Published in final edited form as:

J Biomed Mater Res A. 2009 November ; 91(2): 416–427. doi:10.1002/jbm.a.32236.

Characterization of esterified hyaluronan-gelatin polymer composites suitable for chondrogenic differentiation of mesenchymal stem cells*

Peter Angele¹, Rainer Müller², Detlef Schumann¹, Carsten Englert¹, Johannes Zellner¹, Brian Johnstone³, Jung Yoo³, Joachim Hammer⁴, Johann Fierlbeck⁴, Martin K. Angele⁵, Michael Nerlich¹, and Richard Kujat¹

¹ Department of Trauma Surgery, University Hospital of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

² Institute of Physical and Theoretical Chemistry, University of Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany

³ Department of Orthopedic Surgery, OHSU, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, USA

⁴ Department of Biomechanics, University of Applied Sciences, Galgenbergstr. 30, 93053 Regensburg, Germany

⁵ Department of Surgery, Ludwig Maximilians University, Klinikum Großhadern, Marchioninistr. 15, 81377 München, Germany

Abstract

Composite scaffolds of homogeneously mixed esterified hyaluronan (HY) and gelatin (G) were manufactured with variable component compositions (HY100%; HY95%/G5%; HY70%/G30%). The goals of this study were to analyze the produced composite scaffolds using physical and chemical methods, e.g., scanning electron microscopy, IR-spectroscopy, water contact angle, protein assay, and tensile testing as well as to assess the effects of adding gelatin to the composite scaffolds on attachment, proliferation and chondrogenic differentiation of human mesenchymal stem cells. Numbers of attached cells were significantly higher on the composite material compared to pure hyaluronan at different time points of two-dimensional or three-dimensional cell culture ($p < 0.02$). In composite scaffolds, a significantly greater amount of cartilage-specific extracellular matrix components was deposited after 28 days in culture (glycosaminoglycan: $p < 0.001$; collagen: $p < 0.001$) as compared with 100% hyaluronan scaffolds. Additionally, gelatin containing composite scaffolds displayed stronger promotion of collagen type II expression than pure hyaluronan scaffolds. The mechanism, by which gelatin influences cell adhesion, was examined. The effect was inhibited by collagenase treatment of the composites or by addition of $\alpha 5 \beta 1$ -integrin blocking antibodies to the cell suspension. In summary, the results describe the establishment of a class of composite polymer scaffolds, consisting of esterified hyaluronan and gelatin, which are potentially useful for cell-based tissue engineering approaches using mesenchymal stem cells for chondrogenic differentiation.

*No benefit of any kind will be received either directly or indirectly by the authors.

Correspondence to: P. Angele; e-mail: angelepeter@aol.com., Tel +49-941-944-6805; Fax +49-941-944-6806.

Keywords

gelatin; hyaluronan; tissue engineering; chondrogenesis; mechanical properties; mesenchymal stem cells

INTRODUCTION

Bioresorbable materials are presently being investigated for use as cell-free repair scaffolds, as delivery scaffolds for cells, or as substrates for *in vitro* tissue engineering.¹⁻³ A large variety of new biomaterials with specific properties for the regeneration of different tissues has been developed. Among the many materials employed for the production of scaffolds, hyaluronan has been used by several groups as a substrate for tissue engineering. It is a naturally occurring glycosaminoglycan present in all tissues in varying concentrations, with the highest concentrations in soft connective tissue. Hyaluronan-based implants have been utilized for tissue engineering to repair defects of the skin or of the musculoskeletal system including cartilage, bone, ligament and intervertebral disc.⁴⁻¹⁴ In order to decrease the *in vitro* and *in vivo* biodegradation rates, slow-degrading hyaluronan-based scaffolds were developed by chemical modification.^{6,8,11-13,15} These derivatives, mostly esters, have the advantage that their biodegradation produces nontoxic products after hydrolysis of the ester bonds.^{9,12,16} However, this modification of hyaluronan produces scaffold surfaces that can impede cell attachment¹⁷ and cell (re)-differentiation.⁷ To alleviate this problem, coating of hyaluronan-based scaffolds with fibronectin^{13,18} or covalent binding of RGD to a cross-linked hyaluronan¹⁹ have been performed. As an alternative to this, we^{8,20,21} and others^{4,22-24} have described the combination of gelatin (hydrolyzed collagen) with hyaluronan to facilitate cell adhesion in scaffolds. Collagen and its derivative gelatin have also been extensively used in tissue engineering.¹⁻³ Collagen promotes cell attachment and proliferation²⁵⁻²⁷ and collagen substrates have been linked directly to intracellular signaling pathways by cellular integrin receptors.^{19,28,29} Because of their biocompatibility and cell differentiation potential, collagen-based scaffolds have been used for cartilage and meniscus repair.^{30,31} However, the low biomechanical stability and quick degradation rate of collagen and its substrates have made chemical modifications necessary, using potentially toxic agents that may still be present during *in vivo* usage.^{32,33} The advantages of combining hyaluronan and gelatin as a collagen substrate have been described in our previous work.^{8,20,21,34} We were able to show that the composite scaffolds facilitated chondrogenic differentiation of bone marrow-derived mesenchymal stem cells both *in vitro*^{8,20,21} and *in vivo*.^{8,20,34} Recently, this biomaterial in combination with mesenchymal stem cells has shown successful repair of meniscal defects.³⁵ The goals of this study were to analyze the produced composite scaffolds using physicochemical parameters and to characterize the effect of variants of gelatin content in the scaffolds with regard to cell attachment, proliferation and differentiation of mesenchymal stem cells. We also assessed the mechanism through which gelatin influences cell adhesion.

MATERIAL AND METHODS

Manufacturing process of composite polymer films and scaffolds

The test materials were prepared as two-dimensional polymer films or three-dimensional polymer scaffolds containing either pure esterified hyaluronan (HY100%) or esterified hyaluronan and varying amounts of gelatin (HY95%/G5%; HY70%/G30%). These notations will be maintained throughout the manuscript although the final composition of the hybrid materials may differ from their original composition. Porcine gelatin was purchased by Sigma-Aldrich Chemie GmbH (Germany); the hyaluronic acid benzyl ester (trade name:

“Jaloskin”) was obtained from Fidia farmaceutici S.p.A. (Italy). The polymer films and the three dimensional polymer scaffolds were manufactured following a recently developed manufacturing process^{8,21} in which the polymers were dissolved and homogeneously mixed in hexafluoro-2-propanol (HFIP) which was obtained from Fluka GmbH (Germany). For all compositions, the final concentration of dissolved hyaluronan was 4%. Films were prepared either on smooth polytetrafluoroethylene surfaces (3.5 cm × 3.5 cm), on glass cover slides (1.8 cm × 1.8 cm), or in 24-well plates (1.55 cm diameter, 1.90 cm² area) by air evaporation of the volatile solvent followed by drying in vacuum. To create the three-dimensional scaffolds, salt crystals with a defined grain size (350–450 µm) were added to the polymer solutions and served as a porogen for the scaffolds (mass ratio polymer : salt = 1 : 60). After compressing the constructs in molds and drying in vacuum, the salt crystals were removed by leaching in de-ionized water which was controlled by conductimetric measurement. Finally, the porous scaffolds were dehydrated in acetone-water mixtures of increasing acetone content and dried in vacuum.

