New strategy for chemical modification of hyaluronic acid: Preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels

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Abstract: Biodegradable materials for spatially and temporally controlled delivery of bioactive agents such as drugs, growth factors, or cytokines are key to facilitating tissue repair. We have developed a versatile method for chemical crosslinking high-molecular-weight hyaluronic acid under physiological conditions yielding biocompatible and biodegradable hydrogels. The method is based on the introduction of functional groups onto hyaluronic acid by formation of an active ester at the carboxylate of the glucuronic acid moiety and subsequent substitution with a side chain containing a nucleophilic group on one end and a (protected) functional group on the other. We have formed hyaluronic acid with amino or aldehyde functionality, and subsequently hydrogels with these hyaluronic acid derivatives and bifunctional crosslinkers or mixtures of the hyaluronic acid derivatives carrying different functionalities using active ester- or aldehyde-mediated reactions. Size analysis of the hyaluronic acid derivatives showed that the chemical modification did not lead to fragmentation of the polysaccharide. Hydrogels formed with hyaluronic acid derivatized to a varying degree and crosslinked with low- or high-molecular-weight crosslinkers were evaluated for biodegradability by digestion with hyaluronidase and for biocompatibility and ectopic bone formation by subcutaneous implantation in rats. Several hydrogel formulations showed excellent cell infiltration and chondro-osseous differentiation when loaded with bone morphogenetic protein-2 (BMP-2). Synergistic action of insulin-like growth factor-1 with BMP-2 promoted cartilage formation in this model, while addition of transforming growth factor-β and BMP-2 led to rapid replacement of the matrix by bone. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 47, 152–169, 1999.

Key words: hyaluronic acid; crosslinking; tissue repair; cytokine delivery; bone formation

INTRODUCTION

In orthopedic surgery, defects in articular cartilage present a very complicated treatment problem because of the very limited capacity of cartilage to repair spontaneously. The failure of regenerating persistent hyaline cartilage by surgical procedures prompted investigators to attempt repair using biological strategies.[1] Repair has been induced by transplantation of culture-expanded autologous chondrocytes[2] or by recruitment of mesenchymal stem cells from the synovium using chemotactic and mitogenic factors.[3] The shortcoming of both strategies is the difficulty in stably anchoring the repair-inducing factors, whether cells or growth factors, within the lesion. Hyaluronic acid (HA) is a good candidate for the development of novel biomaterials for local delivery of cells and bioactive factors because of its unique physicochemical properties and its excellent biocompatibility and biodegradability.

Hyaluronic acid, or hyaluronan, is a natural polysaccharide that is most abundant in cartilage and in the vitreous.[4] HA plays a key structural role in the organization of the cartilage extracellular matrix as an organizing structure for the assembly of aggrecan, the large cartilage proteoglycan.[5] The highly negatively charged aggrecan/HA assemblies are largely responsible for the viscoelastic properties of cartilage by immobilizing water molecules. HA is unique among glycosaminoglycans with respect to not being covalently bound to a polypeptide. HA is also unique in having a relatively simple structure of repeating nonsulfated disaccharide units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine (Fig. 1). The molecular mass of HA in extracellular matrices is typically several million Daltons. Besides its structural function in extracellular matrix assembly, HA plays pivotal roles in

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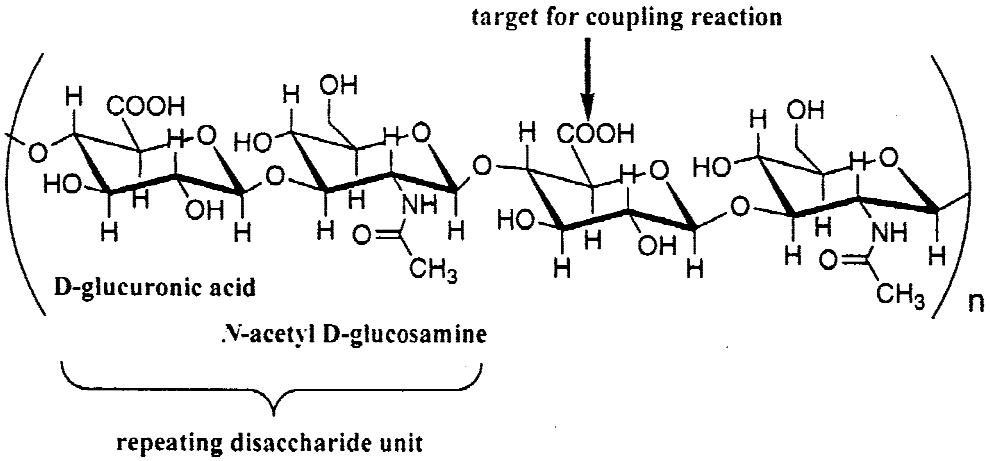


Figure 1. Structure of HA.

promoting cell motility and differentiation in development and wound healing.[4,6] Blocking the interaction of HA with cell-surface receptors, i.e., isoforms of CD44, in prechondrogenic micromass cultures from embryonic limb bud mesoderm inhibits chondrogenesis, indicating that the establishment and maintenance of a differentiated chondrocyte phenotype is at least in part dependent on HA and HA–receptor interactions.[7]

Based on its unique rheological properties, HA is currently being used clinically in viscosupplementation and viscosurgery.[4,8] Viscous solutions of high-molecular-weight HA and its salts are being used in therapy for arthropathies by intraarticular injection,[9] to promote wound healing in various tissues, and as a surgical aid in eye and middle-ear surgery. More recently, HA has been used in derivatized and/or crosslinked form to manufacture thin films which are used to create tissue separations.[10] Extensive efforts have been made by various laboratories to produce derivatives of HA with unique properties for specific biomedical applications. Most of the developments have been focusing on the production of materials such as films or sponges for implantation and the substitution of HA with therapeutic agents[11–13] for delayed release and/or prolonged effect. In fact, the half-life of pharmacological compounds has been shown to be drastically increased when delivered systemically as a HA conjugate.[11,12] Strategies for modification of HA have included esterification of HA,[14,15] acrylation of HA,[16] and crosslinking of HA using divinyl sulfone[17] or glycidyl ether.[18] However, the modified HA molecules show altered physical characteristics such as decreased solubility in water, and/or the chemical reaction strategies used are not designed for crosslinking of HA under physiological conditions, and thus cannot be used to polymerize a biodegradable matrix *in situ* which would be desirable in resurfacing of articular cartilage.

The introduction of functional groups on HA, e.g., amino groups, which could be used for further convenient coupling or crosslinking reactions under mild physiological conditions, is a subject of great interest. Previous methods have produced a free amino group on high-molecular-weight HA by alkaline *N*-deacetylation of its glucosamine moiety.[19,20] However, concomitant degradation of HA via β-elimination in the glucuronic acid moiety was observed under the harsh reaction conditions needed. An early report claimed that carbodiimide-catalyzed reaction of HA with glycine methyl ester, a monofunctional amine, led to the formation of an amide linkage.[21] This, however, has been proven in a number of studies not to be the case.[22,23] Under mildly acidic conditions the unstable intermediate *O*-acylisourea is readily formed, which in the absence of nucleophiles, rearranges by a cyclic electronic displacement to a stable *N*-acylurea[24] (Fig. 2). This O → N migration of the *O*-acylisourea also occurs when the nucleophile is a primary amine[23] and any amide formation that does occur is insignificant, as reported by Ogamo et al.[22] The carbodiimide-mediated introduction of a terminal hydrazido group on HA with a variable spacer has recently been achieved and has led to the ability to conduct further coupling and crosslinking reactions.[13,25]

In this study, we have developed methodology for introducing side chains into HA by carbodiimide-mediated coupling of primary or secondary amines to the carboxyl group of the glucuronic acid moiety using an active ester intermediate. We demonstrate that by "rescuing" the active *O*-acylisourea by formation of a more hydrolysis-resistant and nonrearrangable active ester intermediate, the coupling of primary amines to HA is possible. Using this methodology, we have generated HA derivatives carrying different functional groups for subsequent crosslinking to form hydrogels. Characterization of these hydrogels in different biological assays established that novel biocom-

patible and biodegradable materials are formed with these HA derivatives.

MATERIALS AND METHODS

Synthesis of HA derivatives

General procedure of carbodiimide/HOBt reactions with HA

Sodium hyaluronate (100 mg, 0.25 mmol; MW > 1×10^6; Genzyme Pharmaceuticals, Haverhill, UK) was dissolved in H_2O at a concentration of 3 mg/mL. To this solution was added a 30-fold molar excess of an amine or hydrazide (pK_a 3–8.5; 7.5 mmol), i.e., ethylenediamine, adipic dihydrazide. The pH of the reaction mixture was adjusted to 6.8 with 0.1 *M* NaOH/0.1*M* HCl. 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) (192 mg, 1 mmol; Aldrich Chemical Co.) and 1-hydroxybenzotriazole (HOBt) (135 mg, 1 mmol; Fluka Chemical Corp.) was dissolved in dimethylsulfoxide (DMSO)/H_2O (1:1, 1 mL). After mixing, the pH of the reaction was maintained at 6.8 by the addition of 0.1*M* NaOH and the reaction was allowed to proceed overnight. The pH was subsequently adjusted to 7.0 with 0.1*M* NaOH and the derivatized HA exhaustively dialyzed (Spectra/Por RC 2,

