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Control of swelling properties of polyvinyl alcohol/hyaluronic acid hydrogels for the encapsulation of chondrocyte cells

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ABSTRACT: Hydrogel scaffolds for tissue engineering are important biomaterials. The target in this study was to prepare polyvinyl alcohol/hyaluronic acid hydrogels for the encapsulation of chondrocyte cells by a simple cross-linking reaction. Control of the swelling properties and morphology of the hydrogels for cultivation of chondrocytes was studied. The hydrogels were prepared from polyvinyl alcohol and hyaluronic acid derivatives bearing primary amine and aldehyde functionalities, respectively. The formation of the hydrogel upon mixing the aqueous solutions of the polymer derivatives took place at room temperature in a few seconds. The swelling properties of the hydrogels were found to depend on the polymer concentration and degree of substitution of the modified polymers. Scanning electron microscopy studies showed that the hydrogels had a suitable porous morphology for cell encapsulation. Furthermore, in vitro cell viability tests with the hydrogels showed no cytotoxicity for chondrocytes and that the cells grew well in the hydrogel scaffolds. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42272.

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INTRODUCTION

Hydrogels have a great potential as possible biomaterials, with uses, for example, in tissue engineering and drug release.[1–3] For tissue engineering, hydrogels are useful as their three-dimensional structure resembles extracellular matrix (ECM), which is the key component in tissues.[4] The porous structure of hydrogels enables their use as scaffolds, for example, in the regeneration of cartilage.[5,6] In order to serve as a scaffold, hydrogels must be biocompatible and meet many other requirements such as suitable porosity, swelling, mechanical strength, and degradation properties.[7] All these properties are affected by the type and concentration of polymers employed in hydrogels as well as by the cross-linking type and density.[1,8,9]

Advantages of physically cross-linked hydrogels are that they do not in general contain cytotoxic chemical groups. However, the cross-linking in these hydrogels is often difficult to control, leading to poorly defined scaffolds.[10] Chemical cross-linking, on the other hand, offers uniform scaffolds formed by covalent cross-links, but photoinitiators and cross-linking agents can be cytotoxic, limiting the use of chemical cross-linking in tissue engineering.[11] By a careful selection of the polymers and the chemical cross-linking type, a biocompatible gel-forming process for hydrogels can be achieved. Several studies have been reported on the formation of biocompatible hydrogels from chemoselective multifunctional polymers.[12,13] This approach offers fast gelation times with a well-defined network and a possibility for injectable hydrogels. A Schiff base reaction[12] between aldehyde and amino groups of polymer derivatives is a good example, because the formation of C=N double bond is fast and does not produce any toxic by-products.[14]

Both naturally derived polymers and synthetic polymers, and their combinations, can be used in hydrogels for tissue engineering. Natural polymers offer a good biocompatibility, but often lack sufficient mechanical properties, which can be controlled more adequately with synthetic polymers.[15] Examples of natural and synthetic polymers used often in hydrogels for tissue engineering are hyaluronic acid (HA) and polyvinyl alcohol (PVA).[16] HA is a high-molar-mass polysaccharide with a good biocompatibility and biodegradability and is an important component of extracellular matrix.[17,18] However, the applications of HA hydrogels have often been limited due to lack of sufficient mechanical properties.[19] PVA is a hydrophilic polymer and can

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provide hydrogels with good mechanical properties, but does not promote cell adhesion.[20,21]

In this study, PVA/HA hydrogels are synthesized from complementary reactive hydrazide-modified PVA (PVAHY) and aldehyde-modified HA (HAALD) through a chemical cross-linking reaction between primary amine groups in PVAHY and aldehyde groups in HAALD. The swelling properties of the hydrogels prepared were found to depend on the substitution degree and concentration of the polymer derivatives. An extensive study of the swelling properties for this type of PVA/HA hydrogels has not been reported previously. Finally, the PVA/HA hydrogels were found as suitable scaffolds for bovine knee chondrocyte cell encapsulation and as good hydrogel scaffolds for biomedical applications.

