Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal Name

Journal of Materials Chemistry B

Dynamic Article Links ►

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Highly active porous scaffolds of collagen and hyaluronic acid prepared by suppression of polyion complex formation

Shangwu Chen[a, b], Qin Zhang[a, b], Tomoko Nakamoto[a], Naoki Kawazoe[a], and Guoping Chen[*, a, b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Collagen-hyaluronic acid scaffolds with high bioactivity, good mechanical property and homogeneous pore structures are desirable for their applications in tissue engineering. However, in aqueous condition collagen and hyaluronic acid form polyion complexes (PIC), which results in heterogeneous structures and poor mechanical properties of the scaffolds. In this study, we used low molecular weight salts to suppress PIC formation in collagen-hyaluronic acid suspensions during scaffold preparation. The suppression of PIC formation was studied by using turbidimetry, viscosity measurement and infrared analysis. The effects of PIC formation suppression on the morphology and mechanical properties of the scaffolds were examined with scanning electron microscopy and compression tests. PIC formation was found to be dependent on the ionic strength of the suspension. The secondary structure of collagen was partially altered by its strong electrostatic interactions with hyaluronic acid. The suppression of PIC formation resulted in collagen-hyaluronic acid scaffolds with homogeneous pore structures and remarkably enhanced mechanical properties. Collagen-hyaluronic acid scaffolds prepared under suppression of PIC formation promoted proliferation of fibroblasts and upregulated the expression of genes encoding EGF, VEGF and IGF-1. Using low molecular weight salts to suppress PIC formation could aid in the design of collagen-glycosaminoglycan scaffolds for tissue engineering.

1. Introduction

Three-dimensional porous scaffolds used in tissue engineering act as temporary templates to guide cell ingrowth and tissue regeneration. Apart from the requirements of good biocompatibility and nontoxicity, scaffolds should have an appropriate pore structure to facilitate cell infiltration and metabolite exchange and robust mechanical properties that allow them to endure cell-mediated contraction[1] during cell culture and mechanical load at the transplantation site. Ideally, porous scaffolds should mimic the chemical composition of native tissue and provide suitable mechanical, structural and biological signals to enhance cell activity and tissue regeneration.

Collagen-glycosaminoglycan (CG) scaffolds, whose components are the major structural proteins and polysaccharides found in a natural extracellular matrix, are widely used in tissue engineering due to their good biocompatibility and non-toxic degradation products. Improvements in the biofunctionality and regeneration capacity of collagen-derived scaffolds can be achieved through the incorporation of glycosaminoglycan[2, 3] and CG scaffolds have been used for the regeneration of various tissues such as skin, tendon, nerve, conjunctiva, cartilage and bone[4-8]. However, CG scaffolds prepared by freeze-drying CG slurries have inadequate mechanical properties[9, 10] and uneven matrix distribution, which may limit their practical use, particularly for applications in load-bearing tissues such as cartilage and bone. Attempts to improve the mechanical properties of CG scaffolds have been made by increasing the concentrations of collagen and glycosaminoglycan in the CG slurry. However, high CG concentrations result in a heterogeneous microstructure[10, 11] due to the heterogeneity of the CG suspension. New methods to prepare CG scaffolds with high bioactivity, good mechanical properties and homogeneous pore structures are necessary.

In this study, we used low molecular weight (MW) salts to prepare homogeneous collagen-hyaluronic acid (CH) suspensions for scaffold fabrication. The formation of polyion complex (PIC) between positively charged collagen and negatively charged hyaluronic acid was suppressed by the salts in the CH suspensions[12], making these CH suspensions homogeneous. We hypothesize that PIC suppression in CH suspensions will improve the pore structure, mechanical properties and bioactivity of CH scaffolds. Therefore, the objective of this research is to study PIC formation in CH suspensions with different concentrations of salts and the effects of PIC suppression on the pore structure, mechanical property and bioactivity of CH scaffolds.

2. Materials and methods

2.1. Preparation of CH suspensions

An aqueous solution (pH = 3.0) of type I collagen (1 wt.%) isolated from porcine skin (Nippon Meat Packers, Inc.) and an aqueous solution of hyaluronic acid (HA) (1 wt.%) isolated from rooster comb (Wako Pure Industries, Ltd.) were used to prepare aqueous suspensions of collagen-hyaluronic acid (CH) mixture. Before the two solutions were mixed, sodium chloride (NaCl) granules were added to the collagen and HA aqueous solutions, and the solutions were gently stirred at 4 ºC for 12 h. The collagen and HA aqueous solutions were then mixed at a volume

ratio of 9:1 (collagen:HA) and gently stirred at 4 ºC for 24 h. The mixed aqueous suspensions and subsequently freeze-dried samples were designated as CHx, with x representing the NaCl concentration (x = 0.000-0.200 M). To study the relationship between ionic strength and PIC formation by turbidimetry, we also prepared CH suspensions with a divalent salt (sodium sulfate, Na_2SO_4) at various concentrations. The ionic strength was adjusted to be equivalent to that of NaCl.

2.2. Turbidimetry and viscosity measurements

The transmittance of the CH mixture aqueous suspensions at 500 nm with different concentrations of NaCl and Na_2SO_4 was measured using an UV-Vis spectrophotometer (V-660, JASCO, Inc.). The sheer viscosity of the CH suspensions (within the sheer-speed range of 1-1000 s^{-1}) was recorded at 4 ºC by a MCR301 Rheometer and RheoPlus software (Anton Paar Co.). To observe the gross appearance of PIC, CH suspensions were centrifuged at a speed of 15,000 rpm at 4 ºC for 30 min to precipitate the PIC in these suspensions.