Ultrastructure of polymer scaffolds

Pure hyaluronan and composite scaffolds were prepared for scanning electron microscopy as described previously.^{8,36} Briefly, samples were fixed with 1% glutaraldehyde in phosphate buffer (pH 7.4), osmicated, washed in buffer, dehydrated in increasing concentrations of ethanol and subsequently in acetone. Dehydrated specimens were subjected to critical-point drying using liquid carbon dioxide substitution, mounted on aluminum stubs, and gold-coated using a Polaron sputter coater. Samples were examined and photographed with a Zeiss DSM 940 microscope.

Physicochemical characterization of polymer composites

Physicochemical properties of the composite materials were evaluated with respect to the polymer composition and the effects of the salt-leaching procedure. Air-dried polymer films served as samples for the not salt-leached condition; salt-leached three-dimensional specimens were characterized after condensing to a film by the use of a hydraulic press.

For infrared spectroscopy, films were examined in the transmission mode using a Jasco-610 FTIR spectrometer. To determine the composition of the films, the peak ratios of the ester and the amide-II signals were evaluated. Estimation of residual organic fluorine containing solvent was performed by comparison of the peak ratios of the C-F band at 843 cm⁻¹ and the amide-II signal. Reported values are the average ratios obtained from examination of three films per composition.

Contact angle measurements were carried out by the sessile drop technique.³⁷ A water droplet with a volume of 2 µl was advanced to the surface of each polymer film by means of a microsyringe and the contact angle was measured with a G1 goniometer of Erma Co. (Japan). The films were prepared on the surfaces of glass slides by drying the organic solutions as mentioned before. Reported results are the average contact angles of ten droplets deposited on three individual films. To minimize the effect of film swelling, the values were determined exactly 30 sec after deposition of the water droplet.

The gelatin content of the films was further quantified by measuring the amount of free amino groups which were determined by using 2,4,6-trinitro-benzenesulfonic acid (TNBS, Sigma).³⁸ Light absorption of the diluted solutions was measured at 346 nm against a blank solution in which the reaction of TNBS was suppressed by adding of hydrochloric acid. Three samples of identical composition were examined and the mean values calculated. UV-VIS spectrometry was carried out on a Lambda-18 spectrometer of Perkin Elmer Corp. (USA).

To determine the amount of gelatin that was extracted from the three-dimensional scaffolds during the salt leaching procedure, the bicinchoninic acid protein assay (BCA) was used.³⁹ The washing solutions were aspirated after extraction times of 15, 30, 60, 120, 180, and 360 min. Aliquots (100 μ l) of these solutions were incubated with 1ml BCA working solution (Sigma) at 40 °C for 30 min. The light absorption of the resulting solutions was measured at 562 nm and compared with the values obtained from solutions of defined gelatin concentrations (0.01 to 1 mg/ml). Washing solutions of scaffolds made of esterified hyaluronan were used as blanks. The reported values represent the means of four scaffolds of identical composition but of different weight.

Stress-strain characteristics of the scaffolds

The stress-strain curves of scaffolds were determined by uniaxial measurements using a Zwick-1445 mechanical testing device. Cylindrical bars (6 mm length $\times$ 5 mm diameter) were cut from the matrix sheets using circular punches. Each sample was glued with defined amounts of low melting dental wax (Miltex Inc., USA) between two standardized aluminum discs with a diameter of 10 mm and a thickness of 1 mm. The metal discs allowed a convenient fixation of the samples between two vertically positioned grips. A highly precise positioning of one fixing grip in x,y-direction guaranteed a strictly linear alignment of the specimen axis parallel to the load axis in z-direction. All samples were characterized in the wet state after soaking the specimens in distilled water at room temperature for 10 min (Figure 1). For the tensile experiments, an initial gauge length of 4 mm was used. All samples were deformed to rupture at a deformation rate of 1 mm/min. The displacement and load were recorded continuously by a Lab View 5.0 - software. The Young's modulus was directly determined from the linear slope of the stress-strain curves (n = 6 per group).

Cell culture

The cellular effects induced by the hyaluronan-gelatin composites were examined in two-dimensional films and three-dimensional scaffolds. The polymer films and scaffolds were sterilized via beta irradiation (e-beam) with a dose of 25 kGy by Beta-Gamma-Service GmbH (Germany) according to ISO 11137. Human bone marrow was obtained from bone marrow aspirates of patients undergoing surgery with bone block harvest of the iliac crest (written consent form signed). After Percoll gradient fractionation, Dulbecco's modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) was added to the aspirate and 10×10^6 nucleated cells/100 cm² dish were plated and grown at 37 °C with 5% CO₂ until the cells reached 80% confluency.^{8,21,40-42}

Adhesion and proliferation behavior on polymer films

For analysis of adhesion and proliferation rates, human mesenchymal stem cells (hMSC) were trypsinized, counted and then re-suspended in DMEM containing 10% FBS. Cells were plated (8,000 cells for adhesion analysis and 4,000 cells for proliferation analysis) either in uncoated polystyrene wells (control) of 24-well plates (Sarstedt, Germany) or in wells coated with a film of the polymer composite. The adhesion rate was assessed after 3 h of standard cell culture and the proliferation rate was determined after one, four or seven days of culture. After removal of non-adherent cells the number of attached cells was measured. The quantification of the cells was performed by their mitochondrial dehydrogenase activity using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine ethosulfate (PES) according to the protocol provided by the manufacturer (CellTiter96-Kit of Promega, Germany). Eight samples were examined for each polymer film composition. To assess cell numbers, standard curves were established by plating five dilution steps of cell numbers between 1,000 and 10,000 cells per well and performing the MTS assay. Each standard cell number was used in quadruplicate.

Effect of gelatin on cell adhesion

To further analyze the effect of gelatin on the adhesion and proliferation of mesenchymal stem cells, polymer films were incubated with 0.1% collagenase P (Roche, 1213865) for 4 h prior to cell application. hMSCs (10,000 per polymer film) were incubated for 30 min, 1, 2, 12, or 24 h in DMEM containing 10% FBS and then subjected to immunohistochemical staining for CD44 (Pharmingen, clone IM7, 553130) and β 1-integrin (Chemicon, clone P4C10, MAB 1987 Z). The cell morphology was examined with fluorescence microscopy after application of a FITC-conjugated goat anti-mouse secondary polyclonal antibody (Jackson) and compared between the groups. In replicate films the integrin receptor-ligand binding was blocked by adding a blocking goat anti- α 5 β 1 polyclonal antibody (Chemicon, AB1950; diluted 1:250) to the cell suspension. After 24 h the number of attached cells was measured by cell counting ($n = 5$ per group).

