Functionalization of Hyaluronic Acid with Chemoselective Groups via a Disulfide-Based Protection Strategy for In Situ Formation of Mechanically Stable Hydrogels

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Functionalization of hyaluronic acid (HA) with chemoselective groups enables in situ (in vivo) formation of HA-based materials in minimally invasive injectable manner. Current methods of HA modification with such groups primarily rely on the use of a large excess of a reagent to introduce a unique reactive handle into HA and, therefore, are difficult to control. We have developed the new protective group strategy based on initial mild cleavage of a disulfide bond followed by elimination of the generated 2-thioethoxycarbonyl moiety ultimately affording free amine-type functionality, such as hydrazide, aminooxy, and carbazate. Specifically, new modifying homobifunctional reagents have been synthesized that contain a new divalent disulfide-based protecting group. Amidation of HA with these reagents gives rise to either one-end coupling product or to intra/intermolecular cross-linking of the HA chains. However, after subsequent treatment of the amidation reaction mixture with dithiothreitol (DTT), these cross-linkages are cleaved, ultimately exposing free amine-type groups. The same methodology was applied to graft serine residues to the HA backbone, which were subsequently oxidized into aldehyde groups. The strategy therefore encompasses a new approach for mild and highly controlled functionalization of HA with both nucleophilic and electrophilic chemoselective functionalities with the emphasis for the subsequent conjugation and in situ cross-linking. A series of new hydrogel materials were prepared by mixing the new HA-aldehyde derivative with different HA-nucleophile counterparts. Rheological properties of the formed hydrogels were determined and related to the structural characteristics of the gel networks. Human dermal fibroblasts remained viable while cultured with the hydrogels for 3 days, with no sign of cytotoxicity, suggesting that the gels described in this study are candidates for use as growth factors delivery vehicles for tissue engineering applications.

Introduction

Hyaluronan (HA) is a major glucosaminoglycan component of extracellular matrices (ECM) found in different mammalian tissues. HA plays an important role in the structure and organization of the ECM, regulation of cell adhesion, morphogenesis and modulation of inflammation.[1,2] Native HA is highly biocompatible, biodegradable, and nonimmunogenic which makes it an ideal material for various biomedical applications, such as for treatment of osteoarthritis,[3] in ophthalmic surgery[4] and wound healing.[5] Chemical modifications of HA has allowed preparation of HA-drug conjugates[6] and various HA-based nanocarriers[7] which conferred even more extensive applications of HA in drug delivery. Chemically cross-linked HA hydrogel biomaterials provided control over degradation rates and mechanical stiffness of HA *in vivo*. Matching the mechanical properties of HA hydrogels to biological tissues together with the use of HA hydrogels as carriers for growth factors[8,9] and cells[9−11] resulted in successive regeneration of different tissues including bone,[9,12] cartilage,[10,11] as well as in stimulation of angiogenesis.[8] Our group has recently developed cross-linkable HA-bisphosphonate prodrug that are selectively taken up by cells that produce the HA receptor CD44.[13] We have also demonstrated the hydrogel-mediated delivery of BMP-2 (bone morphogenetic protein-2) to induce local bone formation[14,15] as well as the utility of guanidinium-modified HA hydrogel for gene delivery.[16]

Functionalization of HA with chemoselective groups is advantageous for the preparation of glycoconjugates with complex biomacromolecules and for the performance of chemically cross-linked hydrogel materials. Mild chemoselective reactions, also referred to as click reactions,[17,18] allow mild, quick, and injectable immobilization of growth factors and cells at physiological aqueous environment without formation of toxic side products. This may significantly reduce cell death or lost of bioactivity of the encapsulated growth factors in such chemically formed HA hydrogel systems.[8,9,11,12,14] HA functionalization with chemoselective groups is, however, a challenging task due to multifunctionality and limited solubility of HA in organic solvents. For example, modification of HA with nucleophilic hydrazide groups[19,20] has been accomplished by reacting HA with homobifunctional adipic dihydrazide (ADH), which can potentially act as a cross-linking agent at the stage when only one of the two hydrazide groups is intended to be coupled to the HA carboxylate. To avoid the concomitant cross-linking during hydrazide functionalization, a large excess of ADH was therefore applied. In spite of that, the cross-linking effect of ADH was substantial even at high molar ratios of ADH to polysaccharide carboxylate as well as when using low amount of the condensing reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC).[21] Consequently, acrylation of the HA-ADH derivative was utilized for the installation of an electrophilic methacrylamide group.[9,22] Haloacetate-derivatized HA was prepared via direct acylation of HA hydroxyl in water using a 10-fold excess of anhydride reagent due to a side reaction with HA carboxylate groups and the formation of hydrolytically