2.3. Preparation of CH scaffolds

CH porous scaffolds were prepared by freeze-drying. First, a CH suspension was poured into a silicone frame on a copper plate wrapped with perfluoroalkoxy (PFA) film (Universal Co., Ltd). Copper plates were chosen as substrates to facilitate heat conduction and PFA film was used to ensure the easy detachment of frozen suspensions. A silicone frame (60 mm × 40 mm × 5 mm) on the copper plate was used to confine the suspension to the copper plate and control the thickness. The surface of the suspension was flattened by covering the silicon frame with a glass plate wrapped in PFA film. The entire construct was then frozen at -30 ºC for 9 h. After glass plate and copper plate were removed from the construct, the frozen construct was freeze-dried under a vacuum of less than 5 Pa with a freeze dryer (FDU-2200, Tokyo Rikakikai Co., Ltd.) for 24 h. All samples were frozen and freeze-dried under the same conditions. The non-crosslinked CH scaffolds were used for infrared spectrum measurements, while crosslinked CH scaffolds were used for other experiments.

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Peptide Institute, Inc.) and N-hydroxysuccinimide (NHS, Wako Pure Chemical Industries, Ltd.) were used as crosslinking agents. EDC and NHS were dissolved in an ethanol/water (80/20, v/v) mixture, which was used to protect the CH scaffolds from dissolution during crosslinking. A crosslinking solution containing 50 mM EDC and 20 mM NHS was prepared. CH scaffolds were kept in the crosslinking solution at room temperature for 8 h. The crosslinked scaffolds were washed with MilliQ water for five times. The scaffolds were then soaked in MilliQ water, frozen and freeze-dried again under the same previous conditions. The CH porous scaffolds prepared with 0.000, 0.025, 0.050, 0.075, 0.100, 0.125, 0.150 and 0.200 M NaCl were referred as CH0, CH0.025, CH0.050, CH0.075, CH0.100, CH0.125, CH0.150 and CH0.200, respectively.

2.4. Infrared analysis

The infrared spectra of non-crosslinked CH0, CH0.050, CH0.100 and CH0.150 scaffolds were obtained using a Fourier transformed infrared spectrometer (FTIR-8400S, Shimadzu Corp.) at a resolution of 4 cm-1. For each scaffold, spectra from six different locations were recorded and averaged.

Two-dimensional infrared correlation spectroscopy (2D-IR) was used to study the changes in molecular conformation. Using correlation analysis, 2D-IR can enhance the resolution of an IR spectrum by spreading overlapped IR peaks along two dimensions[13] and 2D-IR has been used to analyze the secondary structures and denaturation of proteins[14-16]. Under an external perturbation of a system (chemical, electrical, thermal, mechanical stimulation, etc.), a sequential set of dynamic IR spectra can be collected and transformed into a 2D correlation map to reveal any spectral changes induced by the perturbation. The 2D-IR map, on which correlation intensity is plotted as a function of two independent wavenumbers, shows peaks corresponding to the structural change caused by the perturbation. Before two-dimensional correlation analysis, the baselines of averaged spectra of different CH scaffolds were corrected. These spectra were then analyzed using a 2D Pocha software provided by Daisuke Adachi (Kwansei Gakuin University) to generate 2D-IR maps for interpretation.

2.5. Scanning electron microscopy (SEM) and compression tests

Crosslinked CH scaffolds were cut with a blade and their cross sections were sputter-coated with platinum. The cross sections were observed at 10 kV with a scanning electron microscope (JSM-5610, JEOL, Ltd.). For compression testing, crosslinked CH scaffolds were cut into disks with a diameter of 8 mm and a height of 4 mm using a biopsy punch. The dry disk samples were compressed at a rate of 0.1 mm/s to generate stress-strain curves with a texture analyzer (TA.XTPlus, Texture Technologies Corp.). The Young's modulus was calculated from the initial linear region of the stress-strain curve and sample dimension. A minimum of six samples were tested for each type of scaffold.

2.6. Culture of dermal fibroblasts in CH scaffolds

The CH0 and CH0.150 scaffolds were used for culture for human dermal fibroblasts. Collagen porous scaffolds prepared by the same procedure as that of CH0 scaffolds were used as a control. The three types of scaffolds were punched into disks with a diameter of 12 mm and cut to a thickness of 2 mm. The discs were sterilized with 70% ethanol for 30 minutes, rinsed three times with sterile Milli-Q water and conditioned with culture medium. Neonatal human dermal fibroblasts (NHDF, Cascade Biologics, Inc.) were subcultured in 75-cm^2 tissue culture flasks in Medium 106 (Life Technologies Corporation) supplemented with low serum growth supplement kit (Life Technologies Corporation) in an incubator equilibrated with 5% CO_2 at 37°C. The adjusted medium was referred as serum medium. The fibroblasts in confluence were rinsed with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, detached from the culture flasks using 0.025% (w/v) trypsin and 0.01% (w/v) ethylenediaminetetraacetic acid (EDTA), and neutralized by HEPES solution containing 10% FBS (Kurabo Industries, Ltd.). The collected cell suspension was centrifuged and suspended in the serum medium to a concentration of 5.0 × 10^6 cells/ml. The cells were then seeded in the three types of scaffolds placed in the wells of 12-well cell culture plates by dropping 200 µl of cell suspension solution onto the top surface of each scaffold and cultured in an incubator equilibrated with 5% CO_2 at 37°C. After 3 h incubation to allow cell attachment, 10 mL serum medium was added to each well of the plates and cultured for 14 days with the serum medium in an incubator equilibrated with 5% CO_2 at 37°C. The medium was exchanged every two days.

