Macromolecular interactions in alginate–gelatin hydrogels regulate the behavior of human fibroblasts

**Bapi Sarker^{1,2}, Raminder Singh^{3,4},
Tobias Zehnder¹, Tim Forgber¹,
Christoph Alexiou³, Iwona Cicha³,
Rainer Detsch¹ and Aldo R Boccaccini¹**

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Abstract

Due to the presence of tripeptide arginine–glycine–aspartic acid, gelatin is considered a very promising additive material to improve the cytocompatibility of alginate-based hydrogels. Two different strategies, physical blending and chemical crosslinking with gelatin, are used in this study to modify alginate hydrogel. As the intermolecular interactions between the polysaccharide and protein in the resulting physically blended and chemically crosslinked hydrogels are different, significant differences in the properties of these hydrogel types, regarding especially their surface topography, degradation kinetics, mechanical properties, and protein release behavior, are observed. Cellular behavior on both types of alginate–gelatin hydrogels is investigated using primary human dermal fibroblasts to elucidate the effects of the different structural, mechanical, and degradation properties of the produced hydrogels on fibroblast attachment and growth. The hydrogel that is chemically crosslinked with gelatin exhibits the highest degree of cytocompatibility regarding adhesion, proliferation, metabolic activity, and morphology of growing fibroblasts.

Keywords

Hydrogels, alginate, gelatin, fibroblast, tissue engineering

¹Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, Erlangen, Germany

²Department of Mechanical Engineering & Materials Science, Washington University in St. Louis, St. Louis, MO, USA

³Cardiovascular Nanomedicine Unit, Section of Experimental Oncology and Nanomedicine, ENT Department, University Hospital Erlangen, Erlangen, Germany

⁴Laboratory of Molecular Cardiology, Department of Cardiology and Angiology, University Hospital Erlangen, Erlangen, Germany

Corresponding author:

Aldo R Boccaccini, Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, 91058 Erlangen, Germany.

Email: aldo.boccaccini@ww.uni-erlangen.de

Introduction

Connective tissue, which supports all different tissues and organs in the body, consists of fibers (collagen and elastic fibers), viscous ground substance, and cells, including fibroblasts, adipocytes, and immune cells. Apart from providing the structural support and resistance to mechanical forces, connective tissue plays an important role in facilitating the exchange of oxygen, essential nutrients, and metabolic waste between cells and blood capillaries.¹ Fibroblasts, the most common cells of connective tissue, play a key role in maintaining the structural integrity of tissues and in the natural processes of tissue regeneration and wound healing.² Predicting the fibroblasts' response to biomaterials is thus one of the fundamental aspects of soft tissue engineering as the resulting tissue architecture and function largely depends on the appropriate cell–matrix interactions.³ Cell behavior on a biomaterial is guided by cell–matrix adhesion complexes which provide the cells with the relevant microenvironmental information through both biophysical (structural and mechanical) and biochemical (soluble and insoluble mediators that act as ligands) signals.⁴ These adhesion complexes, that is, integrin-mediated focal adhesion structures, bind to cell-binding motifs present in the extracellular matrix (ECM) and transmit the information bidirectionally between the ECM and cells.⁵ Specific domains are widely used as cell-binding motifs for modification of the matrix to promote cell adhesion. Tripeptide arginine–glycine–aspartic acid (RGD), which is originally described as the cell-binding motif in fibronectin, is often used to modify biomaterials. In particular, hydrogel-based biomaterials like alginate (ALG) benefit from such modification^{6,7} as polysaccharides including pure ALG lack the cell-binding motifs and do not support cell adhesion.⁸ Although it is well established that RGD-conjugated ALG hydrogel promotes adhesion and proliferation of various cells,^{8,9} our previous study¹⁰ revealed that chemically crosslinked alginate–gelatin (ADA-GEL-x) hydrogel supported adhesion, proliferation, and migration of human osteosarcoma cells to significantly higher degree as compared to RGD-conjugated ALG hydrogels. Thereafter, we investigated human dermal fibroblasts behavior on ADA-GEL-x hydrogels at different compositions.¹¹ The results of that study showed that fibroblast growth, attachment, and proliferation were enhanced on ADA-GEL-x having higher amount of gelatin (GEL). GEL, a hydrolyzed product of collagen, is a biodegradable protein, which contains RGD sequence of collagen.^{12,13} It is therefore not surprising that growth and attachment of fibroblasts were enhanced on ADA-GEL-x hydrogels containing large amounts of GEL.¹¹ However, an open question remained whether it is the chemical crosslinking between ALG and GEL that is necessary for fibroblast adhesion and proliferation, or whether the presence of GEL in ALG hydrogel, for example, by simple blending of the two polymers, would be sufficient for improved fibroblast growth. Moreover, it is unknown what are the effects of physical blending and chemical crosslinking of GEL with ALG in terms of the structural and degradation properties of the hydrogels and the regulation of fibroblast behavior.