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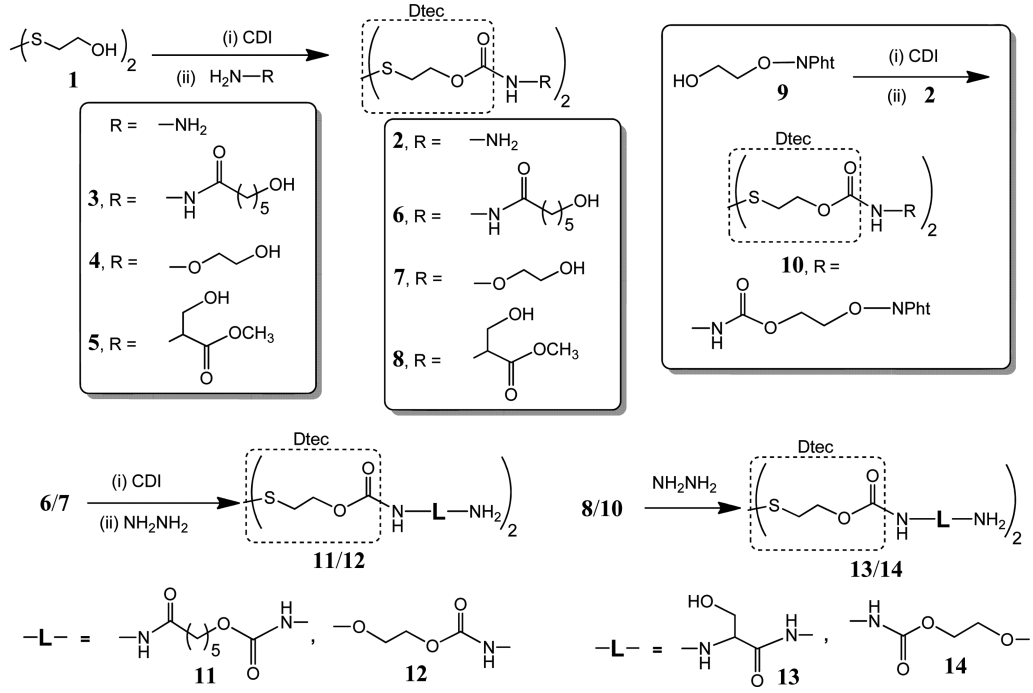
Scheme 1. Difunctional Symmetric Reagents **11**−**14** Protected with 2,2′-Dithiobis(ethoxycarbonyl) Group



unstable mixed anhydride.[23] Partial oxidation of HA with sodium periodate was also used to generate electrophilic aldehyde groups in the oxidized open rings of the glucuronic acid units.[12] Periodate oxidation of HA requires, however, prolonged reaction time due to extensive inter- and intramolecular hydrogen bonding between hydroxyl and carboxylate groups of HA which, as a side reaction, causes degradation of HA backbone and reduction of the molecular weight by at least an order of magnitude.[24] A solution to controlled functionalization of HA with chemoselective groups would be temporal blocking of the group of interest with a suitable protecting group. The repertoire of protecting groups, the removal of which would not be destructive for HA, is, however, quite limited because both highly basic and acidic conditions degrade HA by cleavage of the labile glycosidic bonds. The use of protection strategy for functionalization of HA with chemoselective groups has been limited so far to only the preparation of thiolated HA.[25]

Here we report the synthesis of a series of symmetrical difunctional reagents that have a central divalent protecting group, 2,2′-dithiobis(ethoxycarbonyl) (Dtec), which links two identical molecules. These reagents allow mild and highly controlled functionalization of HA with various types of nucleophilic chemoselective groups, which is exemplified by first time HA modification with aminooxy and carbazate groups. In spite of the cross-linking effect of these reagents during the first amidation coupling, they can be easily cleaved at the central disulfide part afterward, ultimately liberating the masked nucleophilic groups. The novelty with this approach is that no excess of reagents is needed, no intermediate purification is needed, the reaction yields are quantitative without side reactions, and it allows the retention of the molecular weight of the HA. The new HA derivatives were tested in preparation of hydrogel materials by mixing with new electrophilic HA derivative containing aldehyde-terminated side chains to produce a series of HA-based hydrogels with tunable mechanical properties.