2.7. Analysis of cell distribution and viability

After 24 hours, 7 days or 14 days of culture, the scaffolds were washed three times with PBS and fixed with 2.5% glutaraldehyde aqueous solution at room temperature for 1 hour. The scaffolds were then washed three times with pure water, frozen at -30 ºC

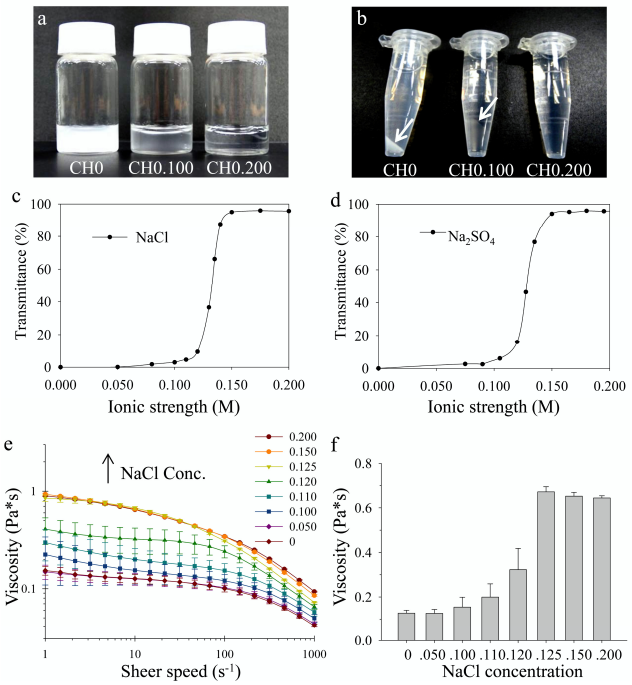

Fig. 1. Formation of polyion complex in CH suspensions. Gross appearance of (a) CH suspensions with different concentrations of NaCl (0, 0.100 or 0.200 M NaCl) and (b) CH suspensions after centrifugation. Arrows mark the boundary between the precipitated PIC and supernatant. Transmittance at 500 nm of CH suspensions prepared with different concentrations of (c) monovalent NaCl or (d) divalent Na_2SO_4.The ionic strength (M) was calculated from molar concentrations of salt ions. (e) Sheer viscosity-sheer speed relationship of CH suspensions prepared with different concentrations of NaCl (0 - 0.200 M). (f) Viscosities of CH suspensions measured at a sheer speed of 10 s^{-1}. Means ± SD, N = 3.

and freeze-dried for 2 days. The cross sections of the freeze-dried samples were coated with platinum for SEM observation to examine cell distribution in the scaffolds.

Live/dead staining was performed to evaluate cell viability using calcein-AM (live: green) and propidium iodide (dead: red) staining reagents (Cellstain Double Staining Kit, Dojindo Laboratories). After two weeks of culture, the cell/scaffold constructs were washed with HEPES buffer and incubated in 2 µM calcein-AM and 4 µM propidium iodide solution in HEPES buffer for 30 minutes. After being rinsed with HEPES buffer, the submerged specimens were observed with a fluorescence microscope.

2.8. Cell proliferation assay and real-time PCR analysis

The cell proliferation in the scaffolds was evaluated by quantifying the DNA amount. After being cultured for 1, 3, 7, or 14 days, the cell/scaffold constructs were washed with pure water, freeze-dried and then digested with papain solution. Papain (Sigma-Aldrich, St Louis, Missouri, USA) was dissolved at 400 µg/ml in 0.1 M phosphate buffer (pH 6.0), with 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraaceticacid (EDTA). An aliquot of papain digests was used to measure the DNA content with Hoechst 33258 dye (Sigma-Aldrich) and an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation wavelength of 360 nm and emission wavelength of 460 nm. Four samples were used to calculate the average and standard deviation.

For analysis of gene expression, the cell/scaffold constructs (3 samples for each type of scaffolds) after the 14-day culture were rinsed with PBS buffer, immersed in 1 ml Sepasol-RNA I Super G solution (Nacalai Tesque, Inc.) and frozen at -80 ºC. The frozen constructs were crushed into powder by an electric crusher and transferred back into the Sepasol solution to isolate the RNA from the cells. Total RNA (1.0 µg) was used as a first strand reaction that included random hexamer primers and murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time PCR was amplified for 18 S rRNA, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1). The reaction was performed with 10 ng of cDNA, 90 nM PCR primers, 25 nM PCR probe, and FastStart TaqMan Probe Master (Roche Diagnostics Japan, Tokyo, Japan). The expression levels of 18 S rRNA were used as an endogenous control, and gene expression levels relative to GAPDH were calculated using the comparative Ct method. The sequences of primers and probes are listed in Table 1. The primers and probes were obtained from Applied Biosystems and Hokkaido System Science (Sapporo, Japan).

Table 1. Primers and probes for real-time PCR analysis.

mRNA	Oligonucleotide
18 S rRNA	Hs99999901_s1
GAPDH	Hs99999905_m1
VEGF	Hs00900054_m1
EGF	Hs00153181_m1
IGF-1	Hs00153126_m1

2.9. Statistical analysis

A one-way analysis of variance followed by pairwise multiple comparison (Tukey Test) was used to compare groups of data. Paired t-tests were used to compare individual sets of data. Significant differences were considered when $p < 0.05$.