Chondrogenic differentiation behavior in polymer scaffolds

Adherent hMSC colonies were trypsinized, counted, and 2×10^6 mesenchymal stem cells were injected into cylindrical polymer scaffolds with defined pore size and cultured at 37 °C in 5% CO₂ in a defined medium, previously shown to induce chondrogenic differentiation of mesenchymal stem cells.^{8,21,40–42}

DNA contents of the polymer scaffolds were assayed using a fluorimetric assay as described previously.^{21,42} Scaffolds were individually digested with 150 µg/ml papain overnight at pH 6.0 at 60 °C. Aliquots of the digest (10 µl) were mixed with 200 µl of diluted Hoechst 33258 Dye Solution (0.1 µg/ml). The fluorescence emission of the samples was measured with Cytofluor at 360 nm excitation, 460 nm emission with a gain of 70. A standard curve was produced with calf thymus DNA (Sigma) and used to determine the DNA content of the experimental samples. The glycosaminoglycan content of the scaffolds, used as an indicator of proteoglycan production, was quantified spectrophotometrically, with a 1,9-dimethylmethylene blue (DMMB) binding assay as described elsewhere.⁴³ Scaffolds were digested in papain solution at 60 °C overnight. In a 96-well plate, 50 µl aliquots of the samples were mixed with 200 µl of 50 µmol/l DMMB (Sigma) dissolved in 40 mmol/l glycine-NaCl at pH 3.0 and optical density was read at 540 nm using the ELISA reader Emax from Molecular Devices (Germany). A standard curve was prepared with papain-treated chondroitin sulfate A (Sigma). For the experimental groups, the proteoglycan content of the negative controls (cell free polymer scaffold) was measured and subtracted to get an estimation of the produced extracellular proteoglycan since the hyaluronan from the scaffold is detectable in the assay, albeit at a low level.

The hydroxyproline content of the scaffolds was measured with a modified version of the standard assay.⁴⁴ Papain digested scaffolds were dried, weighed, and hydrolyzed with 2 mol/l sodium hydroxide solution for 4 h in a pressure apparatus. 50 µl, 25 µl, and 10 µl triplicated aliquots of hydrolysate were incubated for 20 min with 450 µl of chloramine T solution at room temperature. After addition of 500 µl of Ehrlich's reagent, the color was developed for 20 min at 65 °C. Light absorption was read at 560 nm in the ELISA reader. The hydroxyproline content of samples was extrapolated against identically processed hydroxyproline standards between 1 and 20 µg/ml. The collagen content was calculated by multiplying the total hydroxyproline content of the assay with a conversion factor ($\times 7.5$). For the experimental groups the content of the negative controls (cell-free polymer scaffold) was subtracted to get an estimation of the produced extracellular collagen since the gelatin of the scaffolds would influence the measurement. For immunohistochemical analysis of MSC differentiation, cell-matrix constructs were formaldehyde fixed at day 28 of chondrogenic cell culture, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Zoeterwoude, Netherlands), and frozen in liquid nitrogen. Expression of collagen type I and

collagen type II has been demonstrated on 10 μm cryosections by the immunoperoxidase ABC technique (Vector, Burlingame, CA, USA) after Hsu et al. 45, applying predigestion of sections (15 min in 0.1% pepsin at pH 3.5), monoclonal primary antibodies mouse anti-collagen type I (clone col-1, Sigma, St. Louis, MO, USA), collagen II (clone II-4C11, Calbiochem – Merck, Schwalbach, Germany), biotin conjugated polyclonal secondary goat anti-mouse IgG (Jackson, West Grove, PA, USA) antibodies, and the Ni and Co enhanced DAB stain visualization.

Statistical Analysis

Data are reported as the mean $\pm$ SD. One-factor analyses of variance (ANOVAs) with Bonferroni-Dunn, Tukey and Fisher's post hoc tests for multiple comparisons were performed using SPSS 12.0G. Significance was accepted at a level of $p < 0.05$.

RESULTS

Ultrastructure of polymer scaffolds

Hyaluronan-gelatin composite scaffolds were produced following the protocol of the recently developed manufacturing process.^{8,20,21} The addition of gelatin (Figure 2A, 2B) produced no significant ultrastructural changes compared to 100% hyaluronan-based scaffolds (Figure 2C, 2D). Both scaffolds (HY 100%, HY70%/G30%) showed a double-pore structure. In addition to the main pores (mean pore size 350–450 μm) that were formed directly by the porogen (salt crystals), a second class of macropores with a smaller pore size between 50 and 100 μm was produced. Figure 2B and 2D display the microstructure of the pore walls which exhibited no significant differences between pure hyaluronan and composite scaffolds. The scaffolds revealed thin pore walls of about 3 μm in average thickness that were essentially solid throughout the scaffolds. A high degree of interconnectivity between the primary and secondary pores was noted (Figure 2A, 2C).

Physicochemical characterization of polymer composites

Several analytical methods have been used to determine the composition of the polymers and to control unintended changes which may occur during scaffold processing. The infrared spectra of polymer films consisting of esterified hyaluronan and gelatin exhibited signals for the ester and the amide groups (Figure 3A). The peak ratio of the ester to amide-II signals decreased with increased gelatin content of the polymer films (Table 1). Comparable results have been obtained by measuring the water contact angle of the film surfaces. The wetting angle decreased with increasing amounts of the hydrophilic gelatin constituent. The amino group concentration, which could be quantified by the TNBS assay, increased with increasing gelatin content of the examined films (Table 1).

Effects of the salt-leaching procedure

Manufacture of the three-dimensional polymer scaffolds required washing steps with water or buffer solution in order to remove the pore forming particles consisting of sodium chloride. Additionally, washing of the scaffolds with water reduced residual organic solvent that withstands vacuum drying. The qualitative effect of rinsing polymer samples of the composition 70% derivatized hyaluronan and 30% gelatin with water is shown by the IR spectra of Figure 3A. The spectrum of the vacuum dried film exhibited strong signals in the area of 1200 cm^{-1} and between 950 and 840 cm^{-1} which belong to the vibration of C-F groups of the organic solvent HFIP. After leaching the salt crystals from 3-D polymer constructs with water, the signals disappeared from the IR spectrum.