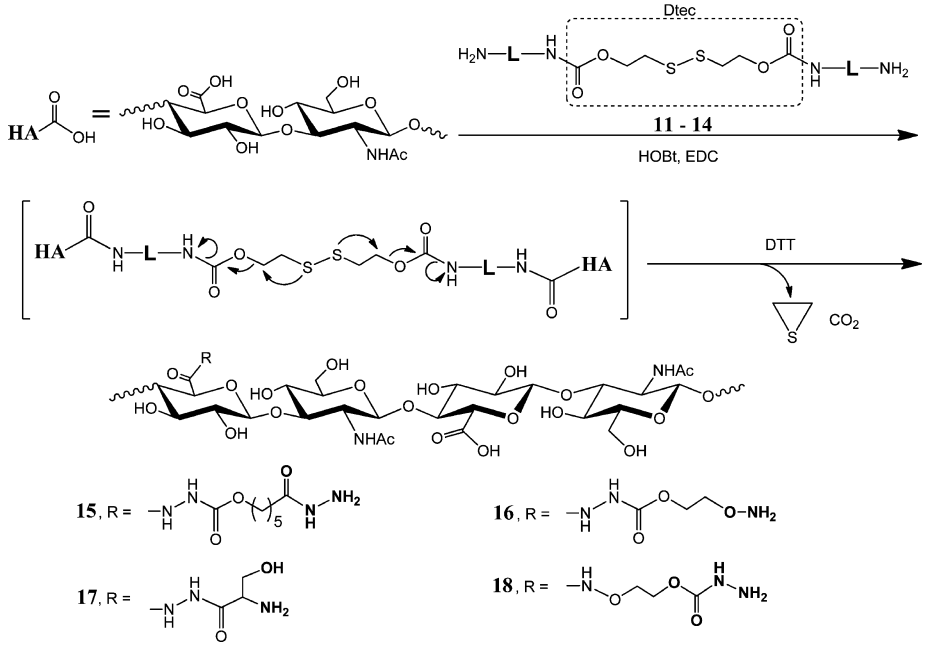
Results and Discussion

Design of New Difunctional Reagents for Mild Incorporation of Chemoselective Nucleophilic Groups. We have elaborated a synthetic method that allows one pot functionalization of HA with various types of orthogonal groups. To accomplish this, we have introduced a protecting group strategy that allows removal of multiple protective groups under the same mild reaction conditions eventually affording different types of orthogonal click functionalities. A series of symmetrical difunctional reagents **11**−**14** have been synthesized (Scheme 1) that have a central divalent protecting group, 2,2′-dithiobis(ethoxycarbonyl) (Dtec), which links two identical molecules.

The synthesis of the reagent **2** (Scheme 1) presenting two hydrazine molecules coupled together through the divalent Dtec protecting group has been reported by us recently.[13] The divalent protecting group has been prepared from 2,2′-dithiodiethanol **1** by activation with 2 equiv of carbonyldiimidazole (CDI) and used in situ for a subsequent carbamate coupling reaction with 2 equiv of an appropriate amine-type compound (hydrazide **3**, aminooxy compound **4**, or serine derivative **5**). This afforded alcohol derivatives **6** and **7**, as well as serine methyl ester **8**. Two hydroxyl groups of the condensation products **6** and **7** were then extended with hydrazinocarbonyls by consecutive activation with CDI and treatment with hydrazine in one pot to give the corresponding reagents **11** and **12**. In a similar way, *N*-(2-hydroxyethoxy)phthalamide **9** was coupled to the reagent **2** to obtain **10**. The serine derivative **8** and pthalimido derivative **10** afforded corresponding hydrazide **13** and aminooxy derivative **14** upon treating with neat hydrazine.

Grafting the Hyaluronic Acid with Side Chains Terminated with Chemoselective Nucleophilic Groups. The symmetrical linkers **11**−**14** have a terminal amine-type functional group that can undergo an amide-type reaction with the carboxylate residue of HA in aqueous solution. The self-immolative central Dtec group of these linkers when treated with external thiol generate unstable thiols that decompose spontaneously, liberating ethylene episulfide, carbon dioxide,

Scheme 2. Functionalization of HA with Nucleophilic Chemoselective Groups



and the corresponding free amine-type click functionality.[26] Amidation of HA with bifunctional reagents **11**−**14** can give rise to either a one-end coupling product or a two-end intra/intermolecular cross-linking of HA chains. However, after subsequent treatment of the reaction mixture with dithiothreitol (DTT), these cross-linkages are cleaved, ultimately exposing free amine-type groups (Scheme 2).