In this study, we therefore compared the behavior of normal human dermal fibroblasts (NHDFs) on ALG-based hydrogels that were modified with GEL either by physical blending or by chemical crosslinking. Both hydrogels were composed of similar amount of polysaccharide phase (ALG or alginate dialdehyde (ADA)) and protein phase (GEL). Since the crosslinked hydrogels with 40:60 ratio of ADA:GEL performed as one of the best formulations regarding the outcomes of viability, adhesion, proliferation, and metabolic activity of fibroblasts,¹¹ the same ratio was chosen for this comparative study, in which ALG:GEL ratio of 40:60 was used in the alginate–gelatin blended (ALG-GEL-b) hydrogel. In line with the previous studies, cytocompatibility aspects were discussed in the context of structural, mechanical, and degradation properties of the hydrogels for the purpose of tissue regeneration applications.

Materials and methods

Materials

Sodium alginate (sodium salt of alginic acid from brown algae, suitable for immobilization of micro-organisms, guluronic acid content 65%–70%) and GEL (Bloom 300, Type A, porcine skin, suitable for cell culture) were obtained from Sigma–Aldrich (Germany). Ethanol, ethylene glycol, sodium metaperiodate, and calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) were purchased from VWR International (Germany).

Preparation of films

Sodium ALG was dissolved in ultrapure water (Direct-Q; Merck Millipore, Germany) at 37°C by constant stirring to prepare 1% (w/v) solution and sterilized by filtration through 0.22 μm filter (Carl Roth GmbH + Co. KG, Germany). The sterilized sodium ALG solution was lyophilized under sterile condition. The required amount of lyophilized sodium ALG was then dissolved in sterile phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific, Germany) to prepare 2.5% (w/v) and 5% (w/v) solutions, separately. A total of 5% (w/v) GEL solution was prepared by dissolving GEL powder in ultrapure water at 50°C and sterilized by filtration through 0.22 μm filter. To prepare ALG-GEL-b hydrogel precursor solution, 5% (w/v) GEL solution was slowly added to 5% (w/v) ALG solution in the volume ratio of 40:60 of ALG:GEL under continuous stirring. ADA-GEL-x hydrogel in the volume ratio of 40:60 (ADA:GEL) was synthesized by covalent crosslinking of ADA and GEL as described elsewhere.¹⁴ Briefly, ADA was synthesized by partial oxidation of sodium ALG. Sodium ALG was dispersed in absolute ethanol and mixed with equal volume of an aqueous solution of sodium metaperiodate (oxidizing agent) at a ratio of 32.1 w% of sodium periodate:sodium ALG. After 6 h stirring in dark conditions at room temperature, the oxidation reaction was stopped by adding ethylene glycol to the reaction mixture. The dispersion was then dialyzed against ultrapure water using a dialysis membrane (molecular weight cutoff of 6–8 kDa; Spectrum Labs, USA) to remove unreacted sodium metaperiodate. The purified ADA solution was lyophilized using a freeze dryer to obtain dry ADA, which was then dissolved in ultrapure water at a concentration of 2% (w/v) and sterilized by filtration through 0.22 μm filter. The sterilized ADA solution was lyophilized under sterile condition and then the required amount of dry ADA was dissolved in sterile PBS to get 5% (w/v) solution. ADA-GEL-x hydrogel was synthesized by mixing sterilized ADA and GEL solutions at the volume ratio of 40:60. After proper mixing, ALG-GEL-b solution and ADA-GEL-x hydrogel were casted in sterile Petri dishes, followed by the addition of sterile calcium chloride solution (0.1 M) and incubation for 15 min to allow ionic gelation. The hydrogels were then washed with serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific) and cut by punching to prepare films of desired size and shape (circular, diameter 13.5 mm and thickness 1.5 mm). As control material, ALG films were also prepared by casting 2% (w/v) ALG solution, followed by ionic gelation with 0.1 M CaCl_2 solution and punching. The whole fabrication process was accomplished under sterile conditions.