3. Results and discussion

3.1. PIC formation and turbidity of CH suspensions

Collagen and HA aqueous solutions were mixed with or without salts to prepare the CH aqueous suspensions. Two kinds of low molecular weight salts, NaCl and Na_2SO_4, were used to prepare the CH suspensions for the study of the relationship between ionic strength and PIC formation. By gross observation, the CH suspensions prepared without salt (CH0) were opaque. CH suspensions prepared with 0.100 M NaCl (CH0.100) were semitransparent, while CH suspensions prepared with 0.200 M NaCl (CH0.200) were clear (Fig. 1a). The CH0, CH0.100 and CH0.200 suspensions were centrifuged to further reveal the PIC formation in different CH suspensions. After centrifugation, the PIC in CH0 settled at the bottom of tube and was white and compact in appearance. The PIC in CH0.100 was watery and loose, while no obvious PIC sediment was observed in CH0.200 (Fig. 1b). The transmittance of the CH suspensions was measured after the suspensions were prepared at ionic strengths of 0.000 to 0.200 M (Fig. 1c, d). Despite the different salts used, the transmittance of CH suspensions remained approximately zero at low ionic strengths, increased gradually as the ionic strength increased and increased rapidly when the ionic strength changed from a 0.100 M to 0.150 M, after which the maximum transmittance was maintained.

With the presence of salts in the CH suspensions, PIC formation between collagen and HA was suppressed due to the ability of cations and anions from low MW salt to screen the charges along the polymer chains. The suppression of PIC formation by different concentrations of salts resulted in the

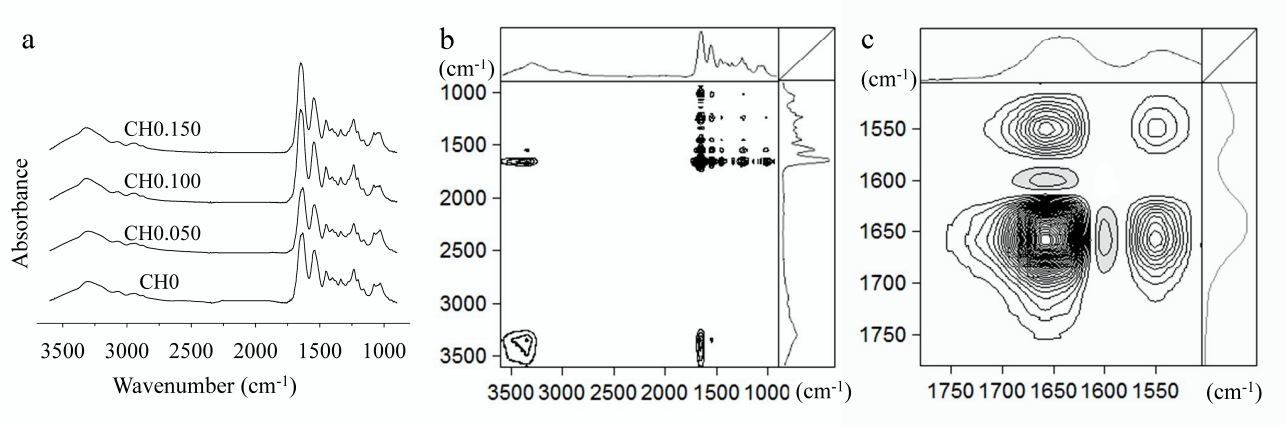

Fig. 2. (a) IR spectra of non-crosslinked CH sponges prepared from CH suspensions with different NaCl concentrations (0, 0.050, 0.100 or 0.150 M NaCl). (b and c) Synchronous 2D-IR maps of non-crosslinked CH sponges prepared from CH suspensions with different NaCl concentrations. The upper right zone (1500-1775 cm^{-1}) of (b) is magnified and shown in (c). The one-dimensional spectra on the top and right columns of the maps are the average spectra used in the correlation analysis.

different transparencies observed in the CH suspensions. When CH suspensions were prepared without salts or with a low concentration of salt, PIC formed and the suspension was opaque, resulting in low transmittance. When high concentrations of salts were used, PIC formation was suppressed and the CH suspensions were clear, resulting in high transmittance. The similarity between the two transmittance curves of CH suspensions prepared with monovalent salts (NaCl) or divalent salts (Na_2SO_4) indicates that PIC formation was dependent on the ionic strength of the CH suspensions rather than the type of salt used, which is consistent with previous studies on PIC formation and ionic strength[17-19]. Therefore, only NaCl was used for the following experiments.

3.2. Viscosity of CH suspensions

The sheer viscosity of CH suspensions prepared with different NaCl concentrations was measured, as shown in Fig. 1e, f. All CH suspensions were sheer-thinning: their viscosity decreased with increasing sheer speed. The viscosities of the CH suspensions prepared without NaCl or with 0.050 M NaCl were the lowest of those measured. The viscosity increased when the NaCl concentration increased from 0.050 M to 0.120 M, and remained unchanged after the NaCl concentration reached 0.125 M.

The observed variations in viscosity can be explained by the electrostatic interactions between collagen and HA molecules in suspension. When the positively charged collagen and negatively charged HA interacted, they formed PIC. The binding affinity between collagen and HA molecules was weakened by the presence of Na^+ and Cl^- ions in the suspensions. With no NaCl or low concentrations of NaCl (< 0.100 M), ionic strength was too low to shield the charges on collagen and HA polymer chains and to weaken their strong binding affinity, resulting in the formation of compact PIC. The formation of compact PIC resulted in decrease of soluble polymer molecules in the suspension and therefore a low viscosity. At intermediate NaCl concentrations (0.100 – 0.125 M), the collagen and HA polyions were partially shielded by Na^+ and Cl^- ions, weakening the binding affinity between the polyions. A weak binding affinity resulted in loose PIC that was not completely separated from the suspension and therefore viscosity of median values. The viscosity increased with NaCl concentration and peaked at high NaCl concentrations (≥ 0.150 M), where the polyions were completely shielded by their counterions and PIC formation was suppressed. Therefore, viscosity reached a stable maximum.