^{1}H NMR spectroscopy provided the evidence that DTT treatment led to the formation of the unstable 2-thioethyl carbamate side chains that underwent subsequent fragmentation reaction (Figure 1). Thus, coupling of the known reagent **19**[25] to HA afforded the expected thiolated HA derivative with clearly visible methylene protons of the HSCH_2CH_2CO- side chains at 2.73 and 2.58 ppm, respectively (Figure 1a). Conversely, when the same procedure was applied using the reagent **11**, ^{1}H NMR of the isolated HA derivative **15** (Figure 1b) showed only α, β, γ, δ, and ε-methylene protons of the reagent **11** that are derived from its synthetic progenitor **3** but not the protons corresponding to the HSCH_2- methylene of the thioethyl carbamate intermediate. This observation demonstrated that the thioethyl carbamate residues were indeed unstable under the basic conditions applied (pH 8.5), underwent the described in the literature[26] fragmentation reaction, and ultimately liberated the hydrazide group.

When reagents **12**, **13**, and **14** were subjected for the EDC-mediated coupling with HA, the thioethyl carbamate moiety was also not detected in the ^{1}H NMR spectra of the corresponding products (see Figure S1 of Supporting Information), demonstrating the generality of our protection strategy for different amine-type functionalities. Thus, we were able to quantitatively functionalize HA with a range of nucleophilic chemoselective

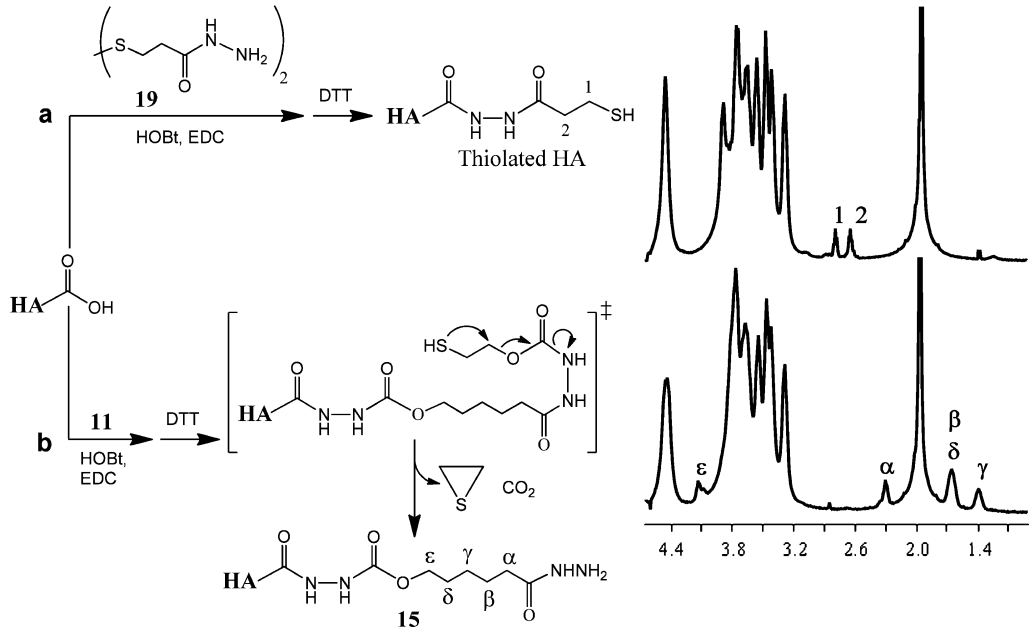


Figure 1. ^{1}H NMR spectra in D_2O of (a) thiolated HA and (b) hydrazide-modified HA **15**. The region from 0.9 to 4.7 ppm for all spectra is compared.

Table 1. Reaction Conditions of HA Modification and Degrees of Substitution (DS) of the Corresponding Products

HA derivative	reagent	molar ratio of HA/reagent/ EDC/HOBt	DS (%)	yield (%)
15	**11**	1:0.15:0.15:1	8.9	95.8
16	**12**	1:0.15:0.3:1	7.5	91.2
17	**13**	1:0.15:0.3:1	6.0	97.9
18	**14**	1:0.15:0.4:1	6.5	98.9

groups including hydrazide (**15**), aminooxy (**16**), serine (**17**), and carbazate (**18**) derivatives using low submolar amounts of the new homobifunctional reagents and carbodiimide coupling reagent (Table 1), unlike previously described adipic dihydrazide (ADH) modification of HA, which normally uses a large excess of ADH to avoid cross-linking.[21]