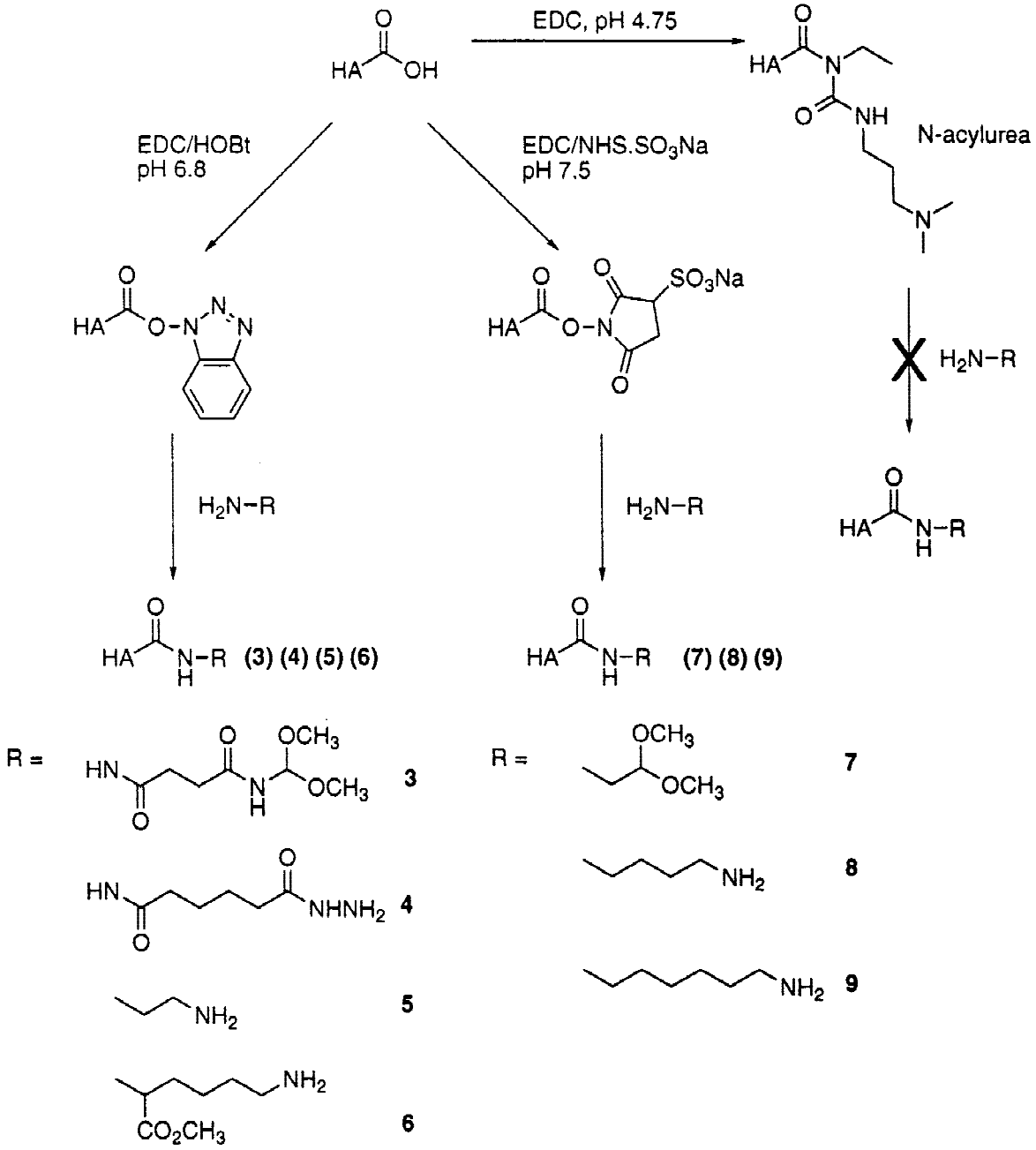


Figure 2. Strategy for coupling of different amines to HA. Generated aldehyde- and amine-functionalized HA derivatives are listed.

MW cutoff 12–14,000; Fisher Scientific) against distilled H_2O. NaCl was added to produce a 5% w/v solution and the modified HA was precipitated by addition of 3 vol equivalents of ethanol. The precipitate was redissolved in H_2O at a concentration of approximately 5 mg/mL and the purified product was freeze-dried and kept at 4°C under N_2. The yield of product was typically ~80%.

Preparation of dimethyl acetal hydrazido-HA (3)

Preparation of N-*(2,2-dimethoxyethyl)-4-(methoxycarbonyl) butanamide* **(1).** EDC (4.98 g, 0.026 mol) was added to a mixture of aminoacetaldehyde dimethyl acetal (2.18 mL, 20 mmol; Aldrich) and methyl monoester of succinic acid (2.64 g, 20 mmol; Aldrich) in 75 mL of dichloromethane, and the reaction mixture was stirred for 24 h at room temperature. The solution was extracted successively with 50 mL each of ice-cold solutions of 0.75 *M* sulfuric acid, 1*M* NaCl, saturated sodium bicarbonate, and 1*M* NaCl. The organic phase was collected and dried with sodium sulfate. The solvent was evaporated under reduced pressure yielding a syrup which showed a single spot on charring upon thin-layer chromatography (TLC) in solvent A (R_f 0.75) and solvent B (R_f 0.24) (see Characterization: Analytical Methods). The apparent yield of **1** was 65% and the ^{1}H-NMR spectra in deuterated chloroform showed the following peaks: δ 5.70 (bs, 1H, N*H*), 4.34 (t, 1H, C*H*-(OCH_3)), 3.67 (s, 3H, $COOCH_3$), 3.43–3.35 (s and t, 8H, CH_3OC and $CHCH_2NH$), 2.38–2.26 (m, 4H, CH_2CO).

Formation of N-*(2,2-dimethoxyethyl)-4-(hydrazido)butanamide* **(2)** *from* **1.** Anhydrous hydrazine (248 μL, 7.9 mmol; Aldrich) was added to a solution of **1** (1.73 g, 7.9 mmol) in 5 mL of anhydrous methanol. The mixture was stirred at room temperature overnight and the solvent subsequently evaporated under reduced pressure, yielding a solid residue. The residue was dissolved in H_2O (6 mL) and extracted three times with an equal volume of dichloromethane. The aqueous solution was evaporated to dryness under reduced pressure and then further dried overnight *in vacuo.* The crystalline solid (1.04 g, 82% yield) was homogeneous on TLC in solvent A (R_f 0.10) when visualized by charring. The ^{1}H-NMR spectrum indicated the loss of the ester methoxy group when compared to **1.**

Sodium hyaluronate was reacted with acyl hydrazide **(2)** using HOBt and EDC as described above, yielding **3** with an apparent degree of modification of ~65%. The ^{1}H-NMR spectra in deuterated water [Fig. 3(B)] showed peaks at δ 3.28 (s, 6H, $CH(OCH_3)_2$), 2.50 (bs, 2H, $CO \cdot CH_2$), 1.9 (bs, 3H, HA-$NHCO \cdot CH_3$), 1.82 (bs, 2H, $CH_2 \cdot CO$).

Preparation of adipic dihydrazide-HA (4)

Sodium hyaluronate was reacted with adipic dihydrazide (1.31 g; Aldrich) using HOBt and EDC as described above. The apparent degree of modification was ~65%: ^{1}H-NMR (D_2O) δ 2.20 (m, 2H, $NHNHCO \cdot CH_2$), 2.10 (m, 2H, CH_2NHNH_2), 1.35–1.55 (m, 4H, CH_2CH_2).

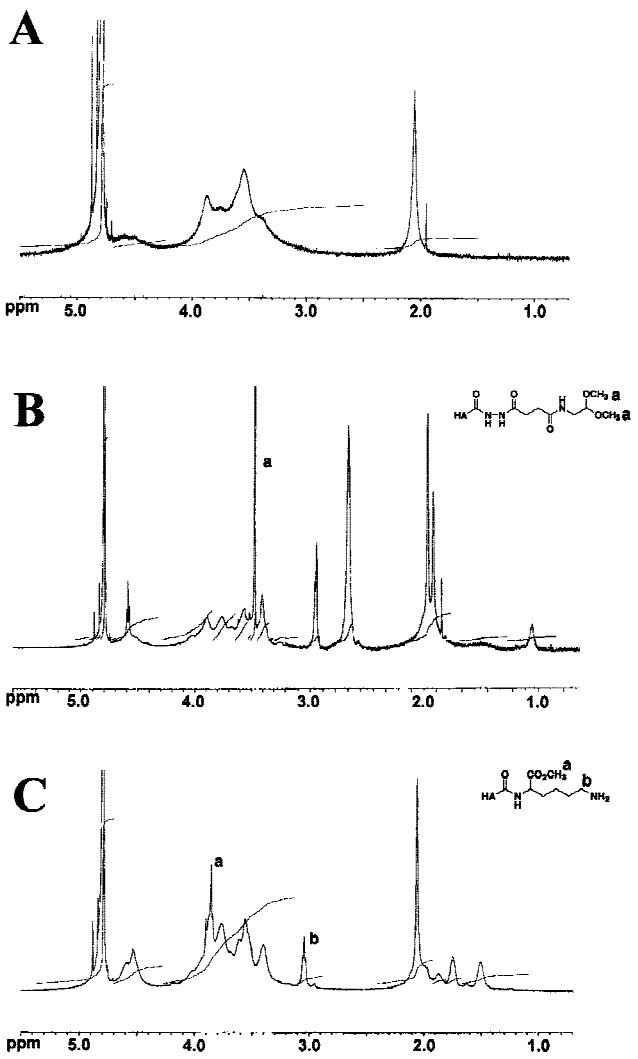


Figure 3. ^{1}H-NMR of native and modified HA in D_2O at 270 Mhz: (A) native HA, (B) *N*-(2,2-dimethoxyethyl)-4-(hydrazido)butanamide-modified HA **(3),** and (C) lysine methyl ester-modified HA **(6).** Peaks are assigned as indicated on the structural formula and detailed in Materials and Methods. ^{1}H-NMR results for other HA derivatives are provided in Materials and Methods.

Preparation of 2-aminoethyl-HA (5).

Sodium hyaluronate was reacted with ethylenediamine dihydrochloride (998 mg; Aldrich) using HOBt and EDC as described above. The apparent degree of modification was ~25%: ^{1}H-NMR (D_2O) δ 3.07 (t, 2H, $CO \cdot NHCH_2$), 2.65 (bs, 2H, CH_2NH_2), 1.9 (bs, 3H, HA-$NHCO \cdot CH_3$).

Preparation of lysine methyl ester-HA (6).

Sodium hyaluronate was reacted with lysine methyl ester (1.748 g; Sigma, St. Louis, MO) using HOBt and EDC as described above. The apparent degree of modification was ~25%: ^{1}H-NMR (D_2O) δ 3.85 (s, 3H, CO_2CH_3), 3.0 (t, 2H, CH_2NH_2), 2.1 (bs, 3H, HA-$NHCO \cdot CH_3$), 2.0–1.83 (dm, 2H,

NHCHCH_2CH$_2$), 1.81–1.70 (m, 2H, CH_2CH$_2$), 1.52–1.43 (m, 2H, CH$_2$CH_2CH$_2$) [Fig. 3(C)].

General procedure of carbodiimide/sulfo-NHS reactions with HA

To an aqueous solution of sodium hyaluronate (3 mg/mL) was added a 30-fold molar excess of an amine (pK_a > 8.5; 7.5 mmol), i.e., 1,4-diaminobutane. The pH of the reaction mixture was adjusted to 7.5 with 0.1*M* NaOH/0.1*M* HCl. EDC (192 mg, 1 mmol) and *N*-hydroxysulfosuccinimide (sulfo-NHS) (217 mg, 1 mmol; Fluka) were dissolved in H_2O (1 mL). After mixing, the pH of the reaction was maintained at 7.5 by the addition of 0.1*M* NaOH and the reaction was allowed to proceed overnight. The HA derivatives were purified and stored as described above.