As a side effect of the washing procedure, the extraction of the gelatin constituent was qualitatively observed by an increase in the peak ratio of the ester to amide-II signals with

IR-spectroscopy (Table 1). For pure hyaluronan-based scaffolds, no differences in infrared band intensities could be detected after the removal of the salt crystals with water. However, for composite scaffolds (HY95%/G5%, HY70%/G30%), a loss of gelatin could be detected with this method. These results were confirmed by contact angle measurements of washed polymer films. The contact angle increased due to dissolution of the more hydrophilic polymer gelatin from the composites (Table 1).

The amount of dissolved gelatin was quantified with the BCA protein assay (Figure 3B). A loss of the gelatin content in composite scaffolds could be detected after the removal of the porogen depending on the original gelatin content. The percentage of dissolved gelatin during washing is higher if a higher amount of gelatin was initially added. Also a time dependency could be detected such that a high percentage of gelatin dissolution was noted at the beginning of the porogen removal with lower dissolution over time. The remaining gelatin content was estimated combining the results of the different characterization techniques including the TNBS assay for primary amino groups (Table 1). For scaffolds with an original gelatin content of 30%, a remaining content of about 15–20% could be estimated after porogen removal. For composites with an original gelatin content of 5%, a final gelatin content of about 3–4% was measured. In the following sections of the manuscript the notations HY95%/G5% and HY70%/G30% will be maintained despite the loss of gelatin described in this chapter.

Biomechanical analysis of the composite scaffolds

Independently of the scaffold composition, no significant swelling or shrinkage of the scaffolds could be detected after cell loading and during culture.

However, the tensile experiments reveal a clear dependency between scaffold composition and mechanical stability (Table 2). With increasing gelatin content, the rupture strength and the maximum Young modulus were decreased. Composite scaffolds (HY70%/G30%) displayed a significant reduction in tensile strength ($p = 0.03$) and Young's modulus ($p = 0.003$) compared with scaffolds made of 100% hyaluronan. Strain at rupture was slightly enhanced with scaffolds containing low amounts of gelatin (HY95%/G5%) compared to the hyaluronan scaffold, whereas for scaffolds with high amounts of gelatin (HY70%/G30%) no difference was detected.

Cell adhesion and proliferation on composite films

Adhesion of human mesenchymal stem cells on films of 100% esterified hyaluronan were significantly lower compared with the polystyrene (PS) reference ($p = 0.002$). On composite films of esterified hyaluronan and gelatin, cell adhesion was significantly greater ($p = 0.006$) as compared to pure hyaluronan and almost identical to that found with PS (Figure 4A). Significant cell proliferation was only found on PS between day 1 and day 7 ($p = 0.002$), while on all other surfaces the cell number remained nearly constant. Cell numbers on films containing gelatin were significantly higher as compared to pure hyaluronan at each time point of the proliferation experiment ($p < 0.001$) (Figure 4B).

Role of gelatin in cell adhesion

To analyze the mechanism by which gelatin is mediating the elevated cell adhesion and proliferation further experiments on 100% hyaluronan-based films and composite films (HY70%/G30%) were performed. CD44 expression on the cell surface was moderately positive in all groups at all incubation time points (Figure 5). $\beta 1$ -integrin expression could be detected on the surface of adherent cells on 100% hyaluronan-based films and composite films. After 30 min of incubation, round mesenchymal stem cells were seen adhering to the composite polymer films (HY70%/G30%). In this group, the first evidence of cell spreading

(spindle-shaped cells) could be detected after 1 h. On 100% hyaluronan-based films or composite films treated with collagenase prior to cell seeding, the cells kept their initial rounded morphology. After 2 h, the cells of the untreated composite films were spread out, whereas in the other groups only a small percentage of spindle-shaped cells could be detected. After 12 and 24 h of cell incubation, cell attachment with a change of cell morphology to fibroblast-like cells could be seen in all groups (Figure 5).

The composite films facilitated significantly higher cell attachment ($p < 0.001$) as detected with dark field microscopy and cell counting (Figure 6). This effect could be inhibited by collagenase treatment of the composite films prior to cell application, giving equivalent cell attachment on both polymer films. Blocking of receptor-ligand binding for $\alpha 5\beta 1$ -integrin on composite films reduced cell adherence to the level seen with 100% hyaluronan-based films or composite and hyaluronan films after collagenase treatment, suggesting a $\alpha 5\beta 1$ -integrin involvement in early cell adhesion of mesenchymal stem cells on gelatin-containing surfaces (Figure 6).

Influence of gelatin on cell differentiation

The effect of adding gelatin on chondrogenic differentiation of mesenchymal stem cells was analyzed in three-dimensional polymer scaffolds. A significantly higher cell adhesion was detected in composite polymer scaffolds ($p = 0.02$). After chondrogenic culture, the cell number between scaffolds with gelatin remained unchanged. However, cells in 100% hyaluronan scaffolds proliferated to reach a number similar to that found in the composite scaffolds (day 14: $p = 0.07$; day 28: $p = 0.7$) (Figure 7A). Both the proteoglycan and collagen contents were higher in composite scaffolds compared with 100% hyaluronan scaffolds. A significant increase in proteoglycan and collagen contents could be observed with addition of gelatin to the polymers ($p < 0.001$ for glycosaminoglycan and collagen for both HY95%/G5% and HY70%/G30%). The inclusion of 30% gelatin led to a significantly higher amount of proteoglycan production compared with 5% gelatin ($p < 0.001$), but no significant differences in collagen content ($p = 0.09$) could be detected (Figure 7B).

Immunohistochemical analysis of cell-matrix constructs disclosed no significant differences in collagen type I expression within pure hyaluronan scaffolds and hyaluronan-gelatin composite scaffolds (HY70%/G30%). In both matrices a weakly pronounced reaction against collagen type I was observed. On the other hand, expression of collagen type II strongly depended on the composition of the scaffold material used. Within composite scaffolds (HY70%/G30%) distinctly higher amounts of expressed collagen type II could be detected compared to scaffolds made from pure hyaluronan (Figure 7C).

DISCUSSION

We have previously described the use of composite polymer scaffolds, consisting of esterified hyaluronan and gelatin, for cartilage tissue engineering with mesenchymal stem cells.^{8,20,21} Here we analyze this new class of composite polymer scaffolds in terms of ultrastructure, biochemical composition and biological effects compared with 100% hyaluronan-based scaffolds.