3.3. Infrared analysis

To examine the effects of the addition of salt on the molecular structures of collagen and HA, we analyzed the infrared spectra of non-crosslinked CH sponges prepared from CH suspensions with different NaCl concentrations (Fig. 2a). Non-crosslinked CH sponges, prepared by freeze-drying CH suspensions, were used for IR analysis to eliminate any effects from the crosslinking reagent. No obvious differences among these spectra were observed because of the same functional groups of collagen and HA in all of the sponges.

The 2D-IR map was used to elucidate the structural change of collagen and HA molecules in CH scaffolds caused by the addition of varying amounts of NaCl. A sequential set of spectra of non-crosslinked CH sponges (CH0, CH0.050, CH0.100, CH0.150) was used for 2D-IR analysis to generate the synchronous 2D-IR maps (Fig. 2b, c). The autopeak (autopeaks: peaks located on the diagonal line) at 1655 cm^{-1} denoted a shift of the amide I band, indicating that collagen had a partially unordered structure at low NaCl concentrations while maintained its α helical structure at high NaCl concentrations. This suggests that the strong electrostatic interactions between collagen and HA molecules in suspensions without NaCl or with low NaCl concentrations could disturb the triple helical structure of collagen. The autopeaks at 3346 cm^{-1} and 1552 cm^{-1} demonstrated that N—H stretching and N—H bending were stronger at higher NaCl concentrations. The more intense stretching and bending vibrations of the amine groups suggested that collagen and HA molecules were less complexed at higher NaCl concentrations. The loss of α helical structure or the increase of unordered structure in proteins interacting with polyions has also been reported in previous works[20-22].

3.4. Scaffold morphology

Fig. 3 displays the SEM images of the microstructures of the crosslinked CH scaffolds. Crosslinked CH sponges exhibited

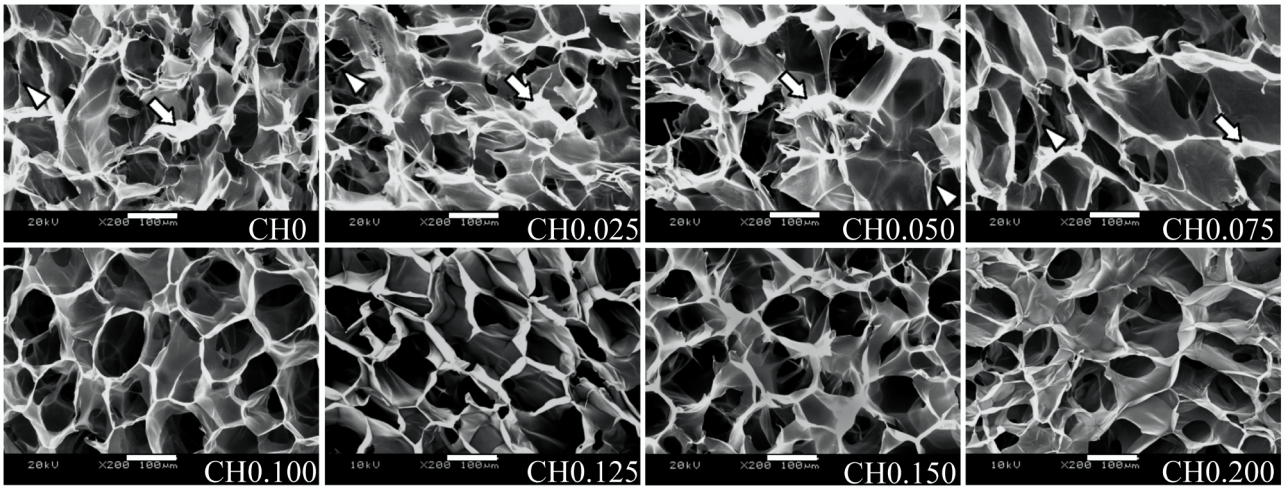

Fig. 3. SEM images of cross sections of crosslinked CH scaffolds prepared with different concentrations of NaCl (0 - 0.200 M). The arrows indicate the regions of high polymer density, and the arrowheads indicate the regions of low polymer density. Scale bar = 100 μm.

flake-like microstructures. There were some matrix blocks indicated by arrows and fibers indicated by arrowheads in the images of CH0, CH0.025, CH0.050 and CH0.075 CH scaffolds. The blocks were polymer matrix-rich regions with a high polymer density, while the fibers were polymer matrix-poor regions with a low polymer density. When NaCl concentrations were lower than 0.075 M, CH scaffolds exhibited heterogeneous pore structures with an unevenly distributed polymer matrix. Scaffolds prepared at intermediate or high NaCl concentrations (≥ 0.100 M) showed well-ordered micropore structures and an even distribution of polymer matrix.

The scaffold morphology was consistent with PIC formation as examined in previous parts. The compact PIC that formed without NaCl or at low NaCl concentrations resulted in the local condensation of the polymer matrix and a heterogeneous pore structure. The blocks CH0, CH0.025, CH0.050 and CH0.075 CH scaffolds should be due to the PIC in the CH suspensions which resulted in generation of polymer matrix-rich and poor regions. However, at higher NaCl concentrations, either PIC became loose or no PIC formed. The CH suspensions without PIC or with loose PIC avoided the local condensation of polymer matrix, resulting in the even distribution of polymer matrix and a homogeneous pore structure.