Preparation of aminoacetaldehyde dimethyl acetal-HA **(7)**

Sodium hyaluronate was reacted with aminoacetaldehyde dimethyl acetal (781 mg) using sulfo-NHS and EDC as described above. The apparent degree of modification was ~10%: ^{1}H-NMR (D_2O) δ 3.30 (s, 6H, CH(OCH_3)$_2$), 1.9 (bs, 3H, HA-NHCO · CH_3).

Preparation of 4-aminobutyl-HA **(8)**

Sodium hyaluronate was reacted with 1,4-diaminobutane dihydrochloride (1.21 g; Aldrich) using sulfo-NHS and EDC as described above. The apparent degree of modification was ~10%: ^{1}H-NMR (D_2O) δ 2.87 (m, 4H, CO · NHCH_2, CH_2NH$_2$), 1.9 (bs, 3H, HA-NHCO · CH_3), 1.55–1.35 (dm, 4H, CH_2CH_2).

Preparation of 6-aminohexyl-HA **(9)**

Sodium hyaluronate was reacted with 1,6-diaminohexane dihydrochloride (1.42 g; Aldrich) using sulfo-NHS and EDC as described above. The apparent degree of modification was ~10%: ^{1}H-NMR (D_2O) δ 2.9 (m, 4H, CO · NHCH_2, CH_2NH$_2$), 1.9 (bs, 3H, HA-NHCO · CH_3), 1.6–1.1 (m, 6H, CH_2CH_2CH_2).

Deprotection of HA-acetals to form HA-aldehydes

The acetal modified HA was dissolved in H_2O at a concentration of 5–10 mg/mL and 1*M* HCl was added to give a final concentration of 0.025*M* HCl. The solution was then allowed to stand at room temperature for 0.5–1.0 h. The solution was neutralized by the addition of 1*M* NaOH, yielding the deprotected HA-aldehyde.

Preparation of polyaldehyde by oxidation of HA

Sodium hyaluronate (300 mg, 0.75 mmol) was dissolved in H_2O at a concentration of 20 mg/mL. Sodium periodate (160 mg, 0.75 mmol; Aldrich) was added and the reaction allowed to proceed for 2 h at room temperature. Low-molecular-weight reaction products were removed by exhaustive dialysis (Spectra/Por RC 2) and the polyaldehyde polymer solution was saturated with nitrogen by bubbling and stored at 4°C.

Characterization of HA derivatives

Analytical methods

Thin-layer chromatography was done with silica gel 60 F_{254} (Merck). The solvent systems used were (a) 4:1 (v/v) ethyl acetate:acetone; and (b) 3:1 (v/v) toluene:methanol. The ^{1}H-NMR spectra were obtained with a Jeol E-270 instrument at 270 MHz. For NMR spectroscopy, HA was dissolved in D_2O/NaOD, pH 14, at a concentration of 1–2 mg/mL. Compound *x* admixed with HA served as a control for HA modified with compound *x*.

Size distribution

Preparations of HA or chondroitin sulfate (type A from bovine trachea, MW ~45,000; Sigma) were dissolved in PBS at a concentration of 1 mg/mL and 1 mL applied on a Superose 6 FPLC column (HR 10/30; Pharmacia) equilibrated in PBS (5.6 m*M* Na_2HPO_4, 1.06 m*M* KH_2PO_4, pH 7.4, 154 m*M* NaCl). The flow rate was 0.25 mL/min and 0.5-mL fractions were collected and the absorbance at 205 nm recorded. Elution profiles of different forms of HA under conditions minimizing HA–HA interactions (50 m*M* Na_2HPO_4, pH 11, 500 m*M* NaCl) were identical. *N*-Deacetylated HA was prepared according to Dahl et al.[20] Briefly, high-molecular-weight (MW > 10^6) HA (10 mg, 0.025 mmol) and hydrazine sulfate (5 mg, 0.038 mmol) were mixed with anhydrous hydrazine (0.6 mL) in a glass tube. The tube was sealed and heated to 100°C for time intervals ranging from 0 to 180 min. After rapid cooling on ice, 0.75 mL of toluene was added and the mixture evaporated to dryness under reduced pressure. HA preparations were digested with 100 U/mL testicular hyaluronidase for 2 h.

Formation of HA hydrogels

General procedure for crosslinking of HA derivatives

Functionalized HA was dissolved by agitation at room temperature in PBS, pH 7.4–8.5, at a concentration of up to 20 mg/mL to study the formation of hydrogels at different

HA concentrations, and at a concentration of 13–15 mg/mL for subsequent experiments. The degree of modification of the HA derivative was derived from the integration of the ^{1}H-NMR peaks. After complete dissolution, the HA solution was transferred into a 1-mL syringe. When reacting HA with low-MW crosslinkers, a slight excess of the compound (1.1 molar equivalent of functional groups) was used in a second 1-mL syringe. Polyethylene glycol bis(succinimidyl propionate) ($(SPA)_2$-PEG) (MW ~3400) and succinimide ester of carboxymethylated four arm polyethylene glycol ($(SC)_4$-PEG) (MW ~20,000) were purchased from Shearwater Polymers (Huntsville, AL), 3,3′-dithiobis (sulfosuccinimidyl propionate) (DTSSP) from Pierce Chemical Co., and glutaraldehyde from Fluka. The solution was freshly prepared in PBS at 1/10 of the HA volume. The syringes were connected while paying special attention to exclude air; the contents were rapidly mixed with 20 passages and allowed to gel. When reacting HA molecules with different functionalities, 0.5–1 equivalent of HA-aldehyde was mixed with 1 equivalent of HA-hydrazide, depending on the degree of modification of the HA derivatives. At room temperature, gelation occurred within 30 s to several minutes, depending on the formulation, and the gel properties did not significantly change after approximately 5 min.

In vitro and *in vivo* testing of HA hydrogels

Hyaluronidase digestion

Hyaluronic acid hydrogels were formed as detailed above in 1-mL syringes. The syringes containing the crosslinked gels were incubated at 37°C for 1 h to ensure the crosslinking reaction was complete, after which identical ~100-μL cylindrical gels were formed by cutting the syringes with a razor blade and extruding the gels. The gels were incubated at 37°C with different concentrations of bovine testicular hyaluronidase (Sigma), 50–5000 U/mL, in 400 μL of 30 m*M* citric acid, 150 m*M* Na_2HPO_4, pH 6.3, containing 150 m*M* NaCl, for the indicated time (0–48 h). Degradation of the gels was determined from the release of glucuronic acid into the supernatant as measured by the carbazole assay.[26]

Rat subcutaneous implantation

Biomaterials were implanted in 4- to 5-week-old male Sprague-Dawley rats. Briefly, a small vertical incision was made on either side of the xiphoid cartilage of the sternum, and the skin undermined with a blunt instrument to separate the skin from the underlying tissue. Biomaterial discs (10 mm in diameter, 3 mm width) were placed into these pockets and the skin incisions closed with sutures. For each test group, four to six biomaterial specimens were implanted. Animal experiments were performed according to NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, Rev. 1985).

Hyaluronic acid hydrogel discs were prepared by crosslinking HA derivatives (12–14 mg/mL) in PBS in 3-mL syringes as described above. The HA solution was supplemented with cell adhesion molecules such as collagen type I fibrils (1 mg/mL) or fibronectin (500 μg/mL) prior to crosslinking when desired to promote cell infiltration. Collagen fibrils were prepared by polymerization from dilute solution (2–3 mg/mL) of acid-solubilized intact bovine collagen type I (retaining telopeptides; Organogenesis, Canton, MA) in PBS and harvested by centrifugation, following standard protocols.[27] For induction of chondro-osseous differentiation, different growth factors including bone morphogenetic protein-2 (BMP-2) (200 μg/mL; Genetics Institute, Cambridge, MA), insulin-like growth factor-1 (IGF-1) (500 ng/mL; Celtrix Pharmaceuticals, Santa Clara, CA), and transforming growth factor-β2 (TGF-β2) (50 ng/mL; Celtrix) were mixed with the HA solution just prior to crosslinking. For inhibition of vascularization, 10 mg/mL suramin (Sigma) was added.

At the indicated time postoperatively, the animals were euthanized, and the implants were exposed through a dorsal midline skin incision and excised with the attached surrounding tissue. The tissue was fixed in 10% formalin in PBS, dehydrated through a graded ethanol series, and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin/eosin and Safranin-O/Fast green.

RESULTS AND DISCUSSION

We focused on the production of hydrogels from HA, since HA matches several of the desired properties for a biomaterial for delivery of bioactive agents such as cells, growth factors, cytokines, and drugs for tissue repair. It is biodegradable, provides an excellent substratum for cell migration, and, most important, has proven its biocompatibility in various forms in clinical practice (for discussion, see Balazs and Laurent.[8]) The relatively simple repetitive structure of HA allows also for specific modification and introduction of a large number of functional groups for subsequent crosslinking (Fig. 1).

Preparation of HA derivatives

Initial studies

It is well known that sugars can be oxidized using periodate,[28] and initially, we followed standard procedures to generate polyaldehydes from HA by periodate oxidation. Periodate treatment oxidizes the proximal hydroxyl groups (at C2 and C3 carbons of glucuronic acid moiety) to aldehydes, thereby opening the sugar ring to form a linear chain. While periodate oxidation allows for the formation of a large number of functional groups, the disadvantage is the loss of the native backbone structure. Consequently, the generated derivative is presumably not recognized as HA by cells. Indeed, hydrogels formed by using periodate-oxidized HA as a crosslinker, e.g., in combination with the HA-amine derivatives described below, showed very limited tissue transformation and poor cellular infiltration in the rat ectopic bone formation assay (see

below) (Table I). Clearly, periodate oxidation of HA was not suitable and our goal had to be to generate an activated form of HA that differs minimally from native HA to conserve its unique physicochemical properties. Also, a minimal change affecting only a relatively small number of dissaccharide units would presumably not alter its property to serve as a cell substratum, since short HA oligosaccharides are recognized by HA receptors.[6] Since deacetylation of the *N*-acetylglucosamine moiety leads to concurrent degradation of the HA chain[19,20] [Fig. 4(A)], our efforts focused on the carboxylate group of the glucuronic acid moiety (Fig. 1). Initially, attempts were made to generate an aldehyde derivative by reduction of the carboxyl groups on the glucuronic acid residues using 9-borabicyclo-3,3-nonane, a method that allows direct conversion of the carboxylic acid into an aldehyde.[29] However, this avenue of investigation was unsuccessful, and we decided to introduce an aldehyde onto HA by means of a side chain using carbodiimide-mediated coupling.