Modification of Hyaluronic Acid with Aldehyde-Containing Side Chains. The use of modifying reagent **13** allowed also facile grafting serine side chains via hydrazide linkages. The 1,2-aminoalcohol motif of the serine moieties is known to participate in selective oxidation with sodium periodate into the corresponding aldehyde. Periodate oxidation of 2-aminoalcohols is a very fast reaction, reportedly 1000-fold faster than the 1,2-diols.[27,28] Periodate, for example, effects quantitative oxidation of *N*-terminal serine when present in only a low molar excess over 2-aminoalcohol.[29] On the other hand, the glycol groups in the glucuronate residues of hyaluronan are resistant to periodate oxidation, although identical structures in similar polymers and environments are easily attacked.[30] The reason to that is the extensive inter- and intramolecular hydrogen bonding between hydroxyl and carboxylate groups of HA, which makes glucuronic hydroxyls at C2 and C3 less accessible for IO_4^- anions. We hypothesized that the attachment of 2-aminoalcohols $-CH(NH_2)CH_2OH$ as side chains to the HA polymer backbone with the following quick periodate oxidation could selectively convert serine side groups into aldehydes without affecting the glycol groups of the HA backbone. To date, the normal way of HA functionalization with aldehyde groups relies on sodium periodate oxidation of the native hyaluronan for 2 h, which is accompanied by glycoside bond cleavage and significant reduction of the molecular weight by at least an order of magnitude.[24]

Addition of the 150 mM solution of sodium periodate in water to the 0.8 wt % aqueous solution of **17** at a 1:1 [HA carboxylate]/[$NaIO_4$] molar ratio led to the formation of a gel, indicating a very fast reaction. The reaction was stopped after 5 min by the addition of a 10-fold excess of ethylene glycol and the dilution of the mixture with water two times. The gel dissolved gradually after stirring the diluted mixture overnight. The oxidized HA product **20** (Figure 2) was isolated from the dissolved mixture by dialysis against water two times followed by lyophilization. The conversion of the serine side chains into aldehyde groups was proved by first examining the ^{1}H NMR spectra of HA derivatives **17** and **20**. Compared to native HA, the spectrum of serine-modified HA **17** showed additional peaks at 4.27 and 4.00 ppm that were assigned to the protons at the carbon A (Figure 2). These signals, corresponding to the proton peak 4 of the reagent **13**, disappeared in the spectrum of the oxidized product **20**. Because aldehydes in water exist in equilibrium with their hydrated forms as well as they can potentially react with numerous hydroxyl groups of HA with the reversible formation of hemiacetals, it should come as no surprise that we did not observe a peak corresponding to the aldehyde proton. Instead, we observed new resonances at 5.43 and 3.63 ppm that were attributed to the protons of the hydrate and hemiacetal forms. The formation of reversible hemiacetal cross-links could be responsible for the gel formation during the oxidation of the HA-serine derivative. The fact that the gel was dissolved when the excess of ethylene glycol was added to the diluted reaction mixture confirms our assumption. The neighboring electron-negative carbonyl group makes the serine-derived aldehyde more reactive toward both alcohols and amines. The amount of aldehyde groups in the obtained derivative was quantified by reductive amination with *tert*-butyl carbazate (TBC) and subsequent integration of the *tert*-butyl peak at 1.4 ppm, which indicated 5% of aldehyde functionalization. It should be noted that the peak at 3.63 ppm disappeared after the reaction with TBC, thus, corroborating its hemiacetal nature.

Regioselectivity of periodate oxidation at the serine side chains under the conditions applied was demonstrated by performing oxidation of the native HA using the same mild conditions (5 min reaction at room temperature, equimolar amount of periodate). No oxidation of the glycol groups of the