Previous studies by Harley et al.[10] and Davidenko et al.[11] reported that a high concentration (≥ 1 wt.%) of collagen and GAG suspension for scaffold preparation would result in heterogeneous CG scaffolds. This was confirmed by the heterogeneous morphology of CH0 scaffolds in our study. With the addition of NaCl to suppress PIC formation, a homogenous distribution of polymer matrix could be achieved. Apart from NaCl, other low molecular weight salts such as KCl and Na_2SO_4 can be used. Therefore, the difficulty in preparing homogeneous CG scaffolds from highly concentrated suspensions can be overcome by suppressing the PIC formation using low MW salts.

3.5. Mechanical property

The compressive Young's modulus of the crosslinked CH scaffolds prepared with different concentrations of NaCl was measured through compression tests and the results are shown in Fig. 4. The compressive modulus increased with NaCl concentration and reached a plateau when the NaCl concentration was higher than 0.100 M. The modulus of a CH scaffold prepared with 0.100 M NaCl was approximately 5-fold higher than that of a CH0 scaffold. The higher mechanical properties of CH scaffolds prepared with high concentrations of NaCl (0.100 - 0.200 M) might result from the homogeneous pore structure of these scaffolds. In contrast, the CH scaffolds prepared without NaCl or low NaCl concentrations showed heterogeneous pore structures and had lower mechanical strength. The correlation between improved mechanical property and a well-defined pore structure in porous scaffolds has also been previously reported[23-25].

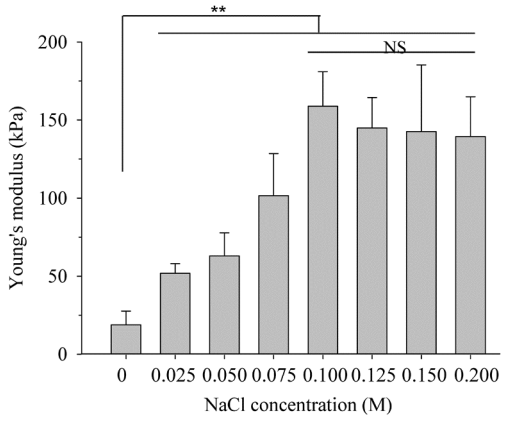

Fig. 4. Compression moduli of crosslinked CH scaffolds prepared with different concentrations of NaCl (0 - 0.200 M). Mean ± SD, N =6; **, $p<0.01$; NS, no significant difference.

After examining the PIC formation in CH suspensions and the properties of scaffolds from these suspensions, we proposed a mechanism to explain the formation of PIC at different ionic strengths and its effects on the morphology and mechanical properties of scaffolds. The interactions between the polyions (collagen and HA) and the different states of PIC formed under different ionic strengths are illustrated in Fig. 5. Because the binding affinity of collagen and HA molecules was determined by the ionic strength of the aqueous environment, polyions of opposite charges are compactly complexed at a low ionic strength (Fig. 5a), or become loosely complexed at an intermediate ionic strength (Fig. 5b) but no complexes are formed at high ionic strengths (Fig. 5c). Compact PIC in CH suspensions interferes with the polymer diffusion during the solidification of the CH

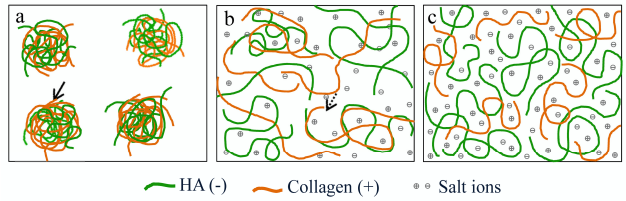

Fig. 5. Speculated schema of (a) compact PIC, (b) loose PIC and (c) no PIC formation in CH suspensions at different concentrations of salt. The solid arrows indicate complete complexation of collagen and HA, and the dashed arrows indicate partial complexation of collagen and HA.

suspensions at a low temperature, which renders the network of polymer solids heterogeneous. CH suspensions with loose PIC or no PIC are able to undergo even polymer diffusion that results in a homogeneous polymer matrix network. The homogeneous micropore structure and the uniform distribution of collagen and HA within the matrix may account for the improved mechanical properties of CH scaffolds.

3.6. Cell adhesion and proliferation in CH scaffolds

Adhesion of fibroblasts in the scaffolds after culture for 24 hour was observed by using SEM. Fibroblasts adhered to all the three types of scaffolds and showed better spread in the CH0.150 than did in CH0 and Col scaffolds (Fig. 6a). The cell proliferated with culture time and filled the spaces in the scaffolds (Fig. 6b-d). Most of the cells were alive and few dead cells were detected (Fig. 6e). DNA Quantification (Fig. 7) showed that the DNA content in