Introduction of a (functionalized) side chain onto HA

Direct carbodiimide-mediated coupling of amines to the carboxyl group of HA in an aqueous environment, e.g., with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), did not yield the predicted product since the *O*-acyl isourea that is formed as a reactive intermediate rearranges rapidly to a stable *N*-acyl urea (Fig. 2). This results in predominant coupling of EDC (>99%) as indicated by the ^{1}H-NMR spectrum which showed the characteristic sharp singlet at $\delta = 2.08$ corresponding to the *N*-methyl protons and the methylene protons of the *N*-ethyl methyl group at $\delta = 1.0$, which is consistent with previous reports.[22,23] We found that the coupling of primary amines to HA is possible via the formation of a more hydrolysis resistant and non-rearrangable active ester intermediate (Fig. 2). We formed active esters of HA with 1-hydroxybenzotriazole (HOBt) or *N*-hydroxysulfosuccinimide (Sulfo-NHS) using the water-soluble carbodiimide EDC for coupling. Nucleophilic addition to the ester formed from HOBt requires the amine to be presented in an unprotonated form at acidic pH (about 5.5–7.0). Only a limited number of amines including hydrazides and "activated" amines, e.g., ethylene diamine, have pK_a values in a suitable range and are consequently relatively unprotonated and reactive with the ester-intermediate formed with HOBt (Fig. 2: **3–6**). Simple primary amines, e.g., putrescine, which have typically pK_a values > 9 do not form significant amounts of adduct under acidic coupling conditions. The Sulfo-NHS-derived intermediate allows for the coupling reaction to be carried out at neutral or slightly basic pH (about 7.0–8.5) and consequently yields products by reaction with simple primary amines (Fig. 2: **7–9**).

Preparation of HA-aldehyde derivatives

A heterobifunctional side chain, *N*-(2,2-dimethoxyethyl)-4-(hydrazido) butanamide **(2),** containing a protected aldehyde in the form of an acetal and an acyl hydrazide for coupling was synthesized using stan-

TABLE I
Evaluation of Biological Properties of Different HA Hydrogels in Rat Subcutaneous Implantation Model

Group	HA Derivative	Crosslinker	Granulomatous Inflammation*	Cell Infiltration†	Chondro-osseous Differentiation‡
1	~25% AD-HA	Glutaraldehyde	+++	−/+	−
2	~25% AD-HA	DTSSP	+	−	−
3	~25% AD-HA	$(SPA)_2$-PEG	+	++	+++
4	~65% AD-HA	$(SPA)_2$-PEG	+	+	+/++
5	~25% AD-HA	Periodate-oxidized dextran	+++	+	−
6	~25% AD-HA	Periodate-oxidized HA	+	+/++	+/++
7	~25% AD-HA	~10% aminoacetaldehyde dimethyl acetal-modified HA	++	+++	+
8	~25% AD-HA	~65% *N*-(2,2-dimethoxyethyl)-4-(hydrazido)butanamide-modified HA	+/++	++	++
9	~25% LME-HA	$(SPA)_2$-PEG	+/++	+++	++

HA hydrogels were formed by crosslinking 12 mg/mL of the HA-amine derivatives (adipic dihydrazide-[AD-HA] or lysine methylester-[LME-HA] modified HA) with a slight excess of crosslinker (groups 1–4 and 9) or by mixing polysaccharide derivatives with different modifications such as to yield the same final polysaccharide concentration (groups 5–8), as described in Materials and Methods. Hydrogels were supplemented with prefibrillized collagen type I and BMP-2 to stimulate endochondral bone formation (see Table II).

*+ = mild inflammation with mononuclear cells; ++ = moderate with occasional foreign-body giant cells; +++ = severe with abundant presence of foreign-body giant cells.

†+ = <10% of implant infiltrated; ++ = >30%; +++ = >80%.

‡+ = occasional foci of cartilage/bone; ++ = thin layer surrounding implant; +++ = >30% of implant replaced.

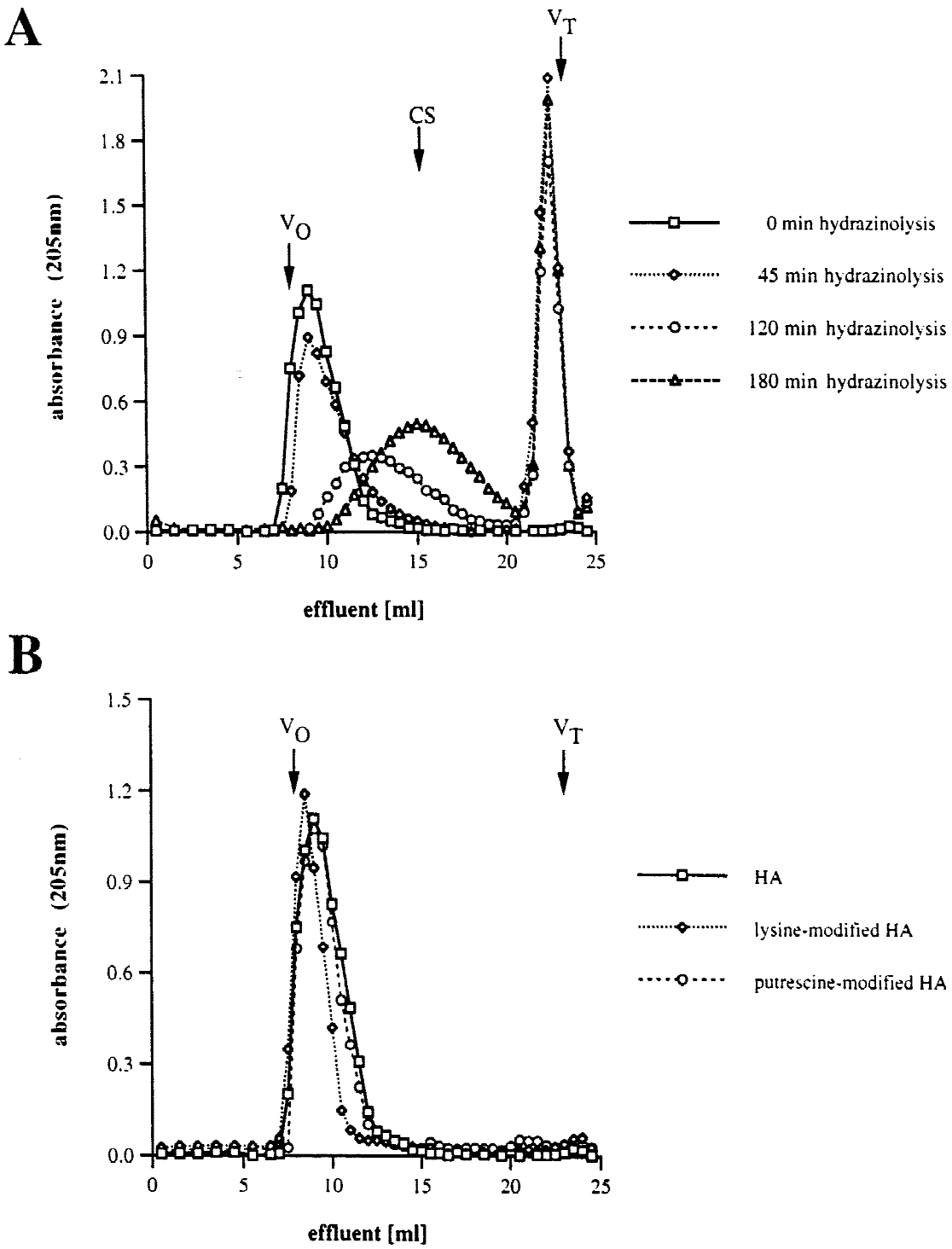
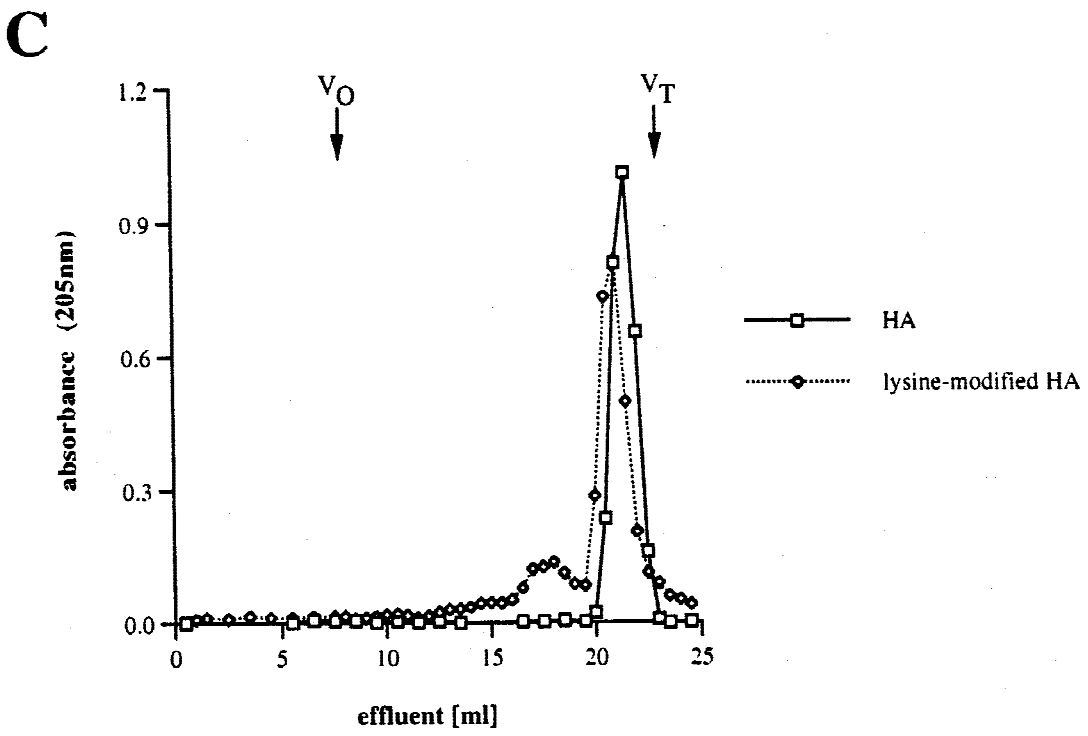


Figure 4. Size distribution of derivatized HA. HA preparations were separated on a Superose 6 FPLC column in PBS and the absorbance at 205 nm was recorded. (A) HA (MW > 10^6) was *N*-deacetylated by hydrazinolyses for the indicated time (0–180 min). (B) HA was derivatized with lysine methyl ester **(6)** or putrescine **(8)** via a benzotriazole or sulfosuccinimide ester intermediate, respectively. (C) HA and HA derivatives were digested with 100 U/mL of testicular hyaluronidase for 2 h (see next page). The void volume (v_0) and total volume (v_T) of the column are indicated by arrows. The elution of chondroitin sulfate (CS) with a molecular mass of ~45 kDa is indicated in (A).

dard chemistry as detailed in Materials and Methods. The side chain was coupled to HA as outlined in Figure 2 **(3).** High-molecular-weight HA (MW > 10^6) was dissolved in water, and a 20-fold molar excess of the side chain was added to ensure efficient coupling. Next, a fourfold molar excess of HOBt was added and the pH adjusted to 6.8. Finally, a fourfold molar excess of EDC was added to the reaction mixture, which re-



sulted in a slowly progressing decrease in pH. The pH was maintained at 6.8 for at least 4 h by the addition of 0.1*M* NaOH and the reaction was allowed to proceed overnight. The progressing slight decrease in pH presumably reflects the consumption of the basic primary amine over time since the reaction does not result in a net change in protons. This is in sharp contrast to the rapid pH increase observed when HA is reacted with EDC alone, which has been attributed to the net consumption of a proton,[23] and suggests that the reaction proceeds through the active ester intermediate.