The composite polymer scaffolds were manufactured with a newly developed procedure using the solvent hexafluoro-2-propanol to homogeneously mix esterified hyaluronan and gelatin in the same solution, which resulted in the formation of primary and secondary pores in the scaffolds. The pore walls consisting of the homogeneously mixed polymers were thin and very compact. The ultrastructure of scaffolds created with this protocol differed from those made with a previously described manufacturing process, in which only primary pores with thicker and foam-like pore walls were created.¹⁶ The structure of the pore walls in the

recently developed manufacturing process was achieved by an air drying step. The drying solution of the hyaluronan spreads over the walls of the crystals of the porogen and condenses to very thin, homogenous, compact layers. By drying, the first solvent evaporates and its volume gets substituted by air yielding the additional secondary pores. With the previously published manufacturing process,¹⁶ the esterified hyaluronan does not rigidify by drying but by flocculation in the second solvent in which it is insoluble. Therefore the ester remained voluminous distributed in the pore interstices and resulted in thick pore walls with foam-like ultrastructure lacking additional secondary pores. The addition of gelatin did not alter these ultrastructural differences in scaffolds manufactured with our manufacturing process.

The rationale behind manufacturing a composite of hyaluronan and collagen was to mimic the embryonic environment for optimized cell attachment and suitable cell differentiation. 9:46 Mixtures of hyaluronan and collagenous components for the development of composite biomaterials have been described before.^{4:22,27,47-54} Because of the fast degradation rate of water soluble hyaluronan *in vivo*, various modifications of hyaluronan (esterification, cross-linking) have been applied.^{16:24,54-57} These modifications resulted in prolonged scaffold stability and reduced degradation rates, however impaired biological properties could be observed. With polymer composites of hydrophobic esterified hyaluronan and hydrophilic gelatin we combined the advantages of esterified hyaluronan scaffolds in terms of scaffold stability, reduced degradation and excellent biocompatibility^{8:9,11-13,58} with the advantages of non cross-linked gelatin in terms for optimized cell adhesion and differentiation.^{24-27,51,54} These composites have been shown to facilitate chondrogenic differentiation of bone marrow-derived mesenchymal stem cells both *in vitro*^{8,21} and *in vivo*.^{8:20,34}

Physicochemical analysis revealed some loss of gelatin during the manufacturing procedure. However, at the end of the manufacturing process, gelatin was still detectable in the scaffolds as seen in Figure 3 and Table 1. The amount of the dissolved gelatin is dependent on the originally added gelatin content. The percentage of dissolved gelatin during washing is higher when a higher percentage of gelatin content was initially added. This can be explained with the different hydrophilicity of the polymers depending on the amount of gelatin. Polymers with a higher amount of hydrophilic components like gelatin facilitate greater scaffold swelling, which is accompanied by a higher elution of water-soluble particles.

The addition of gelatin resulted in a reduction of scaffold stability. This was expected, because gelatin by itself has poor performance in terms of scaffold stability,^{36,59-61} and so should decrease the stability of the polymer composite. Earlier studies have shown that composite polymers of 70% esterified hyaluronan and 30% gelatin provide an appropriate environment for the chondrogenic differentiation of mesenchymal stem cells.^{8:20,21,34} The same polymer composites also showed good structural stability during *in vitro* experiments that included daily applied compressive loading.²¹

Modified hyaluronan showed good biocompatibility with different cell types,^{7,9,10,13,62} however, several methods have been used to improve cell attachment and cell proliferation including addition of RGD sequences,^{19,55} protein coating¹³ or addition of collagen prior to crosslinking.^{4:22,27,47,48,51-54} In the literature, the attachment of fibroblasts was shown to significantly improve only after addition of more than 40% cross-linked gelatin to cross-linked hyaluronan.²⁴ In our study, the addition of about 5% gelatin provided a significant increase in cell adhesion and proliferation in comparison with 100% hyaluronan-based polymers. Why the lower level of gelatin provided such marked improvement in cell attachment in our study compared with those reported by others is unclear, but it could be

due to the derivatization of the gelatin component, the cross-linking of the hyaluronan component or the different cell types used for the analyses. As well as increased cell attachment to two-dimensional films, the addition of gelatin also facilitated a significant increase in initial cell uptake in three-dimensional composite scaffolds compared with 100% esterified hyaluronan scaffolds.

After degradation of the collagen component with collagenase, the increased cell adhesion in the composite films was blocked completely, indicating the specificity of gelatin in promoting the elevated cell adhesion. Early cell attachment on gelatin-containing surfaces is mediated by early expression of β 1-integrin. The higher level of cell adhesion in the composite polymer films compared with 100% esterified hyaluronan-based films was blocked completely with α 5 β 1-integrin blocking antibodies, indicating the involvement of α 5 β 1-integrins, at least in part, in the attachment of mesenchymal stem cells to the composite polymer films. The interaction of cells with their surroundings requires more than a mechanical site for adhesion.⁶³ Receptors for natural extracellular matrix proteins such as members of the integrin receptor family have been linked directly with intracellular signaling events.^{64–66} Therefore, early expression of α 5 β 1-integrin on the cell surface may simultaneously induce important signal transduction pathways for chondrogenic differentiation. α 5 β 1-integrins seem to be of particular importance in the modulation of cartilaginous tissue.⁶⁷

The biological performance of the scaffolds examined in the present study was clearly improved after addition of gelatin to the fundamental component hyaluronic acid benzyl ester. Adhesion as well as chondrogenic differentiation of mesenchymal stem cells in terms of glycosaminoglycan and collagen synthesis and expression of the hyaline-like cartilage-specific collagen type II seem to increase with increasing gelatin. On the other hand, biomechanical properties of the composites decreased with increasing gelatin content. Therefore, composite scaffolds with lower amounts of gelatin ($\leq 5\%$) could be employed in applications in which a higher mechanical toughness of the scaffolding material is recommended, like the engineering of meniscus or vertebral disc structures. Composites with higher gelatin content ($5\% \leq x \leq 30\%$) are more favorable for engineering of cartilage structures which are exposed to a lower degree of mechanical stress, like reconstruction tissues of the ear and nose. The successful use of the new biomaterial in combination with mesenchymal stem cells for the repair of a critical size meniscal defect could be shown recently.³⁵

CONCLUSIONS

A new class of composite polymer scaffolds, consisting of homogeneously mixed hydrophobic esterified hyaluronan and hydrophilic gelatin, was established and thoroughly analyzed by physicochemical means. The composites showed appropriate morphological structure with interconnecting pores and initial biomechanical stability without chemical cross-linking and are, therefore, free of problems usually connected with stabilization procedures. The addition of hydrolyzed collagen significantly improved the biological properties of the esterified hyaluronan polymer. The resulting 3-dimensional composites appear very useful for cell-based tissue engineering approaches with mesenchymal stem cells, especially for their chondrogenic differentiation. Also polylactide- and polyglycolide-based scaffold materials are well-known for their differentiation promoting potential, but in contrast to the composite scaffolds described in the present study these polymers exhibit the risk of generating high amounts of acidic products during biodegradation which could cause adverse effects for the engineered tissue.