MATERIALS AND METHODS

Materials

Polyvinylalcohol with M_w 27,000 g/mol (98.0–98.8% hydrolyzed) was from Aldrich and polyvinylalcohol with M_w 15,000 g/mol (96% hydrolyzed) from Merck. Hyaluronic acid sodium salt from *Streptococcus equi* (M_w 1.63 × 10^6 g/mol) was from Sigma. 1,1′-Carbonyldiimidazole (CDI), glycine ethyl ester hydrochloride, hydrazine solution 35 wt % in H_2O, and *t*-butyl carbazate (TBC) were from Aldrich. Dimethyl sulfoxide (DMSO), 2,4,6-trinitrobenzenesulfonic acid solution 5% (w/v) in H_2O (TNBS), ascorbic acid, sulfadiazine, and propidium iodide were from Sigma. Sodium periodate was from Sigma-Aldrich, and triethylamine and fluorescein diacetate were from Fluka. Sodium acetate, acetic acid, and sodium tetraborate used for buffer solutions were from Merck. Dulbecco's modified Eagle's medium (DMEM) was from Euroclone (Pero, Italy) and fetal bovine serum (10%) as well as L-glutamine were from PAA (Linz, Austria). All solvents were of analytical grade and Milli-Q water was used in synthesis and determinations.

Preparation of Polymer Derivatives and Hydrogels

PVA was functionalized with hydrazide groups according to a previously reported method slightly modified.[10] PVA (400 mg, 9.1 mmol of repeating units) was dissolved in 8 mL of DMSO that had been dried with 4 Å molecular sieves. The solution was heated at 85°C with mixing for 2 h to dissolve the polymer. CDI (730 mg, 4.5 mmol) was added and the solution was mixed for 3 h under nitrogen atmosphere, after which 126 mg (9.0 mmol) of glycine ethyl ester hydrochloride and 125 μL (9.0 mmol) of triethylamine were added. The solution was allowed to react overnight under nitrogen atmosphere at room temperature. Hydrazine solution (8.6 mL, 0.271 mol) was added and the solution was mixed for 24 h at room temperature. After the reaction, 50 mL of water was added and the solution was filtered to obtain a clear solution, which was placed in a beaker and evaporated on a hot plate until about 8 mL of the solution remained. To this, a mixture of diethyl ether and ethanol (80/20 mL) was added at room temperature. Precipitated polymer was separated and dissolved in water (~20 mL). The modified polymer was purified by 72 h dialysis against water using a dialysis membrane of 1000 molecular weight cut-off. After dialysis, the polymer solution was lyophilized and the white solid product was stored in a refrigerator. ^{1}H NMR (400 MHz, D_2O, δ): 5.10 (m, 1H, polymer backbone CH of the modified unit), 4.10–3.95 (m, 1H, polymer backbone CH of the unmodified unit), 1.80–1.50 (m, 2H, polymer backbone CH_2).

Aldehyde-modified HA was prepared by oxidizing HA with sodium periodate,[13] and stored in a refrigerator. ^{1}H NMR (400 MHz, D_2O, δ): 9.59 (2H, —C(O)H), 4.40–4.60 (anomeric H, partially overlapped by H_2O signal), 3.95–3.20 (glucosidic H), 2.03 (3H, —NHC(O)CH_3).

In order to prepare the hydrogels, PVAHY (2.5–4.2 mg) and HAALD (27.5–45.8 mg) were separately dissolved in 1 mL of water, then mixed together (PVAHY/HAALD mass ratio of 1 : 11) at room temperature with initial total polymer concentrations of 15, 20, and 25 mg/mL. The mass ratio 1 : 11 was chosen with the aim of achieving almost an equivalent number of cross-linkable functional groups for hydrogel formation. The hydrogel was formed at room temperature within a few seconds, but it was allowed to stand for 2 h before further handling in order to ensure a proper cross-linking.