A side chain with protected aldehyde functionality on one end and a primary amine for coupling on the other end, aminoacetaldehyde dimethyl acetal, was incorporated into HA in a similar manner (Fig. 2: **7**). HOBt was replaced with a fourfold molar excess of Sulfo-NHS and the pH was adjusted to 7.5. Upon addition of EDC, a similar drop in pH was observed. The pH was maintained at 7.5 and the reaction was allowed to proceed overnight.

The HA derivatives were purified by exhaustive dialysis against water followed by repeated ethanol precipitation to remove unreacted amine and other small reaction products. ^{1}H-NMR confirmed that the modification was successful, with the characteristic appearance of the acetal protons at δ = 3.30 [Fig. 3(B)]. The HA-acetal derivatives were easily activated to the reactive aldehydes by mild acid treatment, and when mixed with dihydrazides or dihydrazide-modified HA (see below) produced hydrogels, further confirming that successful modification had been achieved.

Preparation of HA-amine derivatives

Introduction of homobifunctional amines, i.e., ethylene diamine, lysine methyl ester, histidine methyl ester, and adipic, succinic, or suberic dihydrazide, which contain at least one amino group with a pK_a < 8.0, onto HA was conducted in a similar manner using HOBt and EDC (Fig. 2: **4–6**). The degree of modification could be controlled between ~10% and 25% for amines and ~10% and 70% for hydrazides by adjusting the molar equivalency ratio of HA:EDC from 1:0.5 to 1:4. The HA derivatives were purified as described above and analyzed by ^{1}H-NMR. The spectra revealed that modification was successful. For example, the HA modified with lysine methyl ester showed the characteristic singlet at δ = 3.85 corresponding to the methyl protons of the methyl ester group and the signal at δ = 3.0 corresponded to the methylene protons adjacent to the amino group [Fig. 3(C)]. The remaining methylene protons appeared between δ = 1.4 and 2.0, and showed the expected characteristic splitting patterns. Homobifunctional amines having amino groups with pK_a > 9.0, i.e., 1,4-diaminobutane (putrescine) and 1,6-diaminohexane, were coupled to HA using Sulfo-NHS and EDC (Fig. 2: **8,9**). In this case, the degree of modification with a fourfold excess of EDC was ~10% as assessed by ^{1}H-NMR. The ability of these HA derivatives **(4–6,8,9)** to form hydrogels with amino group specific crosslinkers such as aldehydes and NHS-esters further confirmed the modification of HA with amino groups.

Molecular weight of modified HA

It has been shown that low-molecular-weight fragments of HA, but not the ubiquitous high-molecular-weight form of HA found in the extracellular matrix, is capable of eliciting inflammatory reactions.[30–32] Thus, it is of great concern that the method used for modification of HA does not lead to cleavage of the HA chain and to the production of low-molecular-weight HA fragments. Because of the large size of high-molecular-weight HA and the heterogeneity in size of HA in high-molecular-weight HA preparations, accurate size determination is difficult. We decided to analyze the different HA species by molecular sieve chromatography since the more recently developed electrophoretic methods could not be used because of the changed charge density of derivatized HA species. HA species were analyzed on a Superose 6 FPLC column which has been shown to separate HA and other glycosaminoglycans up to a size of about 2.5×10^5 Da.[33] While high-molecular-weight HA elutes in the void volume of the column, degradation products are resolved. This is exemplified by the cleavage of HA occurring by *N*-deacetylation using hydrazinolysis and the concomittant reduction in size of the generated HA fragments over time [Fig. 4(A)]. The HA derivatives generated by functionalization using either HOBt and EDC or Sulfo-NHS and EDC eluted in the void volume of the column and no cleavage products were apparent, indicating that the size of HA was unchanged [Fig. 4(B)]. In contrast, treatment of native or derivatized HA with testicular hyaluronidase produced HA fragments, presumably tetra-saccharides, that eluted near the total volume of the column demonstrating the sensitivity of the HA species to enzymatic degradation [Fig. 4(C)]. The presence of a variable amount of larger fragments in the digest of the derivatized HA, depending on the degree of modification, which are resistant to further cleavage [Fig. 4(C)], indicates that the derivatization prevents interaction with the enyzme and consequently abolishes cleavage proximal to the sites of derivatization.

Formation and characterization of HA hydrogels

Crosslinking of functionalized HA

The functionalized HA molecules can be crosslinked by reacting HA derivatives with different functionalities or using homo- or heterobifunctional crosslinkers which are commercially available in large variety. Since the generated HA derivatives were very soluble in water, hydrogel formation could be pursued under physiological conditions, i.e., in PBS at pH 7.4–8.5. Crosslinking of the HA derivatives yielded hydrogels with physicochemical properties ranging from a soft pliable material to a tough resilient material. The properties of the hydrogels were dependent on the concentration of the HA derivative, the degree of modification of the HA derivative and, concomittantly, the crosslinking density of the material, and the size of the introduced cross-bridge between the HA molecules. HA-amine derivatives modified to an extent of ~10–70% were crosslinked with a small bifunctional active ester, DTSSP (MW ~600), a large bifunctional active ester, $(SPA)_2$-PEG (MW ~3400), a very large multivalent active ester, $(SC)_4$-PEG (MW ~20,000), and with a small bifunctional aldehyde, glutaraldehyde (Fig. 5). A slight excess of crosslinker functionality over HA functionality was used to maximize crosslinking, but at the same time not generating extensive monovalent crosslinker conjugates (molar ratio of functional groups was 1:1.1, HA derivative:crosslinker). Stable gels could be formed by crosslinking 5–20 mg/mL HA derivative. The pH under which crosslinking occurs depends on the nature of the amine HA is functionalized with. Since over 99% of the hydrazide amino groups (pK_a ~ 2–3) are present as unprotonated nucleophiles at pH 5.0 in contrast to primary amines (pK_a > 8.5), hydrazide functionalized HA **4** could be crosslinked at pH > 5.0, whereas primary amine functionalized HA **5,6,8,9** could be crosslinked efficiently only at pH > 8.0. Gels with good physical properties were generated by crosslinking 10–15 mg/mL HA derivative, modified to a degree of at least 10%. While large changes in the macroscopic physical properties of the formed gels were noted when the degree of modification was varied from ~10% to 70%, i.e., for HA functionalized with adipic dihydrazide **(4),** gels formed from the HA derivative at a given degree of modification with different crosslinkers gave only minimal variations in the gel properties. The nature of the modification, ethylenediamine **(5),** putrescine **(8),** lysine methyl ester **(6),** adipic dihydrazide **(4),** etc., had also no apparent effect on the macroscopic gel properties. Crosslinking of the HA-aldehyde derivatives **3,7** with bifunctional amines, e.g., adipic dihydrazide, produced hydrogels similar to the ones obtained with HA-amine derivatives. Conjugated amines such as hydrazides or benzylamines were required for polymerization of HA in this case to resonance stabilize the unstable Schiff base product formed by reaction of the aldehyde with the primary amine (i.e., hydrazides yield a more stable hydrazone linkage). Crosslinking with simple primary amines such as putrescine did not produce stable hydrogels. After initial gelation, the gels dissolved and a precipitate formed, suggesting that aldol condensation of initially generated Schiff bases took place. Inclusion of reducing agents such as sodium borohydride or pyridine borane in the reaction did not sig-