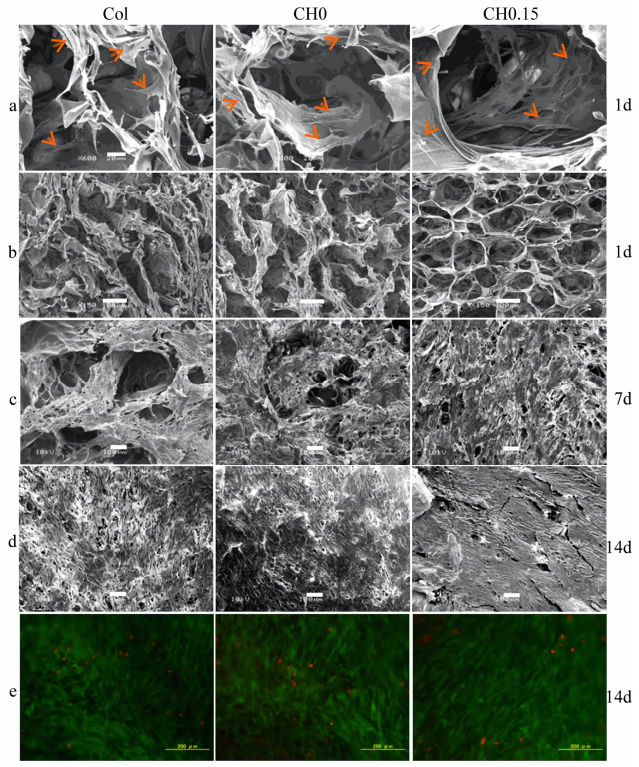

Fig. 6. (a) Adhesion of NHDFs in Col, CH0, and CH0.15 scaffolds after culture for 24 hours. Arrows mark some of the fibroblasts adhered on the walls of scaffolds. (b-d) SEM images showing proliferation of NHDFs and production of ECM during the 14 days culture. (e) Live/dead staining of NHDFs in collagen (Col), CH0, and CH0.15 scaffolds after culture for the 14 days. Green: live cells; red: dead cells. Scale bars, a: 20 μm; b-d: 100 μm; e: 200 μm.

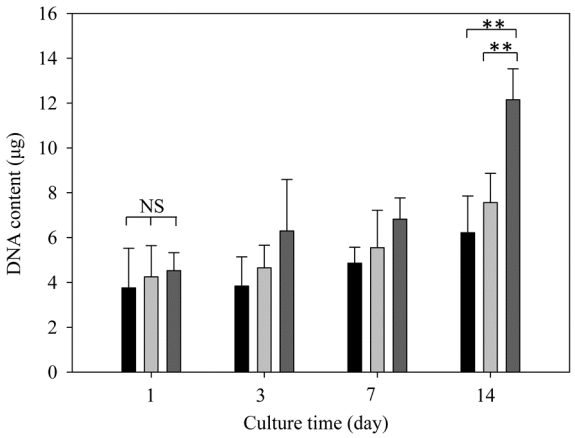

Fig. 7. Proliferation of NHDFs in Col, CH0, and CH0.15 scaffolds during the 14 days culture, as determined by DNA quantification. Mean ± SD, N = 4; **, p<0.01; NS, no significant difference.

the three types of scaffolds was almost the same after one day culture and increased with culture time. The increase of DNA content in the three scaffolds with culture time was in the increasing order of Col<CH0<CH0.15. DNA content in the CH0.15 scaffold after 14 days culture was significantly higher than that in the Col and CH0 scaffolds. The results indicated that CH0.15 scaffold had a higher promotive effect on the proliferation of fibroblasts compared to Col and CH0 scaffolds.

The expression of genes encoding EGF, VEGF and IGF-1 was analyzed after 14 days culture (Fig. 8). Fibroblasts cultured in the CH0.15 scaffold showed the highest expression of these genes. The cells cultured in the CH0 scaffold showed higher expression level of these genes than did in the Col scaffold. These results indicated that the CH scaffolds promoted cell proliferation and expression of the genes encoding EGF, VEGF and IGF-1. The results were in good agreement with previous reports that show that CH scaffolds have a promotive effect on cell proliferation[11, 26]. Cell proliferation was further promoted and the expression of EGF, VEGF and IGF-1 was upregulated when fibroblasts were cultured in the CH0.15 scaffold. Suppression of PIC formation during the scaffolds preparation could make the homogeneous distribution of HA in collagen matrix and maintain collagen's secondary structure, which therefore stimulated cell proliferation in the porous matrices at the highest level. High expression of EGF, VEGF and IGF-1 should have some effects on the promotion of cell proliferation because these genes have been reported to be concerned with proliferation of fibroblasts[27-29]. On the other hand, cells may sense the mechanical property of their substrate via the integrin-actin coupling at focal adhesion complexes[30] and adapt their behaviors such as adhesion and proliferation to different scaffold stiffness[31, 32]. Therefore, the

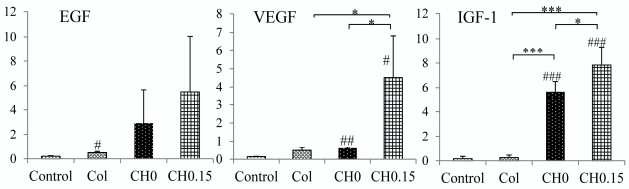

Fig. 8. Gene expression levels of NHDFs cultured in Col, CH0, and CH0.15 scaffolds for 14 days; the NHDFs used for cell seeding at day 1 were used as control group. EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; IGF-1: insulin-like growth factor 1. Mean ± SD, N =3; *, p<0.05; ***, p<0.001; #, p<0.05, compared to Control; ##, p<0.01, compared to Control; ###, p<0.001, compared to Control.

high mechanical property of CH0.15 scaffold might also have promoted the proliferation of fibroblast through mechano-transduction pathways.

4. Conclusions

The interactions between collagen and hyaluronic acid in suspensions of various ionic strengths were examined with turbidimetry and viscosity measurements. Gross appearance observations and turbidimetry showed that PIC formation between collagen and HA was dependent on the ionic strength of their suspension. Viscosity measurements further confirmed the different states of PIC formed under various ionic strengths. The strong binding affinity of collagen and HA can partially induce a change in collagen's secondary structure, as indicated by infrared analysis. The effects of PIC suppression by low molecular weight salts on the morphology and mechanical properties of CH scaffolds were examined with SEM and compression tests. CH suspensions with no PIC or loose PIC resulted in scaffolds with homogeneous micropore structures and markedly improved compressive moduli, compared with those fabricated from CH suspensions with compact PIC. CH scaffolds prepared with no PIC promoted proliferation of fibroblasts and expression of EGF, VEGF and IGF-1 genes. Using low molecular weight salts to suppress PIC in CG suspensions used to prepare scaffolds should aid in the design of highly active CG scaffolds with homogeneous pore structures and good mechanical properties for tissue engineering.