Characterization of Polymer Derivatives and Hydrogels

The degree of substitution (DS, mol %) of PVA was determined by a spectrophotometric method applying TNBS according to a previously reported method slightly modified.[10] PVAHY (~1 mg) was dissolved in 2 mL of borate buffer solution (0.1 M, pH 9.3). The sample (0.1 mL) was diluted with 2.8 mL of borate buffer solution and 75 μL of 0.025 M TNBS solution (prepared from 5% solution by diluting with borate buffer solution) was added. The sample solution was shaken thoroughly and allowed to stand for 30 min at room temperature before measurement of the absorbance (Shimadzu UVmini-1240 UV–vis spectrophotometer) of the red solution at wavelength 505 nm. A solution containing 2.9 mL of borate buffer solution and 75 μL of 0.025 M TNBS solution was used as a reference. Standard solutions were prepared from TBC in the same manner as the sample solution. Reported DS values are the average of three measurements. The DS of HAALD was determined according to the literature.[15]

^{1}H nuclear magnetic resonance (NMR) spectra of the polymer derivatives were recorded with a Bruker Avance-400 NMR spectrometer in D_2O using trimethylsilylpropionate (TSP) as an internal reference. The number of scans was 32 and relaxation delay was 10 s. A Bruker AMX-400 was used to obtain ^{13}C cross-polarization/magic angle spinning (CP/MAS) NMR spectra of the polymer derivatives and a dried and ground (non-swollen) hydrogel. Glycine was used as an external reference, and the spin rate was 10,000 Hz, number of scans 17,500, relaxation delay 4 s, and contact time 2 ms.

Cross-sectional morphology of freeze-dried (Heto Powerdry LL1500) hydrogel samples was investigated with a Hitatchi S-4800 scanning electron microscope at 1 kV. Samples of swollen hydrogels were freeze-dried, cut to expose cross-section, and sputtered with a thin layer (2.5 nm) of gold (Cressington 280HR).

The swelling behavior of the hydrogels was examined by immersing them in water for 24 h, after which they were weighed and the mass of the swollen gel (W_s) was obtained.

The swollen hydrogel was dried and the mass of the dried hydrogel (W_d) was determined by weighing. Swelling ratio (SR) was calculated from $SR = (W_s - W_d)/W_d$. Gel fraction (GF) of the hydrogels was calculated from $GF\ \% = (W_d/W_0) \times 100$, where W_0 is the initial mass of the added polymers.

Determination of Cell Viability

Chondrocyte cells for in vitro experiments were harvested from 1.5-year-old bovine knees, obtained from a local abattoir (Atria, Kuopio, Finland) within 24 h of slaughter, and then isolated in a two-step protocol, as previously described.[22] Cell isolation was performed similarly twice from one animal (once per knee). Viability of the cells was determined before the following investigations.

The cytotoxicity of the individual gel components was evaluated by pipetting PVAHY (2.5 mg/mL) and HAALD (27.5 mg/mL) solutions separately to a 24-well cell culture plate. Chondrocytes in medium (386 μL, 2.5×10^6 cells/gel) were added to the component solutions and mixed well. Cell culture medium (1 mL) containing DMEM, supplemented with 10% fetal bovine serum, 50 μg/mL ascorbic acid, 2 mM L-glutamine, and 50 μg/mL sulfadiazine, was added on top of the sample solutions. Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere for 24 h and 2 weeks. The medium was changed twice a week. Three parallel samples (per time point) were prepared of each polymer component.

Three hydrogels for cell culture were prepared as described above with an initial total polymer concentration of 15 mg/mL and with degree of substitutions (PVAHY/HAALD) 6.3/6.3, 9.8/8.9, and 12.8/10.6. Chondrocytes in medium (386 μL, 2.5 10^6 cells/gel) were added to aqueous PVAHY solution (2.5 mg/mL, 500 μL) in an Eppendorf tube and mixed well. HAALD solution (27.5 mg/mL, 500 μL) was pipetted to a 24-well cell culture plate and mixed with the solution of PVA derivative and cells. The mixing was performed quickly with a spatula taking care to avoid air bubbles. Hydrogel samples were cultured as earlier described. Three parallel samples (per time point) were prepared of each hydrogel.