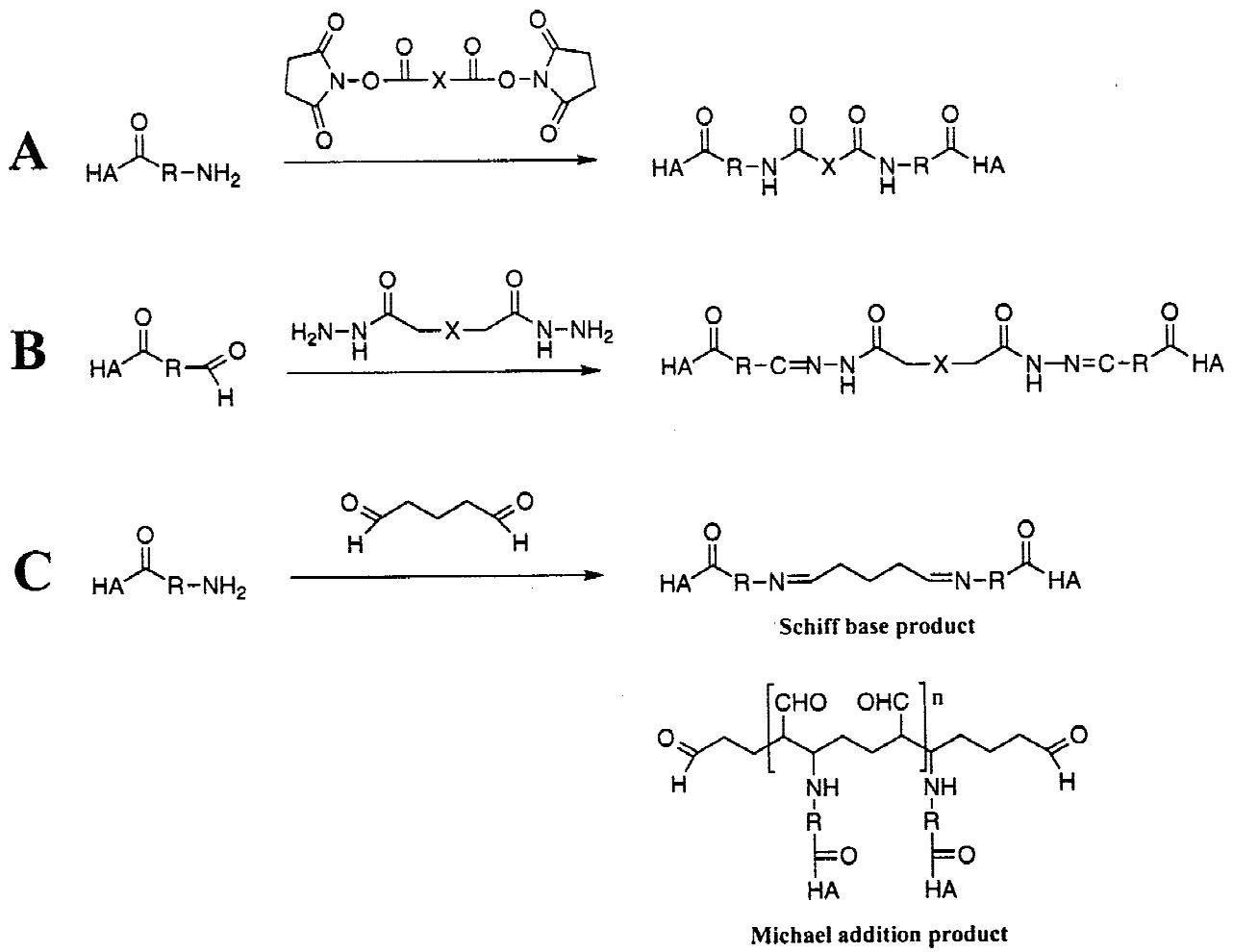


Figure 5. Crosslinking of HA derivatives to form hydrogels: (A) HA-amine derivatives **4–6,8,9** with NHS-esters, (B) HA-aldehydes **3,7** with hydrazides including **4,** and (C) HA-amine derivatives **5,6,8,9** with glutaraldehyde. In addition to the conventional reaction of aldehydes with amines that results in the formation of a Schiff base, glutaraldehyde is also known to undergo polymerization by aldol condensation yielding polymers with α,β-unsaturated aldehydes at neutral or slightly alkaline pH.[48] Subsequent, nucleophilic addition of amines at the ethylenyl double bond creates a stable crosslink [(C), Michael addition].

nificantly alter the results. Hydrogels were also obtained by mixing an equimolar amount of an HA-aldehyde derivative **3,7** with an HA-hydrazide derivative **4.** Gels with good physical qualities [i.e., comparable to 12 mg/mL HA-amine derivative crosslinked with $(SPA)_2$-PEG] were formed when the HA derivatives with different functionality were mixed such as to yield a final HA concentration of approximately 15 mg/mL. For the hydrogels formed from 10–15 mg/mL HA, the reaction mixture started to gel within 30 s as indicated by an increase in viscosity, and gelation was complete within 30 s to 5 min as indicated by the fact that no further changes in the macroscopic physical properties occurred thereafter.

Enzymatic degradation of HA hydrogels

To test whether the generated hydrogels are biodegradable, we determined the sensitivity of the gels to mammalian testicular hyaluronidase, an endohexosaminidase that randomly cleaves HA into polysaccharide fragments with glucuronic acid at the nonreducing end.[34] Initially, hydrogels generated with highly modified HA derivatives were digested with different concentrations of hyaluronidase, 50–5000 U/mL [Fig. 6(A)]. Complete degradation of the hydrogels was achieved with 250 U/mL hyaluronidase over a 48-h period. A linear relationship was seen when comparing the rates of digestion with the hyaluronidase concentration, i.e., the degradation rates with 500 U/mL hyaluronidase being approximately double that for 250 U/mL and 10 times that for 50 U/mL. Subsequently, we digested different hydrogel formulations with 100 U/mL of hyaluronidase to evaluate the effect of the extent of HA derivatization, the nature of the HA derivative, and different crosslinkers [Fig. 6(B)]. HA derivatives which had ~25% of the carboxylate residues modified with adipic dihydrazide **(4)** and ly-

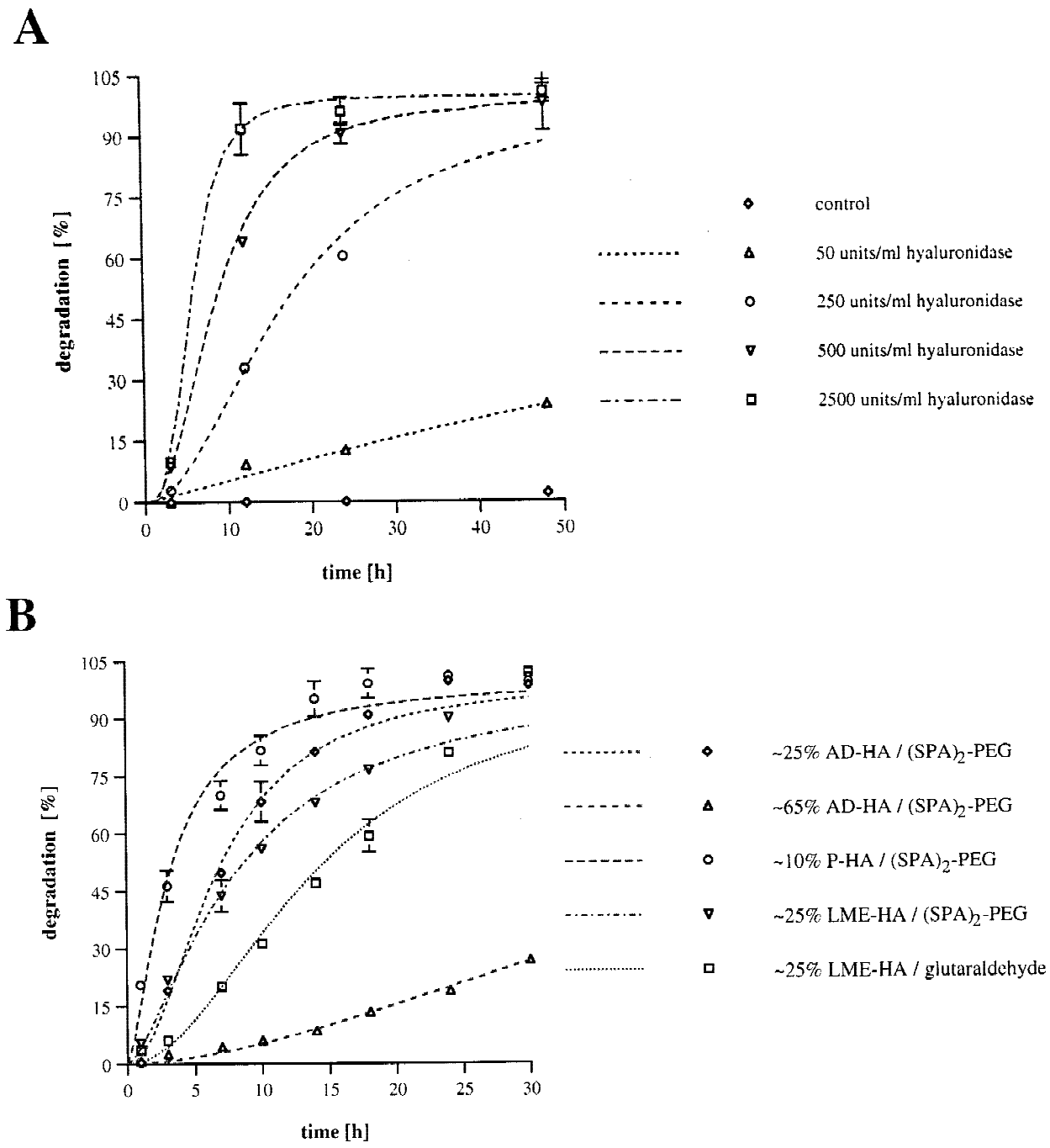


Figure 6. Hyaluronidase digestion of HA hydrogels. (A) HA hydrogels were formed by crosslinking 12 mg/mL ~65% adipic dihydrazide-functionalized HA **(4)** with a bifunctional NHS-ester, $(SPA)_2$-PEG. Gels were incubated with different concentrations of bovine testicular hyaluronidase for the indicated time and the degradation of the gels was measured by the release of glucuronic acid into the supernatant using the carbazole method. (B) HA hydrogels were formed by crosslinking 12 mg/mL ~25% adipic dihydrazide-modified HA **(4)** (AD-HA) with $(SPA)_2$-PEG, 12 mg/mL ~65% adipic dihydrazide-modified HA **(4)** with $(SPA)_2$-PEG, 12 mg/mL ~10% putrescine-modified HA **(8)** (P-HA) with $(SPA)_2$-PEG, and 12 mg/mL ~25% lysine methyl ester-modified HA **(6)** (LME-HA) with either $(SPA)_2$-PEG or glutaraldehyde. Gels were incubated with 100 U/mL of bovine testicular hyaluronidase, and at the indicated time the degradation of the gels determined as above. The values reflect the mean ± standard deviation of three independent measurements.

sine methyl ester **(6),** respectively, and crosslinked with the same amount of $(SPA)_2$-PEG, degraded at very similar rates, indicating that the nature of the introduced side chain had no effect. HA derivatives which had either ~25% or ~65%, respectively, of the carboxylate groups modified with adipic dihydrazide **(4)** and had been crosslinked with $(SPA)_2$-PEG showed the most dramatic difference in degradation, with the

degradation rate for the gel formed with the ~25% derivatized HA being eightfold that formed with the ~65% derivatized HA. In agreement with this, an HA derivative which had ~10% of the HA-carboxylate residues modified with putrescine **(8)** and had been crosslinked with $(SPA)_2$-PEG was degraded at the fastest rate, about twofold that of gels formed with ~25% derivatized HA-amines. This result shows that the degree of modification of HA and thereby of the crosslinking density of the formed hydrogels critically affects the sensitivity of the gels to enzymatic degradation. Finally, ~25% lysine methyl ester-modified HA **(6)** crosslinked with glutaraldehyde was initially slower to degrade than the same HA derivative crosslinked with $(SPA)_2$-PEG, although complete degradation was still reached in the same time period. This is probably due to the smaller size of the crosslinker creating a material with a smaller pore size limiting enzymatic attack only to the very surface of the gel. Complete degradation is achieved in the same time frame because the ratio of surface area to volume rapidly increases over time and becomes the most important factor affecting the rate of degradation besides the ability of the enzyme to infiltrate the gel.