Characterization of hydrogel films

Fourier transform infrared analysis. A Fourier transform infrared (FTIR) spectrometer (Nicolet 6700; Thermo Scientific, USA) was used to evaluate the molecular interactions between the macromolecules in the produced hydrogels. Films were prepared by casting sodium ALG, ADA, GEL,

ALG-GEL-b solutions, and ADA-GEL-x hydrogel into a polystyrene Petri dish and allowed to dry for 3 days at room temperature. The dried films were used to record attenuated total reflectance (ATR)-FTIR spectra.

Surface morphology. The surface topography of the films fabricated from ALG, ALG-GEL-b, and ADA-GEL-x was investigated using a scanning electron microscope (SEM) (Zeiss Auriga; Zeiss, Germany). The samples were prepared for SEM analysis through the three consecutive processes, fixation, dehydration, and drying. Given the protein content in the hydrogel films, these were fixed using glutaraldehyde and paraformaldehyde-based fixative solution and dehydrated in a series of ethanol (from 30 to 100 v%). To dry the samples, a critical point dryer (Leica EM CPD300; Leica, Germany) was used, followed by sputter-coating with gold using the Q150T Turbo-Pumped Sputter Coater/Carbon Coater (Quorum Technologies Ltd, UK) prior to SEM analysis.

Mechanical properties. The mechanical properties of the hydrogels were determined using a ferule-top nanoindenter (PIUMA; Optics11, The Netherlands) at room temperature. A probe with a tip diameter of 91 μm was used. The measurement was taken at different positions of each hydrogel film using a matrix scan function to receive an average value. The loading period was set to 2 s, the holding time to 1 s, and unloading time to 2 s. The reduced Young's modulus (rYm) was calculated based on the load–displacement curves. The measurements were carried out at room temperature, right after the preparation of the hydrogel films, which were directly casted into a single polystyrene Petri dish.

Protein release. Sterile weighed films (125 mg) prepared from ALG-GEL-b and ADA-GEL-x were immersed in 2 mL of L-glutamine, phenol red, and serum-free DMEM and incubated at 37°C. After specific times of incubation, the medium was removed completely and collected for protein release analysis, and fresh DMEM (2 mL) was added to the films. The protein concentration in the released medium was determined by colorimetric protein assay using the Lowry method,^{15,16} with bovine serum albumin (BSA) used as a standard. The absorbance of each solution at 750 nm was measured using an ultraviolet (UV)–visible (Vis) spectrophotometer (Specord 40; Analytik Jena, Germany). The release (%) of protein from the films was calculated as follows

$$\text{Protein release (\%)} = \frac{[\text{Protein}]_{\text{supernatant}}}{[\text{Protein}]_{\text{total}}} \times 100$$

where $[\text{Protein}]_{\text{total}}$ is the initial concentration of GEL (in films) and $[\text{Protein}]_{\text{supernatant}}$ is the final GEL concentration in the medium at different time points. The initial concentration of protein (GEL) in the films was determined by analyzing the amount of protein present in the films per volume of DMEM.

In vitro swelling and degradation behavior. The weighed (W_i) freshly prepared sterile films of ALG, ALG-GEL-b, and ADA-GEL-x were incubated in 3 mL DMEM supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) antibiotic–antimycotic at 37°C with a controlled atmosphere of 5% CO_2 and 95% relative humidity. The medium was changed every 2 days. After selected times of incubation, the culture medium was removed. The samples were weighed (W_t) after the adhered surface medium was removed. The fresh culture medium was then added to the samples. The swelling and degradation of the films were calculated according to the following equation

$$\text{Swelling (w\%)} \text{ or } \text{Degradation (w\%)} = \frac{W_t - W_i}{W_i} \times 100$$

where the positive values are considered as swelling (w%) and the negative values are considered as degradation (w%).

In vitro cell study

Fibroblasts (NHDFs; PromoCell, Germany) were cultured in DMEM (Life Technologies, Germany) supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic–antimycotic, in an incubator at 37°C, with a controlled atmosphere of 5% CO₂ and 95% relative humidity. When ~90% confluence of growing fibroblasts was achieved, the cells were detached using trypsin/1 mM ethylenediaminetetraacetic (EDTA) (Life Tech., Germany), centrifuged, and resuspended in complete DMEM cell culture medium. Cells were counted using trypan blue (Sigma–Aldrich) exclusion method. The prepared circular hydrogel films of pure ALG, ALG-GEL-b, and ADA-GEL-x were placed into the wells of 24-well plates (VWR International) prior to cell seeding. A total of 7.5×10^4 cells within 500 µL of complete DMEM cell culture medium were seeded on each hydrogel film and incubated in an incubator with a controlled atmosphere of 5% CO₂ and 95% relative humidity at 37°C. The cell culture medium was changed on the next day after seeding, and then every 2 days until the further analysis.