The viability of chondrocytes cultivated in the constructs was evaluated using a combination of two fluorescent probes. Specimens were incubated for 5 min in a solution containing 60 μM concentration of cell-impermeable DNA-binding dye propidium iodide and 10 μM concentration of cell-permeable fluorescein diacetate in phosphate buffered saline (PBS). After washing with PBS, the samples were viewed with a confocal scanner (PerkinElmer Life Sciences, Wallac-LSR, Oxford, UK) on a Nikon Eclipse TE300 microscope, using the wavelengths 488/10 nm (excitation) and 525/50 nm (emission) for fluorescein and 568/10 nm (excitation) and 607/45 nm (emission) for propidium iodide.

RESULTS AND DISCUSSION

Characteristics of PVA/HA Hydrogels

PVA/HA hydrogels were synthesized from hydrazide-modified PVA (PVAHY) and aldehyde-modified HA (HAALD) with a mass ratio of 1 : 11 (PVAHY/HAALD) in order to achieve almost an equivalent number of cross-linkable groups. Swelling and porosity properties of prepared hydrogels were examined in order to find out the appropriate substitution degrees (DS) and concentrations of the polymer derivatives that will give suitable hydrogels for chondrocyte cell encapsulation and proliferation.

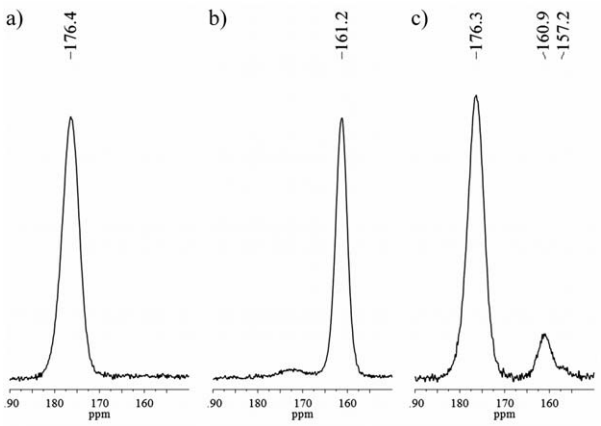

Figure 1. ^{13}C CP/MAS NMR spectra of (a) HAALD, (b) PVAHY, and (c) PVA/HA hydrogel (DS: PVAHY 10.6%, HAALD 7.2%, initial total polymer concentration 15 mg/mL).

The cross-linking through Schiff base reaction between primary amine and aldehyde functional groups producing C=N double bond (Scheme S1, supplementary data) was confirmed with ^{13}C CP/MAS NMR spectroscopy. The solid-state NMR spectra of PVAHY, HAALD, and PVA/HA hydrogel are presented in Figure 1. The signals corresponding to the carbons of the carbonyl groups were observed at 176.3 ppm for HAALD and at 160.9 ppm for PVAHY [Figure 1(c)]. Furthermore, the signal at 157.2 ppm can be due to the carbon of C=N double bond formed in the Schiff base reaction. The low intensity of this signal indicates that the number of C=N double bonds is not high and that the cross-linking reaction may not be complete.

Four series of PVA/HA hydrogels (Table I) were synthesized in order to study the effects of polymer concentration and degree

Table I. Swelling Ratios and Gel Fractions of PVA/HA Hydrogels

Hydrogel[a]	DS mol % (PVA/HA)	Conc. (mg/mL)[b]	SR	GF (%)
Gel-1	4.1/5.0	15	1690	53.0
Gel-2	4.1/5.0	20	1060	45.3
Gel-3	8.6/7.2	15	1000	39.7
Gel-4	8.6/7.2	20	900	32.3
Gel-5	8.6/7.2	25	690	25.6
Gel-6	10.6/9.2	15	640	38.3
Gel-7	10.6/9.2	20	580	38.8
Gel-8	10.6/9.2	25	470	24.8
Gel-9	7.2/7.2	15	840	54.0
Gel-10	7.2/7.2	20	800	43.5

[a] PVA with a molar mass 27,000 g/mol was used in Gel-1–8 and PVA with a molar mass 15,000 g/mol was used in Gel-9–10.
[b] Initial total polymer concentration in water.