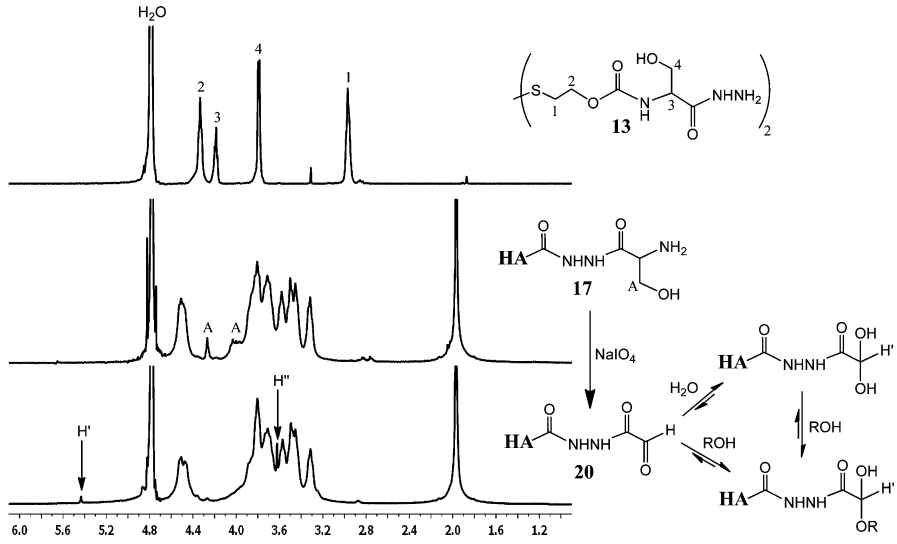


Figure 2. ^{1}H NMR spectra of the reagent **13** (up), HA-serine **17** (middle), and HA-aldehyde **20** (bottom) in D_2O. The region from 0.9 to 6.0 ppm for all spectra is compared.

Table 2. Mechanical Properties of HA Hydrogels

gel composition	$G'_{initial}$[a] (Pa)	$G'_{swollen}$[b] Pa	swelling ratio[c]
15 + **20**	1520 ± 40	247 ± 6	41.8 ± 0.9
16 + **20**	2880 ± 60	259 ± 3	40.3 ± 0.5
18 + **20**	2300 ± 60	218 ± 9	43.4 ± 2.1
15 + **HA-Alox**	765 ± 60	dissolved	dissolved

[a] $G'_{initial}$: elastic modulus measured for the prepared gels after 3 h of setting. [b] $G'_{swollen}$: elastic modulus measured for the swollen gels after 8 days of incubation in PBS (pH 7.4). [c] Swelling ratios were determined after 8 days of incubation in PBS (pH 7.4).

HA glucuronate units was detected under such conditions as was verified by a reaction of the periodate-treated native HA with TBC. Hence, the approach presented here allows the preparation of the HA derivative that is functionalized with electrophilic aldehyde side groups without breaking the HA polysaccharide chain.

In Situ Hydrogel Preparation Using New Hyaluronic Acid Derivatives Modified with Chemoselective Groups. After the new HA derivatives carrying different nucleophilic chemoselective groups had been prepared, we examined them for their ability to form hydrogel materials by mixing with the new HA-aldehyde counterpart. This procedure is free of a low molecular weight cross-linker, it utilizes chemoselective addition cross-linking reactions that are rapid in aqueous solutions without the formation of side products and, thus, can be applied in vivo. Until the present, the only chemical gelling HA-based materials utilizing an aldehyde as one of the cross-linking reactants were hydrazone and imine cross-linked gels. The hydrazone HA gels were, for example, formed from the HA-ADH by reacting with either PEG dialdehyde[31,32] or with HA in which aldehyde groups were generated by sodium periodate oxidation.[12,24,33] Schiff's base cross-linked hydrogels were prepared by mixing of the periodate-oxidized HA with either chitosan[10] or gelatin.[34] Labile imine bonds were also formed by coupling of amine-modified HA with oxidized heparin.[35] Here we sought to prepare new cross-linker free HA hydrogels in which oxime or carbazone bonds serve as cross-linking points to verify the influence of these cross-linkages on the hydrolytic stability of the corresponding gels.

Hydrogels (2 wt % in PBS buffer) were formed by mixing the nucleophilic HA derivatives with the aldehyde-derivatized HA **20** at a 1:1 molar ratio of the cross-linkable functionalities (Table 2). The gelation time was monitored using a test tube inversion assay that showed hydrogel formation within half a minute for all HA derivatives. Slight variations in mechanical properties of the prepared hydrogels ($G'_{initial}$) can reflect variations in degree of functionalization of the HA derivatives as well as different hydrophobicity of the functional side chains. However, after incubation in PBS buffer for 8 days, the gels became softer, as judged by a lower elastic modulus ($G'_{swollen}$; Table 2).

After swelling in PBS buffer for 20 days, none of the HA-derived hydrogels was hydrolytically degraded. In comparison, previously described hydrogels made from hydrazide and aldehyde-modified HA derivatives were highly deformable in spite of much higher degrees of functionalization of the corresponding HAs.[12,24,33] The improved hydrolytic stability of our hydrogel materials can be attributed to the use of the serine-derived aldehyde component which should react with amines forming more stable -C═N- adducts due to the resonance stabilization with the adjacent carbonyl group. This assumption was confirmed by replacing our HA-aldehyde with the well-known HA derivative that is obtained by periodate oxidation of the native HA, named here as HA-Alox.[10,12,24,33,34] We have