Cell viability. Viability of the cells growing on ALG, ALG-GEL-b, and ADA-GEL-x films was determined through the enzymatic conversion of tetrazolium salt (WST-8 assay kit; Sigma–Aldrich) after 1, 3, and 7 days of cultivation. The films were placed into new wells of 24-well plate, and freshly prepared culture medium containing 1 v% WST-8 assay kit was subsequently added into each well. After 2 h of incubation at 37°C, 100 µL of supernatant from each sample was transferred into a well of a 96-well plate and the absorbance was measured at 450 nm with a microplate reader (PHOMO; Autobio Labtec Instruments Co. Ltd, China).

Lactate dehydrogenase activity. For the lactate dehydrogenase (LDH) activity analysis in cell lysates, a commercially available LDH activity quantification kit (TOX7; Sigma–Aldrich) was used. After specific period of incubation (1, 3, and 7 days), the hydrogel films were placed into new wells of 24-well plates and washed with Hanks' balanced salt solution (HBSS). Subsequently, lysis buffer was added to the films (1 mL per film) and incubated for 30 min. Lysates were centrifuged at 2000 r min^{−1} for 5 min and 140 µL from the supernatant solutions was transferred to 1-mL cuvettes. A total of 60 µL of master-mix (equal amounts of substrate solution, dye solution, and cofactor solution for LDH assay) was added to each cuvette and incubated for 30 min. The reaction was stopped with 300 µL 1 N HCl, and 500 µL of water was added. The absorbance of each solution was measured at 490 and 690 nm using a UV–Vis spectrophotometer (Specord 40; Analytik Jena AG).

Fluorescent staining. To visualize viable cells, staining was performed with calcein acetoxymethyl ester (calcein AM; Invitrogen, USA) and nuclei staining was done with blue nucleic acid stain, 4',6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen), after 1, 3, and 7 days of cultivation. To perform the staining, the samples were incubated for 20 min at 37°C in serum-free medium containing 5 µL mL^{−1} calcein AM. Afterward, the samples were washed with HBSS and fixation was performed using 3.7 v% paraformaldehyde composing fixation buffer. The samples were subsequently incubated for 5 min in HBSS containing 1 µL mL^{−1} DAPI. To visualize the cellular morphology and actin cytoskeleton, F-actin of cells was stained with rhodamine phalloidin (Invitrogen)

after fixation and membrane permeabilization with 0.1 v% TritonX in HBSS. Cell nuclei were visualized with green nucleic acid stain, SYTOX, and the staining was performed in the same manner as the DAPI staining. Images of calcein–DAPI and rhodamine phalloidin–SYTOX-stained cells were taken by fluorescence microscope (Axio Scope A.1, Carl Zeiss Microimaging GmbH, Germany).

Cell number. The cell number on the hydrogels films at different cultivation times was calculated by counting the number of cell nuclei using ImageJ software (version 1.47v; National Institutes of Health, Bethesda, MD, USA). Six fluorescent images (DAPI-stained) at two different magnifications (10 $\times$ and 20 $\times$) per sample were used to analyze the cell number using automatic cell counting method. The number of cell nuclei represents the cell number since fibroblasts are mononuclear cells. The entire area of each image was analyzed to calculate the cell number per unit area of sample.

Statistical analysis

Unless specified otherwise, data are reported as mean $\pm$ standard deviation. Statistical differences among the experimental groups (different hydrogels for mechanical properties and incubation time for in vitro cell study) were performed with a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test.