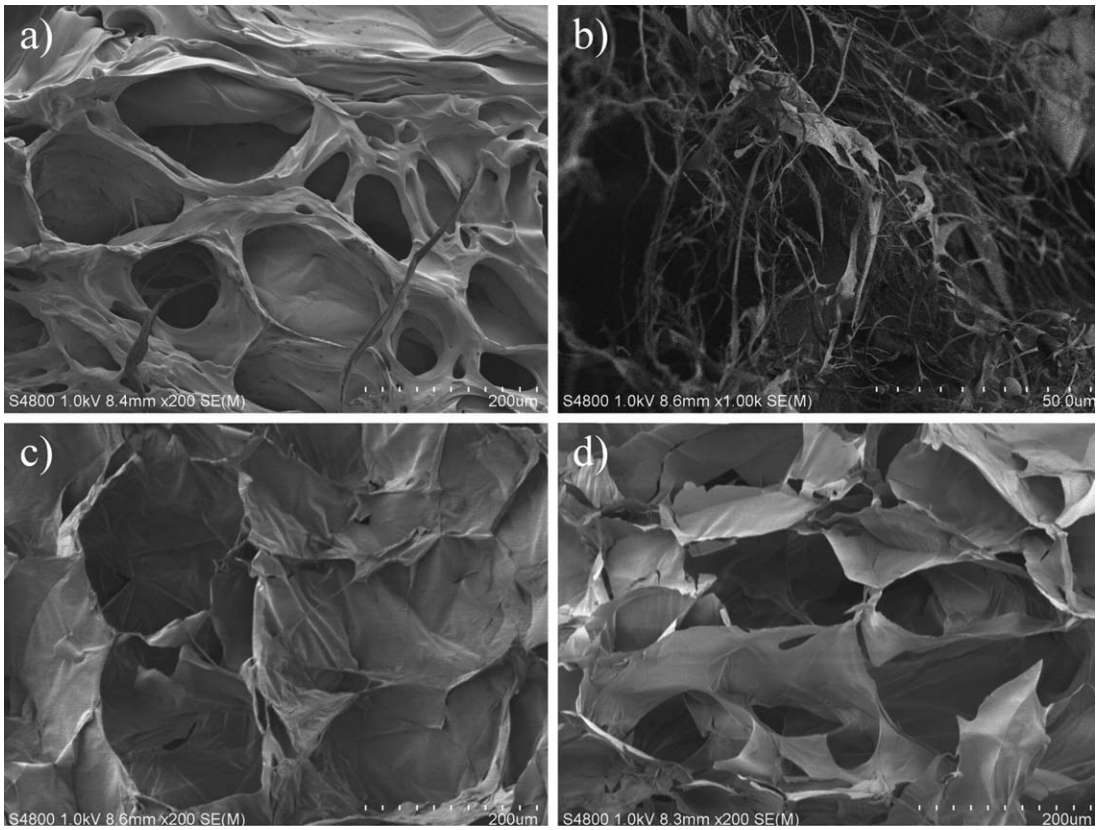

Figure 2. Cross-sectional SEM images of the freshly prepared PVA/HA hydrogel (a) Gel-3, and of the swollen hydrogels (b) Gel-1, (c) Gel-3, and (d) Gel-6 in lyophilized states.

of substitution of the PVA and HA derivatives on the swelling ratio (SR) and gel fraction (GF) of the hydrogels. Within each series, the DS values of PVA and HA were kept the same and the initial total polymer concentration was varied from 15 to 25 mg/mL.

With increasing polymer concentration, the swelling ratios of the hydrogels were found to decrease. For example, the samples Gel-3, Gel-4, and Gel-5 (Table I) had the initial total polymer concentrations of 15, 20, and 25 mg/mL, respectively, and their swelling ratios were 1000, 900, and 690, respectively. The same trend was found for hydrogels having the lowest (Gel-1 and Gel-2) and the highest (Gel-6, Gel-7, and Gel-8) DS values. Typically, a smaller swelling ratio would generate a higher mechanical strength for the hydrogel.[23] The large swelling ratios obtained in this study are due to the low cross-linking density of the hydrogels and due to the hydrophilic nature of the modified polymers.

The gel fraction of the hydrogels was found to decrease with increasing the initial total polymer concentration in all the four hydrogel series. Due to the higher polymer concentration, the volume fraction of water within the gel is reduced and hence the viscosity of the polymer solution increases resulting in poor mixing of the polymer solutions. The reduced free volume of water hinders significantly diffusion of free polymers[24] and their mutual reaction leading to lower gel contents.