In vivo evaluation of HA hydrogels for biocompatibility and growth factor–induced ectopic bone formation

Critical for a potential clinical use of the developed biomaterials is their biocompatibility. The overall efficacy for their use in tissue regeneration rests significantly on the absence of acute or chronic reactions to the biomaterials and their degradation products as well as the capacity of the biomaterial for being infiltrated and degraded by cells. Subcutaneous implantation of biomaterials in rats is the established model for evaluation of biocompatibility of biomaterials[35] and for induction of ectopic bone formation by members of the TGF-β gene family, and BMPs in particular.[36,37]

In a first series of experiments, we formulated a number of different HA hydrogels for *in vivo* testing in this model using different HA derivatives and crosslinkers. BMP-2 was added to the HA hydrogels to induce chondro-osseous transformation.[36] The local inflammation caused by the implanted material, expressed as granulomatous inflammation, as well as the infiltration of the material with cells, and growth factor–induced cartilage and bone formation were evaluated histologically 10 or 12 days postimplantation (Table I). At this time point, all implants were surrounded by a thin fibrous capsule, showed a general mild inflammation of the surrounding stroma with mononuclear cells, and active implants showed a ring of tissue remodeling and transformation at the periphery of the implant. The width of this reactive zone was dependent on the nature of the implanted material [Fig. 7(A–C)].

The first study evaluated the crosslinking of an HA-amine derivative **4,6** with various bifunctional NHS-esters, i.e., DTSSP and $(SPA)_2$-PEG, and aldehydes, i.e., glutaraldehyde and periodate oxidized dextran (Table I, groups 1–5). Little inflammation was observed with active ester-crosslinked HA-amine derivative **4,** while a foreign-body giant cell reaction indicative of chronic inflammation was seen when the same HA-amine derivative was crosslinked with glutaraldehyde or dextran-aldehyde [Fig. 7, compare (D) with (E, F)]. Crosslinking of the HA-amine derivative **4** with $(SPA)_2$-PEG, a high-MW NHS-ester crosslinker, gave the best result with 30–40% chondro-osseous transformation of the implant [Fig. 7(B)]. The absence of cellular infiltration and cartilage and bone formation with the materials crosslinked with low-molecular-weight crosslinkers such as DTSSP and glutaraldehyde showed that the size of the crosslinker strongly affected resorption and cellular infiltration of the material [Fig. 7(C)]. This was presumably due to the difference in pore size of the material formed with crosslinkers of different sizes. The degree of modification of the HA-amine derivative **4,** and thereby of the crosslinking density of the material, also affected the resorption and transformation rate of the materials [Fig. 7, compare (A) and (B)]. Nevertheless, limited cartilage and bone formation was seen even with a material formed from an HA-amine derivative **4** modified to an extent of ~65% [Fig. 7(A)]. The results obtained with different HA-amine derivatives, i.e., adipic dihydrazide-modified HA **(4)** and lysine methyl ester-modified HA **(6)** were similar (Table I).

In a second study, we evaluated the crosslinking of the HA-amine derivative **4** with different HA-aldehydes, i.e., periodate-oxidized HA, aminoacetaldehyde dimethyl acetal-modified HA **(7),** and *N*-(2,2-dimethoxyethyl)-4-(hydrazido)butanamide-modified HA **(3)** (Table I, groups 6–8). All implants were well tolerated and foreign-body giant cells were only occasionally found in individual implants. Chondro-osseous differentiation was seen in all groups of materials crosslinked with different HA-aldehyde derivatives, including periodate-oxidized HA, which is in sharp contrast with the results obtained when the same HA-amine derivative **4** was crosslinked with periodate-oxidized dextran or glutaraldehyde. The extent of cellular infiltration and cartilage and bone formation varied somewhat between groups. However, the relatively small differences cannot be considered significant at present since the degree of modification differed among the different HA-aldehyde derivatives, and the materials formed presumably differed in their crosslinking density.

In a second series of experiments, we selected one HA hydrogel and used it to deliver different biological

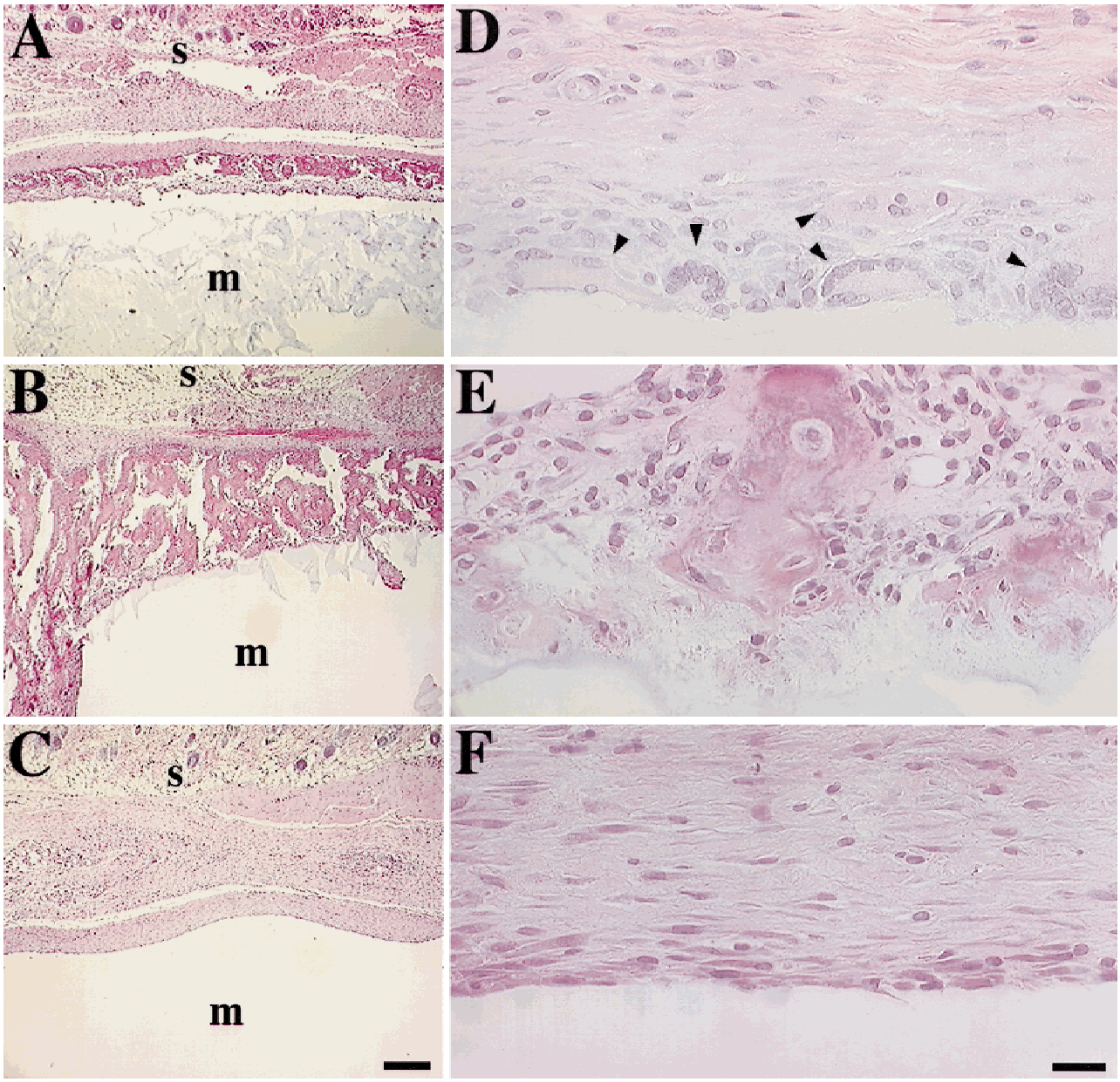


Figure 7. *In vivo* evaluation of different HA hydrogels. HA hydrogels were polymerized and implanted in rats subcutaneously. Hydrogels consisted of 12 mg/mL ~65% adipic dihydrazide-modified HA **(4)** crosslinked with $(SPA)_2$-PEG (A), or 12 mg/mL ~25% adipic dihydrazide-modified HA **(4)** crosslinked with $(SPA)_2$-PEG (B,E), with DTSSP (C,F), or with glutaraldehyde (D). The hydrogels also contained 1 mg/mL prefibrillized intact collagen type I, 200 μg/mL BMP-2, and 50 ng/mL TGF-β2 to induce bone formation. Tissue specimens were harvested 10 days postimplantation and paraffin embedded, and sections were stained with hematoxylin and eosin. Arrowheads in (D) indicate foreign-body giant cells. m = HA matrix (note: HA hydrogel shrinks during processing for histology); s = skin. Bars = 300 μm (A–C), and 25 μm (D–F).

signals by addition of different bioactive factors into the material to demonstrate that we are able to modulate the biological activity of the material (Table II). In a first study, we examined the synergistic effect of TGF-β2 and IGF-1 with BMP-2 on chondro-osseous differentiation, and compared it to implants containing BMP-2 alone, and implants containing no growth factor. Osteogenesis was absent and cellular infiltration into the material markedly reduced in the absence of growth factor [Fig. 8(E)]. Implants with BMP-2 alone showed residual islands of cartilage with most of the cartilage being replaced by bone 10 days postimplantation. This is consistent with published data suggesting that in this model chondrogenesis occurs between days 5 and 7, and endochondral ossification is complete by days 12–14.[36,37] The infiltration and transformation rate of BMP-2/IGF-1– and BMP-2/TGF-β2–loaded materials was similar to that with BMP-2 alone indicating that the resorption rate is a material property. However, 10 days postimplantation

TABLE II
Growth Factor Delivery Using HA Hydrogels

Matrix Proteins	Growth Factors	Antiangiogenic Factor	Tissue Transformation*
None	None	None	No
None	BMP-2	None	No
Collagen type I	None	None	No
Collagen type I	BMP-2	None	Bone (cartilage)
Collagen type I	BMP-2/IGF-1	None	Cartilage (bone)
Collagen type I	BMP-2/TGF-β2	None	Bone (cartilage)
Collagen type I	BMP-2/IGF-1	Suramin	Cartilage
Collagen type I	BMP-2/TGF-β2	Suramin	Connective tissue
Fibronectin	BMP-2/IGF-1	None	No

HA hydrogels were formed from 12 mg/mL ~25% adipic dihydrazide-modified HA, crosslinked with $(SPA)_2$-PEG and supplemented as indicated below with 1 mg/mL prefibrillized collagen type I, 500 μg/mL fibronectin, 200 μg/mL BMP-2, 500 ng/mL IGF-1, 50 ng/mL TGF-β2, and 10 mg/mL suramin.