Results and discussion

Materials characterization

FTIR analysis. The ATR-FTIR spectrum of sodium ALG (Figure 1) exhibits its characteristic absorption bands, 947 cm^{-1} (C–O stretching vibration), 1024 cm^{-1} (C–O–C stretching vibration), 1126 cm^{-1} (C–C stretching vibration), and 1318 cm^{-1} (C–O stretching vibration), and the bands at 1408 and 1590 cm^{-1} correspond to symmetric stretching vibration and asymmetric stretching vibration of carboxyl group, respectively.^{17–20} The broad absorption band at 3700–3000 cm^{-1} resulted from the stretching vibration of O–H. All characteristic peaks of ALG are clearly visible in the spectrum of ADA, which proves that its polysaccharide characteristic was not altered due to oxidation. The spectrum of GEL demonstrates the characteristic absorption bands of GEL at 1630 cm^{-1} for amide I (C=O and C–N stretching vibration), 1543 and 1452 cm^{-1} are assigned to amide II (N–H bending vibration and C–N stretching vibration) and 1236 cm^{-1} corresponds to amide III (N–H bending vibration and C–N stretching vibration).^{21–23} Due to interaction with N–H, the wide absorption band of O–H (stretching vibration) shifted to lower wavenumber and centered at around 3302 cm^{-1} in the spectrum of GEL. Although ALG-GEL-b contains higher amount of GEL than ALG, the spectrum shows all representative bands of pure ALG. The bands of GEL are not clearly visible in the spectrum of ALG-GEL-b. However, due to the presence of GEL, the characteristic absorption bands of carboxylate group of ALG at 1408 and 1590 cm^{-1} shifted toward higher wavenumber at 1414 and 1604 cm^{-1} , respectively. This is possibly due to electrostatic interactions between the carboxyl groups of ALG and the amino groups of GEL.²⁴ In contrast, most of the characteristic bands of both GEL and ADA were present in the spectrum of ADA-GEL-x. More interestingly, the band of amide I at 1630 cm^{-1} shifted toward lower wavenumber and the band of amide II at 1543 cm^{-1} shifted toward higher wavenumber and became weaker, which proves the involvement of amide I and amide II of GEL in the crosslinking reaction with ADA.²⁵ Moreover, the absorption bands at 1621 and 1557 cm^{-1} corresponding to $\nu(\text{C}=\text{N})$ of Schiff's base prove the

Figure 1. ATR-FTIR spectra of sodium ALG, ADA, GEL, ALG-GEL-b, and ADA-GEL-x exhibit the characteristic absorption bands of the materials. The wavenumbers of the major band positions of the materials are labeled accordingly. The clear difference in the positions of the corresponding bands of the blended (ALG-GEL-b) and crosslinked (ADA-GEL-x) materials proves the presence of different intermolecular bonding, in the two hydrogels.

covalent crosslinking between ADA and GEL.¹⁴ Since not all GEL is covalently bound with ADA, due to the presence of uncrosslinked GEL in ADA-GEL-x the band at 1621 cm^{-1} overlapped with the band at 1630 cm^{-1} (amide I of uncrosslinked GEL) becoming broad.¹⁴

Surface morphology. The surface morphology of the films composed of ALG, ALG-GEL-b, and ADA-GEL-x was analyzed by SEM and the results are presented in Figure 2. As expected, ALG and ALG-GEL-b films exhibited very similar morphology, folding in regular pattern that is a typical polysaccharide-like structure. However, a surface masking behavior and more dense morphology were observed for ALG-GEL-b which could be the effect of GEL, which is not chemically linked to ALG. On the other hand, the covalently crosslinked ADA-GEL-x films exhibited completely different surface topography, an uneven fibrous porous structure with porosity at submicron level. This morphology is similar to the structure of collagen, which is a key component of the ECM. It can be expected that due to this topography, ADA-GEL-x will support cell adhesion and promote other cellular activities, acting similar to an exogenous ECM.

Mechanical properties. The mechanical properties of the films made of ALG, ALG-GEL-b, and ADA-GEL-x were investigated using a nanoindenter. The results of the calculation of the rYm from the load-displacement curves and representative curves are shown in Figure 3. Pure ALG exhibited a rYm of 33 kPa, which was found to increase to 43 kPa due to blend with GEL. The rYm

Figure 2. SEM images of hydrogel films derived from (a) ALG, (b) ALG-GEL-b, and (c) ADA-GEL-x showing different surface topographies. The covalently crosslinked hydrogel exhibits more porous structure compared to the other two hydrogels.

of ADA-GEL-x drastically decreased to 8 kPa. Considering the observations from the SEM investigation, the closer values of rY_m of ALG and ALG-GEL-b are consistent as they exhibit a very similar surface topography. The increase in the rY_m for ALG-GEL-b could possibly be explained by the densification of the ALG network by the GEL as it appears in Figure 2(b). The lower mechanical properties of ADA-GEL-x can be explained by the more fibrous and porous morphology of the crosslinked composition as shown in Figure 2(c). As the amount of GEL in ALG-GEL-b and ADA-GEL-x is the same and the GEL is not modified, the drop of the mechanical properties of the crosslinked composition could be explained by the oxidation of ALG during preparation of ADA-GEL-x. The oxidation of ALG causes a lower molar mass of the intermediated product (ADA) due to cleaving of pyranose ring of ALG, and moreover oxidation occurs preferentially in guluronate units of ALG, which reduces the ionic gelation properties^{11,14} that could be the cause of lower mechanical properties of ADA-GEL-x. Moreover, the chemical crosslinking reaction could partially inhibit the ionic gelation resulting in a hydrogel network with reduced mechanical stability.