The higher DS values of the polymer components lead to a decrease in the swelling ratio regardless of the polymer concentration. For example, Gel-1 (PVA/HA DS values 4.1/5.0), Gel-3 (8.6/7.2), and Gel-6 (10.6/9.2) with polymer concentration of 15 mg/mL had swelling ratios of 1690, 1000, and 640, respectively (Table I). With other polymer concentrations (20 and 25 mg/mL), a similar decrease in the swelling ratios was observed with the increasing DS values.

The effect of the molar mass of PVA on the swelling ratio of the hydrogels was studied by comparing Gel-3 and Gel-4 (PVA molar mass 27,000 g/mol) with Gel-9 and Gel-10 (PVA molar mass 15,000 g/mol). The hydrogels containing PVA with the lower molar mass had a slightly lower swelling ratio. Similar results have been obtained with studies of chemically cross-linked poly(ethylene glycol) hydrogels showing that lower polymer molar mass gives a higher cross-linking density and a lower swelling ratio.[25]

The morphology of the freeze-dried PVA/HA hydrogels was studied by SEM. The pore size in the freshly prepared Gel-3 after the freeze-drying varied from 20 to 200 μm [Figure 2(a)]. Figure 2(b–d) shows the cross-sectional SEM images of the swollen hydrogels Gel-1, Gel-3, and Gel-6, respectively, in lyophilized states. Gel-1 with the lowest DS values showed a porous structure formed from polymer fibers, whereas Gel-3 and Gel-6 with higher DS values had more dense porous structure based on polymer platelets. The porous network of the