Acknowledgments

The authors thank Tanja Weinfurter, Daniela Drenkard, Tom Böttner, Dalila Bouakline and Ronny Mai for excellent technical assistance. This work was supported by grants of the Bavarian State Ministry of Sciences, Research and the Arts within the program “High Tech Offensive Bayern” and NIH-NIAMS (1R01AR48132).

References

1. Ibarra C, Koski JA, Warren RF. Tissue engineering meniscus: cells and matrix. *Orthop Clin North Am.* 2000; 31:411–418. [PubMed: 10882467]
2. Sweigart MA, Athanasiou KA. Toward tissue engineering of the knee meniscus. *Tissue Eng.* 2001; 7:111–129. [PubMed: 11304448]
3. Buma P, Ramrattan NN, van Tienen TG, Veth RP. Tissue engineering of the meniscus. *Biomaterials.* 2004; 25:1523–1532. [PubMed: 14697855]
4. Chang CH, Liu HC, Lin CC, Chou CH, Lin FH. Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. *Biomaterials.* 2003; 24:4853–4858. [PubMed: 14530082]
5. Crevensten G, Walsh AJ, Ananthakrishnan D, Page P, Wahba GM, Lotz JC, Berven S. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. *Ann Biomed Eng.* 2004; 32:430–434. [PubMed: 15095817]
6. Cristino S, Grassi F, Toneguzzi S, Piacentini A, Grigolo B, Santi S, Riccio M, Tognana E, Facchini A, Lisignoli G. Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11(R)-based prototype ligament scaffold. *J Biomed Mater Res A.* 2005; 73:275–283. [PubMed: 15789422]
7. Aigner J, Tegeler J, Hutzler P, Campoccia D, Pavesio A, Hammer C, Kastenbauer E, Naumann A. Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester. *J Biomed Mater Res.* 1998; 42:172–181. [PubMed: 9773813]
8. Angele P, Kujat R, Nerlich M, Yoo J, Goldberg V, Johnstone B. Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge. *Tissue Eng.* 1999; 5:545–554. [PubMed: 10611546]
9. Campoccia D, Doherty P, Radice M, Brun P, Abatangelo G, Williams DF. Semisynthetic resorbable materials from hyaluronan esterification. *Biomaterials.* 1998; 19:2101–2127. [PubMed: 9884052]
10. Grigolo B, Roseti L, Fiorini M, Fini M, Giavaresi G, Nicoli Aldini N, Giardino R, Facchini A. Transplantation of chondrocytes seeded on a hyaluronan derivative (hyaff-11) into cartilage defects in rabbits. *Biomaterials.* 2001; 22:2417–2424. [PubMed: 11511039]
11. Radice M, Brun P, Cortivo R, Scapinelli R, Battaliard C, Abatangelo G. Hyaluronan-based biopolymers as delivery vehicles for bone-marrow- derived mesenchymal progenitors. *J Biomed Mater Res A.* 2000; 50:101–109.
12. Milella E, Brescia E, Massaro C, Ramires PA, Miglietta MR, Fiori V, Aversa P. Physico-chemical properties and degradability of non-woven hyaluronan benzylic esters as tissue engineering scaffolds. *Biomaterials.* 2002; 23:1053–1063. [PubMed: 11791908]
13. Solchaga LA, Gao J, Dennis JE, Awadallah A, Lundberg M, Caplan AI, Goldberg VM. Treatment of osteochondral defects with autologous bone marrow in a hyaluronan-based delivery vehicle. *Tissue Eng.* 2002; 8:333–347. [PubMed: 12031121]
14. Balazs EA, Denlinger JL. Clinical uses of hyaluronan. *Ciba Found Symp.* 1989; 143:265–275. [PubMed: 2680347]
15. Baier Leach J, Bivens KA, Patrick CW Jr, Schmidt CE. Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds. *Biotechnol Bioeng.* 2003; 82:578–589. [PubMed: 12652481]
16. Valentini, R.; Kim, H. WO-Patent WO 97/45532. 1997. Hyaluronan based biodegradable scaffolds for tissue repair.
17. Kito H, Matsuda T. Biocompatible coatings for luminal and outer surfaces of small-caliber artificial grafts. *J Biomed Mater Res.* 1996; 30:321–330. [PubMed: 8698695]
18. Solchaga LA, Yoo JU, Lundberg M, Dennis JE, Huibregtse BA, Goldberg VM, Caplan AI. Hyaluronan-based polymers in the treatment of osteochondral defects. *J Orthop Res.* 2000; 18:773–780. [PubMed: 11117300]

19. Glass JR, Dickerson KT, Stecker K, Polarek JW. Characterization of a hyaluronic acid-Arg-Gly-Asp peptide cell attachment matrix. *Biomaterials*. 1996; 17:1101–1108. [PubMed: 8718970]
20. Angele P, Kujat R, Faltermeier H, Schumann D, Müller R, Nerlich M. Biodegradable Hyaluronsäureester/Gelatine Kompositmatrix zur osteochondralen Differenzierung mesenchymaler Vorläuferzellen. *Biomaterialien*. 2003; 4:11–18.
21. Angele P, Schumann D, Angele M, Kinner B, Englert C, Hente R, Fuechtmeier B, Nerlich M, Neumann C, Kujat R. Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds. *Biorheology*. 2004; 41:335–346. [PubMed: 15299266]
22. Alini M, Li W, Markovic P, Aebi M, Spiro RC, Roughley PJ. The potential and limitations of a cell-seeded collagen/hyaluronan scaffold to engineer an intervertebral disc-like matrix. *Spine*. 2003; 28:446–454. [PubMed: 12616155]
23. Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D, Glowacki J. Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds. *J Biomed Mater Res A*. 2001; 55:13–19.
24. Shu XZ, Liu Y, Palumbo F, Prestwich GD. Disulfide-crosslinked hyaluronan-gelatin hydrogel films: a covalent mimic of the extracellular matrix for *in vitro* cell growth. *Biomaterials*. 2003; 24:3825–3834. [PubMed: 12818555]
25. Elisseeff JH, Lee A, Kleinman HK, Yamada Y. Biological response of chondrocytes to hydrogels. *Ann N Y Acad Sci*. 2002; 961:118–122. [PubMed: 12081878]
26. Doillon CJ, Silver FH, Olson RM, Kamath CY, Berg RA. Fibroblast and epidermal cell-type I collagen interactions: cell culture and human studies. *Scanning Microsc*. 1988; 2:985–992. [PubMed: 3399861]
27. Srivastava S, Gorham SD, Courtney JM. The attachment and growth of an established cell line on collagen, chemically modified collagen, and collagen composite surfaces. *Biomaterials*. 1990; 11:162–168. [PubMed: 2350552]
28. Enomoto M, Leboy PS, Menko AS, Boettiger D. Beta 1 integrins mediate chondrocyte interaction with type I collagen, type II collagen, and fibronectin. *Exp Cell Res*. 1993; 205:276–285. [PubMed: 8387015]
29. Scully SP, Lee JW, Ghert PMA, Qi W. The role of the extracellular matrix in articular chondrocyte regulation. *Clin Orthop*. 2001; 391(Suppl):S72–89. [PubMed: 11603727]
30. Stone KR, Steadman JR, Rodkey WG, Li ST. Regeneration of meniscal cartilage with use of a collagen scaffold. Analysis of preliminary data. *J Bone Joint Surg Am*. 1997; 79:1770–1777. [PubMed: 9409790]
31. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am*. 1994; 76:579–592. [PubMed: 8150826]
32. van Luyn MJ, van Wachem PB, Damink LO, Dijkstra PJ, Feijen J, Nieuwenhuis P. Relations between *in vitro* cytotoxicity and crosslinked dermal sheep collagens. *J Biomed Mater Res*. 1992; 26:1091–1110. [PubMed: 1429758]
33. van Wachem PB, van Luyn MJ, Olde Damink LH, Dijkstra PJ, Feijen J, Nieuwenhuis P. Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. *J Biomed Mater Res*. 1994; 28:353–363. [PubMed: 8077250]
34. Angele P, Johnstone B, Kujat R, Nerlich M, Goldberg V, Yoo J. Meniscus repair with mesenchymal progenitor cells in a biodegradable composite matrix. *Trans Orthop Res Soc*. 2000:605.
35. Angele P, Johnstone B, Kujat R, Zellner J, Nerlich M, Goldberg V, Yoo J. Stem cell based tissue-engineering for meniscus repair. *J Biomed Mater Res A*. in press. 10.1002/jbm.a.31480
36. Angele P, Abke J, Kujat R, Faltermeier H, Schumann D, Nerlich M, Kinner B, Englert C, Ruszczak Z, Mehrl R, Müller R. Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices. *Biomaterials*. 2004; 25:2831–2841. [PubMed: 14962561]
37. Neumann AW, Good GR. Techniques of measuring contact angles. *Surf Colloid Sci*. 1979; 11:31–91.