Protein release. Diffusion and degradation are the two major mechanisms for protein release from polymeric matrices. Given the low degradation of ALG, proteins are expected to release from ALG matrices by diffusion through the hydrogel pores into the physiological medium. The kinetics of GEL release from ALG-GEL-b films through the pores of ALG matrix showed a burst release in the initial 24 h, and then the release rate slowed down, as shown in Figure 4. The release rate of protein from ALG by diffusion is generally controlled by the pore size of the ALG polymeric network.²⁶ Therefore, protein molecules on the surface of ALG-GEL-b films were released initially at a burst rate due to ease of diffusion from the surface, as compared to the molecules present in the interior of the hydrogel films. In contrast to ALG-GEL-b, protein release from ADA-GEL-x films involves both diffusion and degradation, since ADA is a degradable matrix. Interestingly, however, protein release rate from ADA-GEL-x was found to be lower compared to that from ALG-GEL-b. This result can be attributed to the covalent crosslinking between the ϵ -amino groups of lysine or hydroxylysine of GEL and the available aldehyde groups of ADA in ADA-GEL-x that impedes protein release.¹⁴ Similar results were observed in our previous study,¹² where a microcapsule model was used. Due to the covalent carbon–nitrogen double bonds of Schiff's base between ADA and GEL, more GEL was retained in the ADA-GEL-x compared to ALG-GEL-b. Therefore, it can be stated that the amount of GEL released by diffusion from ADA-GEL-x was comparatively lower than ADA-GEL-b. The initial burst release of GEL from ALG-GEL-b proves that the release rate of GEL by diffusion was higher for ALG-GEL-b compared to ADA-GEL-x since no difference was observed in the degradation behavior (Figure 5(a)) between these two hydrogels at this initial

Figure 3. Mechanical properties of the hydrogels, measured as (a) reduced Young's modulus (rYm) using a nanoindenter and (b) representative load–displacement curves. Asterisks denote significant difference of pairwise comparison, where $***p < 0.001$ (Bonferroni's post hoc test was used).

Figure 4. Cumulative protein release from the films composed of ALG-GEL-b and ADA-GEL-x, as a function of incubation time in DMEM, determined by colorimetric protein assay using the Lowry method.

stage of incubation. Therefore, although the ADA-GEL-x is more degradable, protein release from the hydrogel was found to be relatively low due to the covalent linkages between ADA and GEL.

In vitro swelling and degradation behavior. In vitro swelling and degradation of ALG, ALG-GEL-b, and ADA-GEL-x films were investigated by evaluating the weight gain and weight loss, respectively, of the hydrogel films during incubation in DMEM, as described in section “Materials and methods.” Within 42 days of incubation, pure ALG films did not undergo degradation compared to

Figure 5. (a) Swelling and degradation of films fabricated from ALG, ALG-GEL-b, and ADA-GEL-x as a function of incubation time in DMEM. (b) Photograph of the hydrogel films after 28 days of incubation, showing the reduction in size of ALG-GEL-b and ADA-GEL-x films, which is in accordance with the results of the quantitative analysis of the degradation study.

their initial state (as-prepared) and exhibited strong swelling properties, as shown in Figure 5(a). On the contrary, both GEL-containing hydrogels (ALG-GEL-b and ADA-GEL-x) exhibited degradation phenomena upon prolonged incubation in medium, although they initially showed swelling behavior. The covalently crosslinked formulation (ADA-GEL-x) exhibited faster degradation characteristics as compared with ALG-GEL-b. This behavior can be associated with the fact that the degradation of ALG-GEL-b is only attributed to the release of GEL over the incubation period since ALG does not undergo degradation. On the other hand, the faster degradation of ADA-GEL-x results from both the release of GEL and the degradation of ADA. Periodate oxidation during synthesis of ADA cleaves the vicinal diols of ALG, which enhances hydrolytic degradation of the hydrogel.¹¹ Despite the fact that the protein release from ADA-GEL-x was low due to covalent linkage between ADA and GEL, ADA enhanced the degradation kinetic of ADA-GEL-x films, as compared to ALG-GEL-b. In Figure 5(b), ALG, ALG-GEL-b, and ADA-GEL-x films after 28 days of incubation are shown. It is clearly visible that the size of the pure ALG film is larger than that of

Figure 6. Viability of NHDFs on ALG, ALG-GEL-b, and ADA-GEL-x films after 1, 3, and 7 days of cultivation analyzed by WST-8 assay.