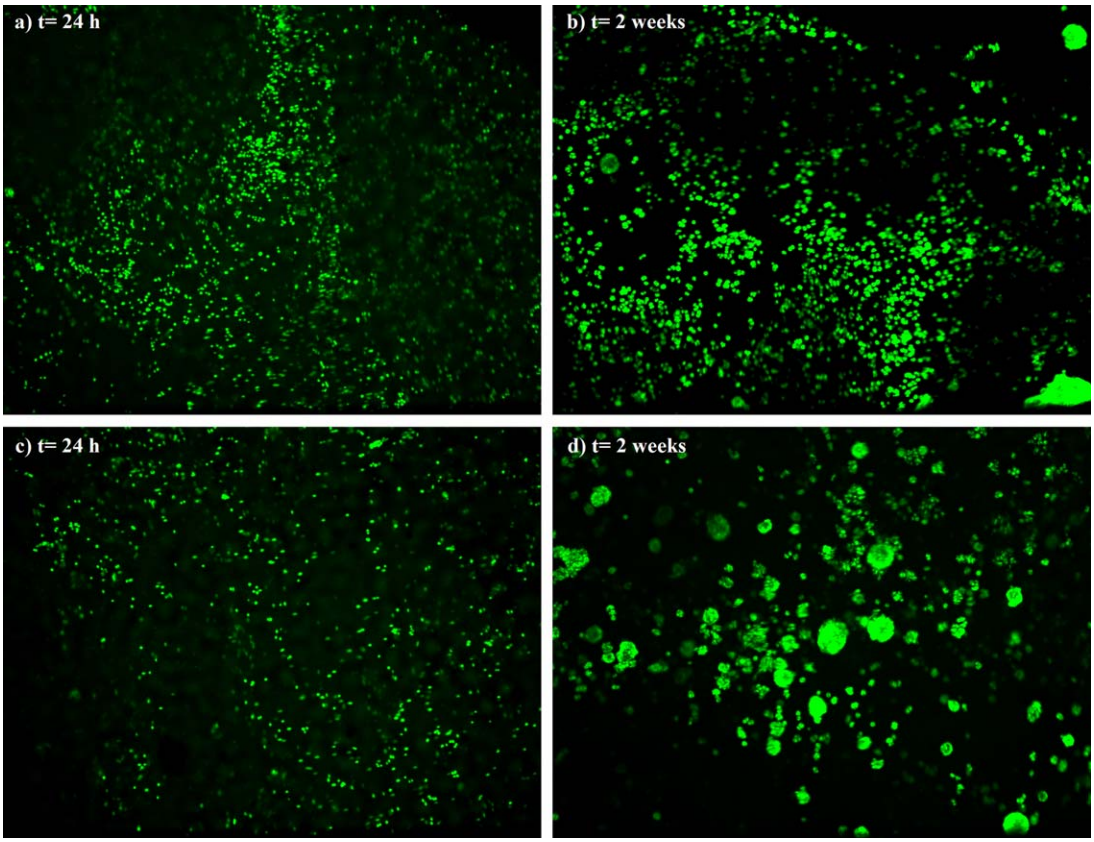

Figure 3. Live/dead staining of chondrocytes in hydrogel with DS values 9.8/8.9 when (a) $t = 24$ h and (b) $t = 2$ weeks, and in hydrogel with DS values 12.8/10.6 when (c) $t = 24$ h and (d) $t = 2$ weeks. Stained living cells appear green and dead cells would have stained in red (4× magnifications). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PVA/HA hydrogels revealed in SEM images can be due to the freeze-drying process, which can change the morphology of the hydrogel,[26] and so the actual porosity of the hydrogel in water can be different.

The characterization results of the chemically cross-linked PVA/HA hydrogels show that the porosity as well as the swelling ratio and gel fraction (and hence the mechanical strength) of the hydrogel can be controlled by polymer concentration and degrees of substitution of the polymer derivatives. The control of these properties is necessary in order to achieve suitable hydrogel scaffolds for cells to proliferate.[7] In order to find suitable PVA/HA hydrogel for the cell encapsulation, three hydrogels with varying DS values of the polymer derivatives were prepared and studied in the cultivation of chondrocyte cells as described in the next section.

In Vitro Cell Viability

The cell viability of bovine knee chondrocytes was studied in the presence of the individual polymer components and PVA/HA hydrogels. A decrease in cell viability has been reported for human dermal fibroblasts exposed to HAALD.[13] In this study, a loss in cell viability of bovine knee chondrocytes was observed in the presence of HAALD (Figure S1, supplementary data). All cells died within 2 weeks. On the other hand, chondrocytes remained viable in PVAHY during 2-week period and thus PVAHY was not considered to be cytotoxic to the cells.

Three PVA/HA hydrogels with varying DS values (initial total polymer concentration 15 mg/mL) were used to study the *in vitro* cell viability of chondrocytes. With the lowest DS values (6.3/6.3, PVA/HA), hydrogel was not formed in the presence of the cells. With polymers having higher DS values (9.8/8.9 and 12.8/10.6), hydrogels were formed and the chondrocyte cells were found to grow well and the cells maintained their typical spherical shape during the 2-week period (Figure 3). However, after 2 weeks, agglomeration of the cells [Figure 3(d)] was observed especially in the hydrogel with the highest DS values (12.8/10.6). The probable reason for the agglomeration was the denser gel structure due to higher DS values. The cell agglomeration has been found to be an important factor in the formation of cartilage from chondrocytes,[27] but the large agglomerates present in our hydrogel having the highest DS values (12.8/10.6) could indicate a limited space for the cell growth. Although HAALD showed cytotoxicity for chondrocytes, the cross-linking reaction with PVAHY produced hydrogels without any significant decrease in cell viability as has also been reported for human fetal chondrocytes in a previous study.[28]

CONCLUSIONS

PVA/HA hydrogels from PVA and HA derivatives bearing complementary reactive primary amine and aldehyde groups were

prepared. A decrease in the swelling ratio was achieved by increasing the degrees of substitution and concentration of the modified polymers. Hydrogels with the low swelling ratios were obtained with the highest polymer concentration and DS values. Cell viability tests indicated that PVA/HA hydrogels can provide scaffolds for bovine knee chondrocytes to proliferate. These results show that the properties of the chemically cross-linked PVA/HA hydrogels can be controlled and that these hydrogels are potential biomaterials for tissue engineering. Furthermore, the cross-linking method applied provides a simple way to encapsulate cells in hydrogel scaffolds.

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