38. Bubnis WA, Ofner CM 3rd. The determination of epsilon-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. *Anal Biochem.* 1992; 207:129–133. [PubMed: 1489085]
39. Smith PK, Krohn RL, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985; 150:72–85.
40. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.* 1998; 238:265–272. [PubMed: 9457080]
41. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am.* 1998; 80:1745–1757. [PubMed: 9875932]
42. Angele P, Yoo JU, Smith C, Mansour J, Jepsen KJ, Nerlich M, Johnstone B. Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated *in vitro*. *J Orthop Res.* 2003; 21:451–457. [PubMed: 12706017]
43. Hoemann CD, Sun J, Chrzanowski V, Buschmann MD. A multivalent assay to detect glycosaminoglycan, protein, collagen, RNA, and DNA content in milligram samples of cartilage or hydrogel-based repair cartilage. *Anal Biochem.* 2002; 300:1–10. [PubMed: 11743684]
44. Stegemann H, Stalder K. Determination of hydroxyproline. *Clin Chim Acta.* 1967; 18:267–273. [PubMed: 4864804]
45. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex(ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981; 29:577–580. [PubMed: 6166661]
46. Maleski MPK. Matrix accumulation and retention in embryonic cartilage and *in vitro* chondrogenesis. *Conn Tissue Res.* 1996; 34:75–86.
47. Doillon CJ, Silver FH. Collagen-based wound dressing: effects of hyaluronic acid and fibronectin on wound healing. *Biomaterials.* 1986; 7:3–8. [PubMed: 3955155]
48. Doillon CJ, Wasserman AJ, Berg RA, Silver FH. Behaviour of fibroblasts and epidermal cells cultivated on analogues of extracellular matrix. *Biomaterials.* 1988; 9:91–96. [PubMed: 3349126]
49. Hong SR, Chong MS, Lee SB, Lee YM, Song KW, Park MH, Hong SH. Biocompatibility and biodegradation of cross-linked gelatin/hyaluronic acid sponge in rat subcutaneous tissue. *J Biomater Sci Polym Ed.* 2004; 15:201–214. [PubMed: 15109098]
50. Liu H, Mao J, Yao K, Yang G, Cui L, Cao Y. A study on a chitosan-gelatin-hyaluronic acid scaffold as artificial skin *in vitro* and its tissue engineering applications. *J Biomater Sci Polym Ed.* 2004; 15:25–40. [PubMed: 15027841]
51. Liu LS, Thompson AY, Heidaran MA, Poser JW, Spiro RC. An osteoconductive collagen/hyaluronate matrix for bone regeneration. *Biomaterials.* 1999; 20:1097–1108. [PubMed: 10382825]
52. Srivastava S, Gorham SD, French DA, Shivas AA, Courtney JM. *In vivo* evaluation and comparison of collagen, acetylated collagen and collagen/glycosaminoglycan composite films and sponges as candidate biomaterials. *Biomaterials.* 1990; 11:155–161. [PubMed: 2161687]
53. Zhang J, Senger B, Vautier D, Picart C, Schaaf P, Voegel JC, Lavalle P. Natural polyelectrolyte films based on layer-by layer deposition of collagen and hyaluronic acid. *Biomaterials.* 2005; 26:3353–3361. [PubMed: 15603831]
54. Segura T, Anderson BC, Chung PH, Webber RE, Shull KR, Shea LD. Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern. *Biomaterials.* 2005; 26:359–371. [PubMed: 15275810]
55. Taguchi T, Ikoma T, Tanaka J. An improved method to prepare hyaluronic acid and type II collagen composite matrices. *J Biomed Mater Res A.* 2002; 61:330–336.
56. Shu XZ, Ghosh K, Liu Y, Palumbo FS, Luo Y, Clark RA, Prestwich GD. Attachment and spreading of fibroblasts on an RGD peptide-modified injectable hyaluronan hydrogel. *J Biomed Mater Res A.* 2004; 68:365–375. [PubMed: 14704979]