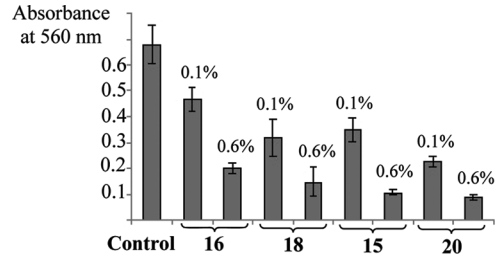


Figure 3. Human dermal fibroblast viability after incubation for 48 h in the presence of either 0.1 or 0.6% HAs modified with nucleophilic and electrophilic chemoselective groups. The left bar corresponds to the untreated control. The columns represent the mean ± S.D.; $n = 3$.

prepared the HA-Alox with 10% of aldehyde content from 1.3 MDa HA by treatment with 1 mol equiv of sodium periodate per HA repeating unit for 2 h. The 2% hydrogel formed from HA-Alox and HA-hydrazide **15** was completely dissolved on day 8 (Table 2). The stability of the hydrogel networks prepared from the serine-derived HA-aldehyde **20** was confirmed by monitoring the swelling ratios over the time of incubation in PBS buffer (Figure 2S of Supporting Information). After 8 days, the newly prepared gels had reached a plateau of swelling ratio ranging from 40 to 45, while the swelling ratio for the (**15** + HA-Alox) hydrazone gel was steadily increasing ultimately leading to the gel dissolution. This allows the design of gel materials that are degraded by cell-directed endogenous processes only and not by hydrolysis.

Degradation of Hyaluronic Acid-Based Hydrogels. The hydrogels were completely degraded by 500 U/mL hyaluronidase (Hase) within 24 h. The amount of Hase added to the solution was much higher than that of physiological conditions. These experiments clearly demonstrated that Hase can recognize the cross-linked HA, proposing that the prepared gels would be resorbable in vivo by native degradation.

Cytotoxicity of the Modified HA Derivatives and the Corresponding Hydrogels. Cytocompatibility of the nucleophilic HA derivatives was evaluated by culturing human dermal fibroblasts (hDFns) for 48 h in the presence of the respective HA macromolecules (0.1 or 0.6% w/v, Figure 3). For comparison, cell viability in presence of electrophilic aldehyde-derivatized HA **20** was examined. MTT assay indicated that HA derivatives modified with nucleophilic groups (**15**, **16**, **18**) were less toxic than the aldehyde-modified HA **20** (Figure 3). Higher toxicity of HA-aldehyde **20** might originate from higher chemical reactivity of the aldehyde group that can potentially react with amino groups of proteins. All the HA derivatives showed dose-dependent cytotoxicity. It should be stressed, however, that cells would not be exposed to the free functional HA components for a long time in the case of in vivo injection due to fast gelling kinetics. To confirm this, hDFn cells were cultured for 3 days, while sharing medium with the hydrogels via cell culture inserts. The corresponding gel-forming solutions were premixed and immediately put in contact with the cell culture medium. The results presented in Figure 4 clearly show that sharing medium with the gels in all cases showed no significant decrease in cell viability. It can therefore be concluded that all potentially toxic components are efficiently cross-linked in the hydrogels during formulation and therefore could be prevented from exposure to endogenous cells while injecting the gels in vivo.

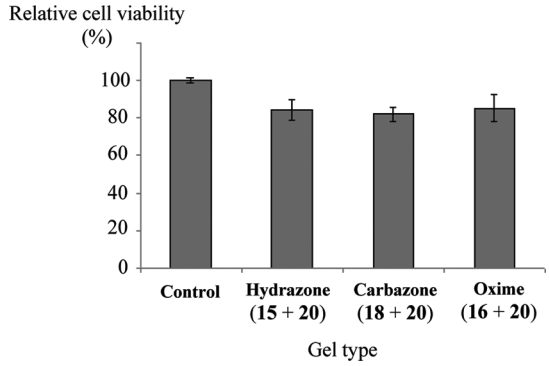


Figure 4. Human dermal fibroblast viability after incubation for 3 days in the presence of 2% w/v gels that were formed by combination of aldehyde-modified HA **20** with different nucleophile-modified HA derivatives. The left bar corresponds to the untreated control. The columns represent the mean ± S.D.; $n = 3$.