*10 days postimplantation; predominant tissue type is indicated and presence of occasional foci of other tissue (endochondral bone formation) given in parentheses (see Fig. 8).

the newly formed tissue was primarily cartilage in the BMP-2/IGF-1 group and primarily bone in the BMP-2/TGF-β2 group [Fig. 8(A–D)]. The angiogenic activity of TGF-β[38,39] induced rapid replacement of the cartilage by bone, and ossification was consequently accelerated as compared to BMP-2 alone. This result is consistent with other studies which have shown that TGF-β2 is a potent stimulator of osteogenesis *in vivo*,[40] presumably by synergistic action with BMPs including BMP-2.[41] IGF-1 is an essential anabolic growth factor for chondrocytes and supports continued chondrocyte replication which favors the maintenance of a chondrocytic phenotype.[42,43] At a later time point (21 days), there was no difference between the groups and ossification was complete, demonstrating that the synergistic action of the growth factors changed the progression of endochondral ossification but not the end result. To test whether the acceleration of bone formation by TGF-β2 was in fact mediated by a stimulation of angiogenesis rather than by affecting the chondro-osseous differentiation of mesenchymal cells, we repeated the experiment with BMP-2/IGF-1 and BMP-2/TGF-β2, but included suramin, which is a potent inhibitor of angiogenesis.[44] Bone formation in the BMP-2/TGF-β2 group was completely abolished by suramin and formation of an elaborate collagenous connective tissue was observed instead 10 days postimplantation [Fig. 8(F,G)]. This reaction is reminiscent of the fibroblastic response elicited by TGF-βs on their own in this model.[38,45] In contrast, the results in the BMP-2/IGF-1 group were very similar in the absence or presence of suramin [data not shown; similar to Fig. 8(A,C)]. Cartilage developed normally, and the only difference was that bone formation was completely absent in the presence of suramin. These results suggest that the TGF-β signal is predominant and that if osteogenesis is prevented by inhibition of angiogenesis, the stimulation of scar tissue formation precludes chondrogenesis.

The differential biological activities observed with the different growth factors demonstrate that the HA materials do not significantly alter their activity and are thus well suited for delivery of bioactive factors to stimulate tissue regeneration. Local delivery of bioactive factors is achieved by physical or chemical incorporation into the porous HA hydrogels, and yields sustained release of the factors by chemical, enzymatic, and physical erosion of the materials. Since the growth factors were mixed with the HA derivative prior to crosslinking, they may in part have become covalently bound to the matrix. It has previously been shown that TGF-β2 retains its activity after conjugation to collagen with the $(SPA)_2$-PEG crosslinker,[45] and our experiments suggest that BMP-2, TGF-β2, and IGF-1 are not inactivated by this crosslinking process (Table II and Fig. 8). On the other hand, the growth factors were inactive in the absence of collagen type I fibrils (Table II), which may be due to the instability of the members of the TGF-β family in physiological salt solutions. The lack of activity of implants supplemented with another cell adhesion molecule, fibronectin, showed that the effect on growth factor activity is specific to collagen (Table II). Collagen binds TGF-βs and BMPs,[46,47] and thus may prevent inactivation of the growth factors by stabilizing them in an active conformation. Alternatively, the kinetics of the release may be significantly different for the collagen-bound growth factors.

General considerations and perspectives

In contrast to existing strategies for preparation of HA hydrogels,[14–18] the hydrogels described in this study can be polymerized *in situ* and/or in the pres-

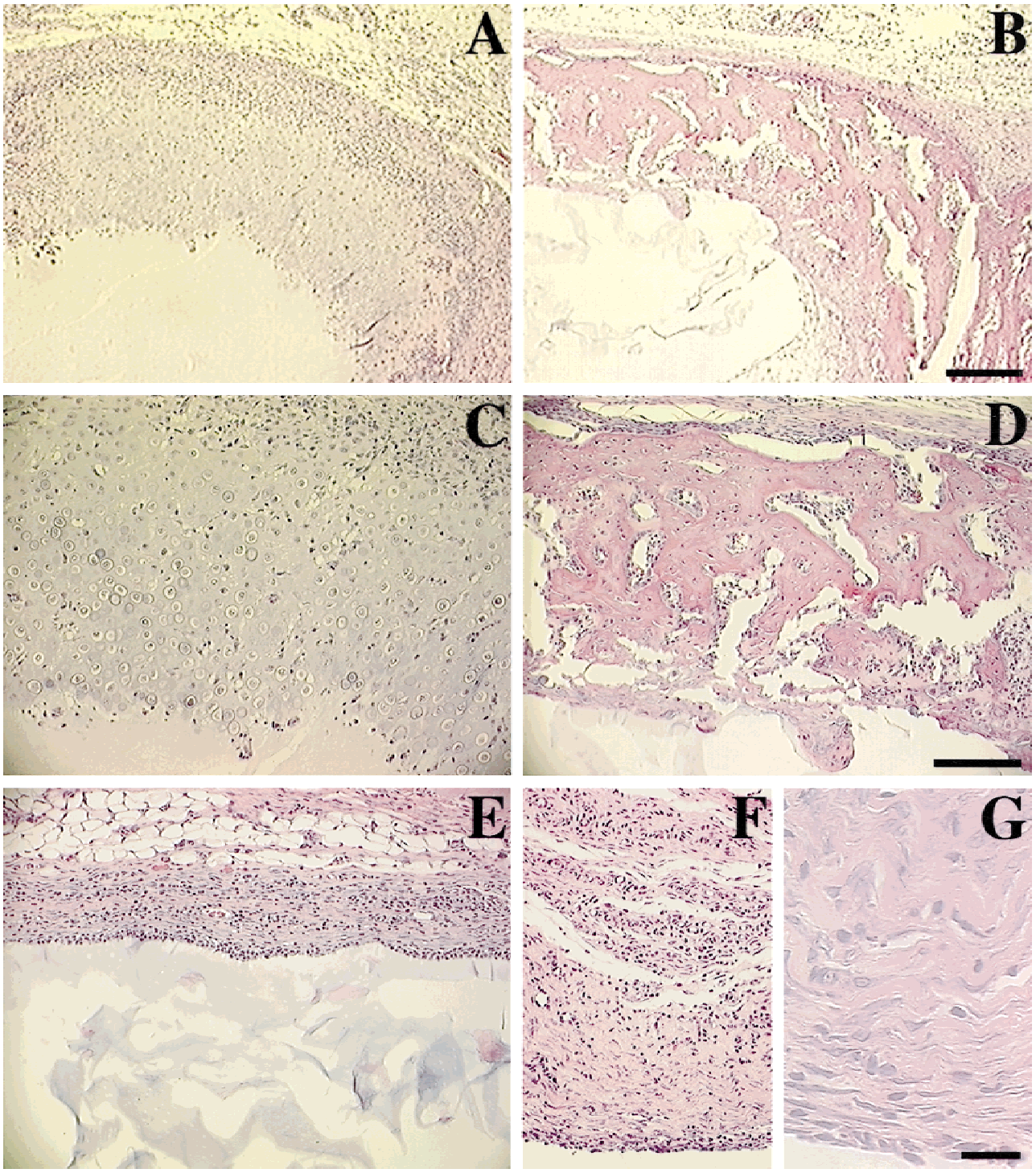


Figure 8. Differential effect of growth factors on tissue transformation. HA hydrogels were polymerized and implanted in rats subcutaneously. Hydrogels were formed from 12 mg/mL ~25% adipic dihydrazide-modified HA **(4)** crosslinked with $(SPA)_2$-PEG, contained 1 mg/mL prefibrillized intact collagen type I, and were supplemented with 200 μg/mL BMP-2 and 500 ng/mL IGF-1 (A,C), 200 μg/mL BMP-2 and 50 ng/mL TGF-β2 (B,D), 200 μg/mL BMP-2, 50 ng/mL TGF-β2 and 10 mg/mL suramin (F,G), or no growth factor (E). Tissue specimens were harvested 10 days postimplantation and paraffin embedded, and sections were stained with hematoxylin and eosin. Bars = 300 μm (A,B), 150 μm (C–F), and 25 μm (G).

ence of isolated cells since the HA derivatives are water soluble and the pursued crosslinking strategies can be carried out under physiological conditions. This allows for the use of these materials as a scaffold for cells, either to produce artificial tissues in culture, e.g., when serving as a dermal equivalent in manufacture of skin grafts, or for transplantation of culture-expanded cells, e.g., when providing an injectable me-

dium for anchorage of autologous chondrocytes in cartilage repair.[2] We have successfully used active ester-crosslinked HA-amine as a scaffold for culture of chondrocytes in a three-dimensional culture system (unpublished results). The employed crosslinking strategies are well tolerated by the cells; thus, isolated cells can be dispersed into the polymerizing HA matrix prior to complete setting. Furthermore, the use of cleavable crosslinkers, e.g., reducible DTSSP, provides an easy method for depolymerization of the matrix to harvest cells or isolate extracellular matrix constituents from such a culture system. Finally, other HA derivatives and crosslinking strategies including, e.g., maleimides that react specifically with sulfhydryls or arylazides for photocrosslinking may be developed in the future using the described technology for conjugation of functionalized amines to HA and yield promising new materials.

CONCLUSIONS

In the present study, we describe a method for carbodiimide/active ester-mediated coupling of amines to high-molecular-weight HA. The degree of derivatization of HA could be controlled and varied from 10% to 70% of the carboxyl groups depending on the nature of the nucleophile used for coupling. A wide range of functionalized amines is commercially available which allows for the introduction of a wide variety of functional groups useful for further crosslinking reactions onto HA besides the HA-amine and HA-aldehyde derivatives described in this article. The mild conditions used for derivatization of HA prevented fragmentation of the polysaccharide, which had been the drawback of other methods for derivatization of HA and is of particular concern since low-molecular-weight HA fragments can elicit inflammatory responses.

The ability to generate HA derivatives with a variety of different functional groups greatly facilitates the crosslinking of HA to form hydrogel materials and the conjugation of biologically active factors such as drugs, growth factors, cytokines, etc., to HA for controlled release. The employed aldehyde- and active ester-mediated crosslinking strategies allowed for rapid polymerization of HA derivatives under physiological conditions. Hydrogels formed by crosslinking HA derivatized to a degree of 10–25% with high-molecular-weight crosslinkers produced materials that were infiltrated by cells and supported growth factor–induced tissue remodeling in the rat ectopic bone formation model. In this model, BMP-2–induced bone formation was accelerated by synergistic action of TGF-β2 and abolished by an anti-angiogenic factor. Synergistic action of IGF-1 with BMP-2 promoted chondrogenesis, independent of angiogenesis. This demonstrates that BMP-induced chondrogenic differentiation is independent of bone formation and that signals released from invading blood vessels are required for progression of endochondral ossification. The induction of different biological responses by these biomaterials holds great promise for their use in tissue regeneration. Finally, hydrogels formed with highly derivatized (>25%) HA preparations and low-molecular-weight crosslinkers were antiadhesive to cells and could be used to generate tissue separations and prevent adhesions following surgery in analogy to existing HA materials.

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