Asterisks denote significant difference of pairwise comparison in each cultivation period, *** $p < 0.001$ (Bonferroni's post hoc test was used).

both GEL-containing films, which confirms the results obtained from the quantitative analysis of swelling and degradation study shown in Figure 5(a).

In vitro cytocompatibility of hydrogels

Cell viability. The viability of cells on ALG, ALG-GEL-b, and ADA-GEL-x films over the cultivation period was investigated by WST-8 assay. As shown in Figure 6, the viability of NHDFs on ALG-GEL-b was significantly higher compared to that on ALG after 3 and 7 days of cultivation. As expected, the viability of NHDFs on ADA-GEL-x was found to be significantly higher compared to that on ALG during all cultivation periods. Most importantly, NHDFs exhibited significantly higher viability on ADA-GEL-x compared to ALG-GEL-b after 1 and 7 days of cultivation. Therefore, it can be stated that the covalently crosslinked hydrogel (ADA-GEL-x) supported cell growth and attachment better than the blended composition (ALG-GEL-b).

To visualize the viability and morphology of living cells, fluorescence staining was performed using calcein AM that stains the cytoplasm of living cells.²⁷ Cell shape and integrity of the cell membrane, the hallmarks of normal living cell equilibrium, were also evaluated by calcein staining. As shown in Figure 7, cells on ALG films were found to be aggregated, and more aggregated cells were observed at longer cultivation times. Since ALG does not contain cell adhesion ligands, cell–matrix interactions were very poor in this hydrogel, and cells formed clusters due to cell–cell interactions, which were stronger than cell–matrix interactions in this case. More viable cells were observed on ALG-GEL-b films. However, the number of cells was found to decrease with time,

Figure 7. Fluorescence microscopy images of fibroblasts adhered on ALG, ALG-GEL-b, and ADA-GEL-x films after 1, 3, and 7 days of cultivation. Living cells were stained with calcein AM (green) and nuclei were counterstained with DAPI (blue). Similar cell behavior (cell clusters) is observed on ALG and ALG-GEL-b after 7 days. Comparatively higher number of living cells with spreading morphology is observed on ADA-GEL-x at all time points.

and also here aggregated cell morphology was observed after 7 days of cultivation. This phenomenon can be explained by the GEL release from the ALG-GEL-b films over the incubation time. It is known that GEL possesses the RGD sequence of collagen which promotes cell adhesion.^{13,28} In ALG-GEL-b, there is no chemical bond between ALG and GEL. Therefore, less amount of GEL remained in the films due to the high release rate of GEL from the blended hydrogel (Figure 4), which had a negative effect on cell adhesion. Moreover, over the relatively long incubation period, most of the GEL was released from the ALG-GEL-b hydrogel surface, which results in nearly similar surface properties like pure ALG. Therefore, similar cellular morphology was observed on ALG and ALG-GEL-b films upon longer incubation time. In contrast, more viable cells were observed on ADA-GEL-x films and the number of cells increased with cultivation time. This phenomenon can be ascribed to the availability of cell-binding peptides of GEL in the ADA-GEL-x hydrogel over the longer cultivation period due to the covalent Schiff's bonds between ADA and GEL. Moreover, the fibrous porous structural morphology of ADA-GEL-x (Figure 2) could play a key role in promoting cell viability.

LDH activity and cell number. LDH activity of fibroblasts grown on ALG, ALG-GEL-b, and ADA-GEL-x films was analyzed after different cultivation periods, as presented in Figure 8(a). LDH is a cytosolic enzyme, which catalyzes the reversible reduction in pyruvate into lactate in the presence of reduced form of nicotineamide adenine dinucleotide (NADH).²⁹ LDH activity directly correlates with the metabolic activities of cells, which has also been used in preclinical investigations into the biocompatibility of the materials for use as surgical implants.³⁰ Corresponding to the

Figure 8. (a) LDH activity of fibroblasts on ALG, ALG-GEL-b, and ADA-GEL-x films and (b) number of cells per unit area of ALG-GEL-b and ADA-GEL-x hydrogel films after 1, 3, and 7 days of cultivation. Fibroblasts could not be counted on ALG due to the aggregation of cells.

