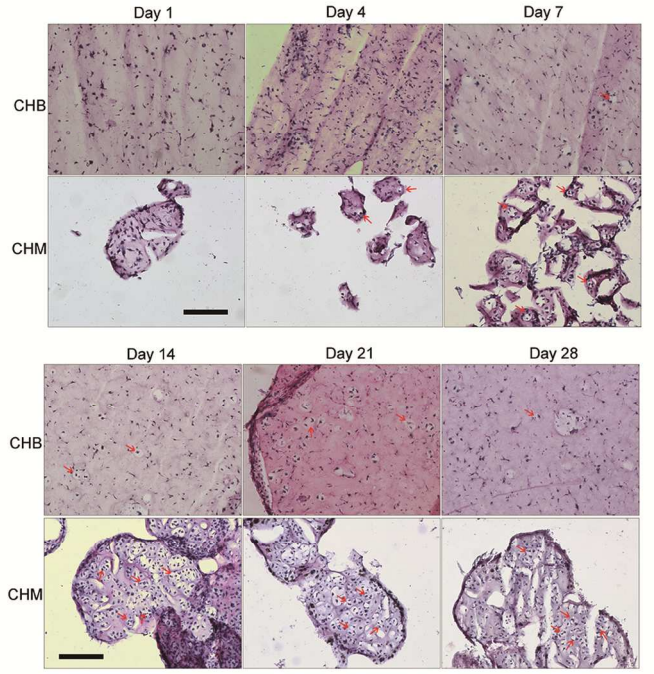

Figure 7. Histological observation of CHB and CHM after *in vitro* culture, staining by haematoxylin and eosin (HE). Scale bars represent 200 μm.

TB staining of CHM and CHB were shown in the Figure 8. On day 1 and day 4, TB staining in both the inner area of CHB (CHBi) and outer area of CHB (CHBo), as well as the CHM was negative. The first positive staining in the CHM was observed on day 7. The color kept on becoming deeper and more homogeneous with the increase of culture time from day 7 to day 28, which meant the increase of GAG contents in CHM. Positive staining was also found in the CHBo on day 7, but the color is light and inhomogeneous. In contrast, the CHBi showed almost no positive staining even on day 28. Only few areas around the lacunae were positively stained. Homogeneous positive TB staining in the CHM also reflected the phenotype recovery of chondrocytes. It was reported that matrix secreted by chondrocytes also owned the ability to promote the recovery of phenotype,[11] and chondrocytes needed the matrix support to retain the new matrix.[29] According to the results of TB staining and biochemical analyses in our study, we could infer that the chondrocytes in CHM have a better function expression than that in CHB.

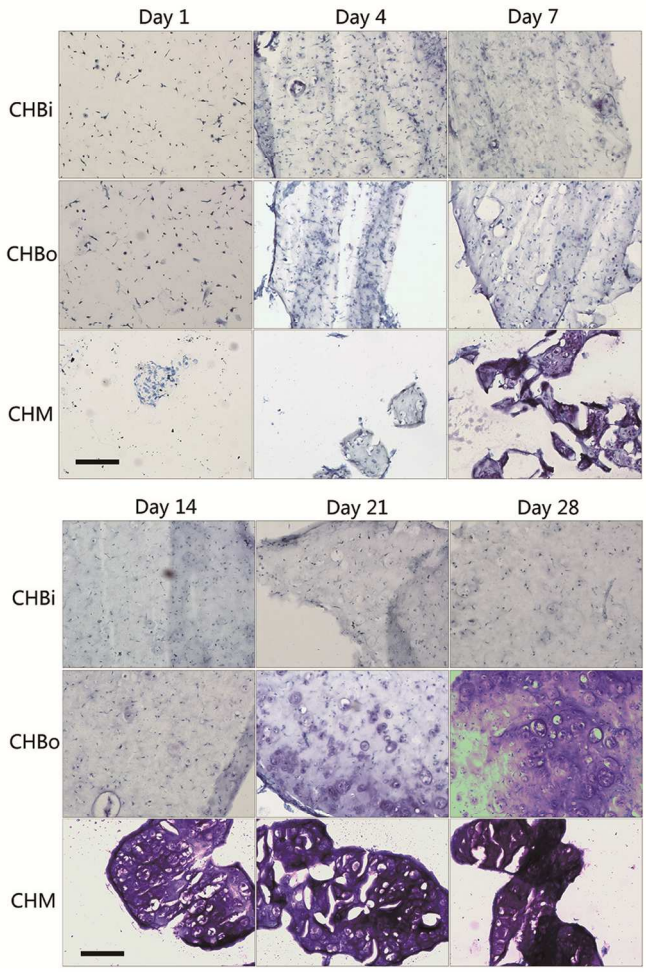

Figure 8. Histological observation of the inner area of CHB (CHBi), outer area of CHB (CHBo) and CHM after *in vitro* culture, staining by toluidine blue (TB). Scale bars represent 200 μm.

The specific extracellular matrix secreted by chondrocytes plays a crucial role in the cartilage regeneration and neo-cartilage formation.[30] It is important to supply a nice microenvironment for chondrocytes to keep the phenotype, since the dedifferentiated cells are apt to lose the capability to secrete expectable ECM to repair the defect.[31,32,33] The inadequate or inappropriate secreted ECM cannot enhance the mechanical property of engineered cartilage tissue.[8,15,34] Farrell compared the mechanical property of different areas of the hydrogel (with a size of 4 mm in diameter, 2.25 mm in depth) and found 2-fold decrease in compressive strain from outer area to inner at 21 day.[35] Mass transfer property, which will affect nutrient supply and metabolic product diffusion, might be the reason that gives rise to the difference of the proliferation and morphology of chondrocytes [2,18,36], and further leads to the mechanical differences of the engineered cartilage. Lots of studies have been made on chondrocyte-collagen hydrogel mixture in the latest decade, but most of which were bulk ones.[4,5,27] There were a few studies focusing on the permeability of hydrogels, even less investigation executed on the relationship between mass transport and cells response in *in vitro* culture. Besides the structure factors, the hydrogel size is one of the most obvious factors which have great influence on the solute transport. Based on the study of microspheres in this paper, we were trying to reveal the size effect of hydrogels and guide the future research and application of hydrogels for tissue regeneration.

We got a cartilage-like tissue with homogeneous specific extracellular matrix as shown in TB staining results. In the study of Valentin Dhote, they mentioned that the diffusion of matrix secreted by chondrocytes will make the matrix more homogeneous, which is the key factor in cartilage regeneration.[37] The results of our studies discovered a similar phenomenon. Therefore, the collagen microspheres of this study promoted the proliferation of cells at the early stage, and accelerated the function expression at the late stage of *in vitro* culture. The superior permeability might be a positive factor, which could accelerate cartilage regeneration.

4. Conclusion

CHM loaded with chondrocytes were fabricated using emulsion method at mild conditions, and they were further compared with CHB via 4-week culture *in vitro*. With the help of outstanding mass transfer property of CHM, the chondrocytes in CHM could not only proliferate more quickly, but also regain normal morphology and phenotype faster than in CHB. As a consequent, more specific matrix secreted and homogeneously distributed in CHM. Lot of lacunae are observed when the chondrocytes loaded CHM are cultured for 4 weeks. On the contrast, limited matrix is accumulated in the outer area of CHB and fewer in the inner area. Therefore, the size would obviously influence the mass exchange of hydrogels and further affect the behavior of embedded cells. Development of cartilaginous constructs base on CHM for cartilage regeneration is under investigation both *in vitro* and *in vivo*.

Acknowledgements

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