Acknowledgements

This work was supported by the World Premier International Research Center Initiative on Materials Nanoarchitectonics and a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Notes and references

[a] *Tissue Regeneration Materials Unit, International Center for Materials Nanoarchitectonics, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan.*
[b] *Department of Materials Science and Engineering, Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan.*
[*] *E-mail: Guoping.Chen@nims.go.jp; Fax: +81-29-860-4706; Tel: +81-29-860-449*

1. K. A. Corin and L. J. Gibson, *Biomaterials*, 2010, **31**, 4835-4845.
2. C. M. Tierney, M. J. Jaasma and F. J. O'Brien, *J Biomed Mater Res A*, 2009, **91A**, 92-101.
3. L. A. S. Callahan, A. M. Ganios, D. L. McBurney, M. F. Dilisio, S. D. Weiner, W. E. Horton and M. L. Becker, *Biomacromolecules*, 2012, **13**, 1625-1631.
4. I. V. Yannas and J. F. Burke, *J Biomed Mater Res*, 1980, **14**, 65-81.
5. L. K. Louie, I. V. Yannas, H. P. Hsu and M. Spector, *Tissue Eng*, 1997, **3**, 187-195.
6. W. C. Hsu, M. H. Spilker, I. V. Yannas and P. A. D. Rubin, *Invest Ophth Vis Sci*, 2000, **41**, 2404-2411.
7. E. Farrell, F. J. O'Brien, P. Doyle, J. Fischer, I. Yannas, B. A. Harley, B. O'Connell, P. J. Prendergast and V. A. Campbell, *Tissue Eng*, 2006, **12**, 459-468.
8. T. Douglas, S. Heinemann, C. Mietrach, U. Hempel, S. Bierbaum, D. Scharnweber and H. Worch, *Biomacromolecules*, 2007, **8**, 1085-1092.
9. B. P. Kanungo and L. J. Gibson, *Acta Biomaterialia*, 2010, **6**, 344-353.
10. B. A. Harley, J. H. Leung, E. C. C. M. Silva and L. J. Gibson, *Acta Biomaterialia*, 2007, **3**, 463-474.
11. N. Davidenko, J. J. Campbell, E. S. Thian, C. J. Watson and R. E. Cameron, *Acta Biomaterialia*, 2010, **6**, 3957-3968.
12. T. Taguchi, T. Ikoma and J. Tanaka, *J Biomed Mater Res*, 2002, **61**, 330-336.
13. I. Noda, *Applied Spectroscopy*, 1990, 550-561.
14. Y. Ozaki, K. Murayama, Y. Wu and B. Czarnik-Matusewicz, *Spectrosc-Int J*, 2003, **17**, 79-100.
15. N. Demirdoven, C. M. Cheatum, H. S. Chung, M. Khalil, J. Knoester and A. Tokmakoff, *J Am Chem Soc*, 2004, **126**, 7981-7990.
16. A. Filosa, Y. Wang, A. Ismail and A. M. English, *Biochemistry-Us*, 2001, **40**, 8256-8263.
17. Y. S. Xu, M. Mazzawi, K. M. Chen, L. H. Sun and P. L. Dubin, *Biomacromolecules*, 2011, **12**, 1512-1522.
18. B. Kim, C. N. Lam and B. D. Olsen, *Macromolecules*, 2012, **45**, 4572-4580.
19. Z. L. Wu, T. Kurokawa, S. M. Liang and J. P. Gong, *Macromolecules*, 2010, **43**, 8202-8208.
20. K. Henzler, A. Wittemann, E. Breininger, M. Ballauff and S. Rosenfeldt, *Biomacromolecules*, 2007, **8**, 3674-3681.
21. P. Schwinte, V. Ball, B. Szalontai, Y. Haikel, J. C. Voegel and P. Schaaf, *Biomacromolecules*, 2002, **3**, 1135-1143.
22. F. Gilde, O. Maniti, R. Guillot, J. F. Mano, D. Delphine Logeart-Avramoglou, F. Sailhan and C. Picart, *Biomacromolecules*, 2012.
23. Q. L. Zhou, Y. H. Gong and C. Y. Gao, *J Appl Polym Sci*, 2005, **98**, 1373-1379.
24. S. J. Hollister, R. D. Maddox and J. M. Taboas, *Biomaterials*, 2002, **23**, 4095-4103.
25. L. Draghi, S. Resta, M. G. Pirozzolo and M. C. Tanzi, *J Mater Sci-Mater M*, 2005, **16**, 1093-1097.
26. S. N. Park, H. J. Lee, K. H. Lee and H. Suh, *Biomaterials*, 2003, **24**, 1631-1641.
27. B. A. Harley, A. Z. Hastings, I. V. Yannas and A. Sannino, *Biomaterials*, 2006, **27**, 866-874.
28. B. A. Harley, M. H. Spilker, J. W. Wu, K. Asano, H. P. Hsu, M. Spector and I. V. Yannas, *Cells Tissues Organs*, 2004, **176**, 153-165.
29. L. Marinucci, C. Lilli, M. Guerra, S. Belcastro, E. Becchetti, G. Stabellini, E. M. Calvi and P. Locci, *J Biomed Mater Res A*, 2003, **67A**, 504-509.
30. D. E. Ingber, *Annu Rev Physiol*, 1997, **59**, 575-599.
31. M. G. Haugh, C. M. Murphy, R. C. McKiernan, C. Altenbuchner and F. J. O'Brien, *Tissue Eng Pt A*, 2011, **17**, 1201-1208.
32. C. R. Lee, A. J. Grodzinsky and M. Spector, *Biomaterials*, 2001, **22**, 3145-3154.