Asterisks denote significant difference of pairwise comparison at each cultivation period, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (Bonferroni's post hoc test was used).

cell viability results (Figure 6), fibroblasts grown on ADA-GEL-x exhibited significantly higher LDH activity compared to that on ALG and ALG-GEL-b after 3 and 7 days of cultivation. LDH assay can also reflect cell proliferation due to the linear relationship between cell number and LDH activity of mammalian cells.³⁰ In this study, LDH assay was not used to determine the cell number. However, the indication of cell proliferation rate can be assessed from the LDH activity of cells. From Figure 8(a), it is apparent that ADA-GEL promoted cell proliferation, reflected also by the results of the fluorescence staining shown in Figures 7 and 9. In this study, cell counting on the three hydrogels after different cultivation periods was done with image processing software and the results are shown in Figure 8(b). Using this technique, it was not possible to count the cell nuclei on ALG hydrogels due to aggregation of cells as shown in Figures 7 and 9. However, the number of cells on ADA-GEL-x significantly increased compared to ALG-GEL-b at all three cultivation time points, which is in accordance with the result of the LDH assay that is presented in Figure 8(b). It is interesting to note that ADA-GEL-x supported fibroblast viability and proliferation to a significantly higher degree as compared to ALG-GEL-b, especially at longer cultivation times.

Cell morphology. Cell morphology was visualized by actin cytoskeleton staining with rhodamine phalloidin, as shown in Figure 9. As expected, there was a markedly lower number of cells, which were also aggregated, on ALG compared to ALG-GEL-b and ADA-GEL-x. On ALG-GEL-b, the typical bipolar morphology of fibroblasts was observed initially (after 1 and 3 days), which was no longer seen after 7 days, when the cells became polygonal and formed colonies. This behavior can be ascribed to the lack of cell-binding peptide in the hydrogel due to the release of GEL over the incubation time, as there was no strong chemical interaction between ALG and GEL. Moreover, due to the release of GEL, it can be expected that the surface topography of ALG-GEL-b becomes even more similar to that of pure ALG over the cultivation period. This can result in fibroblasts developing similar cellular morphology on both types of hydrogels over longer incubation periods. Additionally, the typical aligned growth behavior of fibroblasts was not observed on the ALG-GEL-b hydrogel at any stage of cultivation. In accordance with the results of our previous study,¹¹ the typical elongated (spindle-shaped) morphology of fibroblasts was observed on ADA-GEL-x at all cultivation time points. Moreover, after 7 days of cultivation, cells proliferated and covered the

Figure 9. F-actin (red) staining with rhodamine phalloidin and nuclei (green) staining with SYTOX shows the orientation of stress fiber of fibroblasts grown on ALG, ALG-GEL-b, and ADA-GEL-x films after 1, 3, and 7 days of cultivation. The typical elongated (spindle-shaped) morphology of growing fibroblasts is observed for ADA-GEL-x after all time points and this morphology is not observed for ALG-GEL-b at longer culturing period. Furthermore, the entire surface of ADA-GEL-x film is covered by growing fibroblasts with typical parallel oriented cells after 7 days.

whole surface of the hydrogel with parallel orientation to each other and to the substrate, which is an inherent population behavior of fibroblasts.^{31,32}

Conclusion

In this study, ADA-GEL-x produced by chemical crosslinking and ALG-GEL-b obtained by physical blending were investigated and compared. ADA-GEL-x, prepared through chemical crosslinking between oxidized ALG and GEL, promoted an enhanced growth and proliferation of fibroblasts. On the physically blended ALG–GEL hydrogel (ALG-GEL-b), this phenomenon was found to be inhibited over longer incubation times. ADA-GEL-x exhibited porous fibrillar surface topography that was found to be completely different from ALG-GEL-b and pure ALG. Moreover, ADA-GEL-x hydrogel exhibited slower release rate of protein and higher degradation kinetic compared to ALG-GEL-b hydrogel, whereas pure ALG did not exhibit degradation. It is plausible that the higher degradation of the hydrogel and the resulting porous surface topography, together with better availability of cell adhesive ligand due to the lower release rate of GEL, contributed to the better adhesion, proliferation, spreading, and metabolic activity of fibroblasts on the ADA-GEL-x hydrogel. Therefore, the oxidation of ALG and the intermolecular interactions between oxidized ALG and GEL are considered to cause the changes in morphology, mechanical properties, release kinetic of GEL, swelling, and degradation properties of ALG that were found to be optimal for fibroblasts' attachment and growth on the hydrogel. This behavior was not achieved by simple physical blending between ALG

and GEL. The results of this study indicate that compared to simple blending of pure ALG and GEL, the chemical crosslinking between oxidized ALG and GEL is a superior strategy to develop advanced ALG- and GEL-based hydrogels for tissue engineering applications.

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Declaration of conflicting interests

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