57. Park SN, Park JC, Kim HO, Song MJ, Suh H. Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. *Biomaterials*. 2002; 23:1205–1212. [PubMed: 11791924]
58. Benedetti L, Cortivo R, Berti T, Berti A, Pea F, Mazzo M, Moras M, Abatangelo G. Biocompatibility and biodegradation of different hyaluronan derivatives (Hyaff) implanted in rats. *Biomaterials*. 1993; 14:1154–1160. [PubMed: 8130320]
59. Ma L, Gao C, Mao Z, Zhou J, Shen J. Enhanced biological stability of collagen porous scaffolds by using amino acids as novel cross-linking bridges. *Biomaterials*. 2004; 25:2997–3004. [PubMed: 14967532]
60. Osborne CS, Reid WH, Grant MH. Investigation into the biological stability of collagen/chondroitin-6-sulphate gels and their contraction by fibroblasts and keratinocytes: the effect of crosslinking agents and diamines. *Biomaterials*. 1999; 20:283–290. [PubMed: 10030605]
61. Takahashi K, Nakata Y, Someya K, Hattori M. Improvement of the physical properties of pepsin-solubilized elastin-collagen film by crosslinking. *Biosci Biotechnol Biochem*. 1999; 63:2144–2149. [PubMed: 10664847]
62. Campoccia D, Hunt JA, Doherty PJ, Zhong SP, O'Regan M, Benedetti L, Williams DF. Quantitative assessment of the tissue response to films of hyaluronan derivatives. *Biomaterials*. 1996; 17:963–975. [PubMed: 8736730]
63. Lin CQ, Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. *Faseb J*. 1993; 7:737–743. [PubMed: 8330681]
64. Giancotti FG, Ruoslahti E. Integrin signaling. *Science*. 1999; 285:1028–1032. [PubMed: 10446041]
65. Ruoslahti E. Stretching is good for a cell. *Science*. 1997; 276:1345–1346. [PubMed: 9190676]
66. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science*. 1987; 238:491–497. [PubMed: 2821619]
67. Lee JW, Qi WN, Scully SP. The involvement of beta1 integrin in the modulation by collagen of chondrocyte-response to transforming growth factor-beta1. *J Orthop Res*. 2002; 20:66–75. [PubMed: 11853092]

Figure 1. Biomechanical analysis of three-dimensional composite scaffolds: Using a heatable mold (A), the cylindrical specimens were fixed between two aluminum discs (B, C), and finally transferred into the grips of a mechanical tester (D), where the scaffolds were loaded with uniaxial tension (FM = force meter).

Figure 2. Scanning electron micrographs of polymer scaffolds (magnifications: A, C: 200-fold; B, D: 10,000-fold). No structural and ultrastructural differences were observed after adding of 30% gelatin to hyaluronan scaffolds (A, B) compared to 100% hyaluronan-based scaffolds (C, D). Primary pores are marked with an asterisk (*), regions of secondary pores with a circle (o).

Figure 3. Influence of the salt leaching procedure on the polymer composition and on the organic solvent content of composite scaffolds. Infrared spectroscopy (A) of composites (HY70%/G30%) before and after removal of the porogen. Note the loss of the C-F bands after washing indicating distinct reduction of the solvent HFIP in the polymer composite. Determination of the dissolved gelatin content by BCA-analysis (B). The values indicated represent mean $\pm$ SD, where $n = 4$.

Figure 4.

Adhesion and proliferation of human mesenchymal stem cells from bone marrow on composite films of different composition ratios of derivatized hyaluronan and gelatin. The results of the composite films were compared to polystyrene (PS). Cells were harvested and counted after 3 hours to determine cell adhesion (A) and after 1, 4 and 7 days in culture for cell proliferation (B). The values indicated represent mean $\pm$ SD, where $n = 8$.

Figure 5. Morphology of mesenchymal stem cells attached to films of the composition HY100%, HY95%/G5%, and HY70%/G30% after 1 hour, 2 hours, and 24 hours of 2-dimensional cell culture. In the upper three rows, cells were stained against CD44 expression and the lower row shows expression of β 1-integrin of stem cells cultured on HY70%/G30% composite films. Space bar represents 200 μ m.

Figure 6.

Attachment of mesenchymal stem cells (24 hours incubation) on pure hyaluronan-based (HY100%) and composite polymer films (HY70%/G30%). (A) Dark field microscopy after 24 hours of cell culture with (+ $\alpha 5\beta 1$ -Ab) and without (- $\alpha 5\beta 1$ -Ab) addition of $\alpha 5\beta 1$ -integrin blocking antibody. (B) Cell count of attached cells (after 24 hours) on pure hyaluronan-based (HY100%) and composite polymer films (HY70%/G30%) without further treatment, after collagenase treatment prior to cell seeding or cell seeding with $\alpha 5\beta 1$ -integrin blocking antibodies. The values indicated represent mean $\pm$ SD, where n = 5.

Figure 7.

Chondrogenic differentiation of mesenchymal stem cells. DNA-contents on day 1, 14 and 28 (A) and extracellular matrix deposition under chondrogenic culture condition on day 28 (B) of mesenchymal stem cells in hyaluronan-based (HY100%) and composite scaffolds (HY95%/G5%, HY70%/G30%). The values indicated represent mean $\pm$ SD, where at least $n = 3$. Immunohistochemical analysis of collagen type I and collagen type II expression after 28 days of chondrogenic cell culture in pure hyaluronan and hyaluronan-gelatin (HY70%/G30%) composite scaffolds (C).

Table 1

Physicochemical properties of polymer composites depending on composition ratios of derivatized hyaluronan and gelatin (HY100%, HY95%/G5HY70%/G30%) with and without the influence of the salt leaching procedure. Free amino groups were determined by the TNBS-method. The values indicated represent mean $\pm$ SD, where at least $n = 3$.

	IR peak ratio (ester/amide-II)	Water contact angle [°]	Free amino groups [μ mol/g]
Without washing			
HY100%	2.01 $\pm$ 0.04	51.5 $\pm$ 1.5	0
HY95%/G5%	1.78 $\pm$ 0.05	37.5 $\pm$ 3.0	27.8 $\pm$ 5.8
HY70%/G30%	1.21 $\pm$ 0.05	24.0 $\pm$ 1.5	111.9 $\pm$ 12.0
After salt leaching			
HY100%	1.96 $\pm$ 0.10	48.5 $\pm$ 1.5	0
HY95%/G5%	1.92 $\pm$ 0.03	39.5 $\pm$ 0.5	22.5 $\pm$ 8.5
HY70%/G30%	1.29 $\pm$ 0.03	35.5 $\pm$ 1.0	77.2 $\pm$ 5.0

Table 2

The mechanical properties of composite scaffolds with varying gelatin contents were obtained in wet conditions. The values indicated represent mean $\pm$ SD, with a sample size of $n = 6$. Statistical analysis was performed using SPSS 11.0G One way ANOVA with post hoc Bonferroni test. Significance was accepted with a level of $p < 0.05$ (see text).

	Rupture strength [kPa]	Max strain at rupture [%]	Max Young modulus [kPa]
HY100%	25.3 $\pm$ 8.6	36.2 $\pm$ 8.4	9.7 $\pm$ 2.2
HY95%/G5%	20.7 $\pm$ 5.1	43.7 $\pm$ 7.4	7.1 $\pm$ 1.6
HY70%/G30%	13.8 $\pm$ 7.3	38.9 $\pm$ 9.8	5.7 $\pm$ 2.8