Experimental Section

General. 1,1′-Carbonyldiimidazole (CDI), serine methyl eater hydrochloride, ε-caprolactone, DL-dithiothreitol, hydrazine, *N*-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 2,2′-dithiobisethanol **1** were purchased from Aldrich Chemical Co. Hyaluronic acid (HA) sodium salt of the molecular weight 130 kDa was purchased from Lifecore Biomedical. The reagents were used as received. 2,2′-Dithiobis(ethanol-*O*-hydrazinocarbonyl) **1**,[13] 2-aminooxyethanol **7**,[36] *N*-(2-hydroxyethyloxyphthalimide) **9**,[37] and 3,3′-dithiobis(propionic hydrazide) **19**[25] were synthesized according to the literature procedures. All solvents were of analytical quality (p.a.) and were dried over 4 Å molecular sieves. Dialysis membranes Spectra/Por 6 (3500, g/mol cut off) were purchased from VWR International. The NMR experiments (δ scale; *J* values are in Hz) were carried out on Jeol JNM-ECP Series FT NMR system at a magnetic field strength of 9.4 T, operating at 400 MHz for ^{1}H.

6-Hydroxycaproic Acid Hydrazide 3. ε-Caprolactone (4.4 mL, 41.23 mmol) was dissolved in 40 mL of ethanol. Hydrazine (2 mL, 41.23 mmol) was added dropwise to the obtained solution under vigorous stirring. The reaction mixture was heated to reflux for 20 h and then cooled to room temperature. The obtained colorless crystals were filtered off and dried under vacuum. Yield: 3.87 g (26.45 mmol, 64.2%). ^{1}H NMR (D_2O): 3.47 (2H, t, $HOC\underline{H}_2$-, J = 6.6 Hz), 2.10 (2H, t, -$C\underline{H}_2CONHNH_2$, J = 7.3 Hz), 1.52−1.39 (4H, m, -$C\underline{H}_2CH_2C\underline{H}_2$-), 1.25−1.17 (2H, m, -$CH_2C\underline{H}_2CH_2$-).

Disulfanediylbis(ethane-2,1-diyl) Bis(2-(6-hydroxyhexanoyl)hydrazinecarboxylate) 6. 2,2′-Dithiobisethanol **1** (771 mg, 5 mmol) was placed in a round-bottom flask that was degassed and then filled with argon. A total of 15 mL of dichloromethane (DCM) was added to the flask followed by CDI (1.7 g, 10.5 mmol) under stirring. Stirring was continued for another 2 h under argon at room temperature. 6-Hydroxyhexanoic hydrazide **3** (1.46 g, 10 mmol) was dissolved in hot DMF (15 mL) in a separate flask. After cooling to room temperature, DMAP (61 mg, 0.5 mmol) followed by pyridine (0.81 mL, 10 mmol) were added to the DMF solution. The solution of the CDI-activated 2,2′-dithiobisethanol was added dropwise to the solution of 6-hydroxyhexanoic hydrazide over 1 h at room temperature. The mixture was stirred for 18 h and DMF was evaporated under vacuo. The concentrate was coevaporated with toluene and the residue was triturated with a mixture of DCM (200 mL) and diethyl ether (50 mL). The insoluble waxy product was collected by decantation, and dried under vacuo overnight. Yield: 2.34 g (4.69 mmol, 94%). ^{1}H NMR (CD_3OD): 4.35 (4H, t, 2 × -$OC\underline{H}_2CH_2S$-, J = 6.2 Hz), 3.54 (4H, t, 2 × -$C\underline{H}_2OH$, J = 6.4 Hz), 2.98 (4H, m, 2 × -$OCH_2C\underline{H}_2S$-), 2.22 (4H, t, 2 × -$C\underline{H}_2C(O)$-, J = 7.3 Hz), 1.65 (4H, m, 2 × -$C\underline{H}_2CH_2OH$), 1.55 (4H, m, 2 × -$C\underline{H}_2CH_2C(O)$-), 1.40 (4H, m, 2 × -$C\underline{H}_2CH_2CH_2OH$). ^{13}C NMR: 157.0, 63.2, 61.4, 37.0, 33.4, 32.0, 25.1, 25.0.

Disulfanediylbis(ethane-2,1-diyl) Bis(2-(6-((hydrazinecarbonyl)oxy)-hexanoyl)hydrazinecarboxylate) 11. The compound **6** (2.34 g, 4.69 mmol) was coevaporated with dry pyridine two times and finally dissolved in the same solvent (25 mL). CDI (1.83 g, 11.27 mmol) was added to the solution and the reaction mixture was stirred at room temperature for 2 h under an argon atmosphere. The reaction mixture was then cooled to −5 °C on an ice/NaCl bath and hydrazine (455 μL, 9.39 mmol) was added to the mixture in three portions over 40 min while maintaining the temperature at −5 °C. The mixture was stirred at −5 °C for 1.5 h and then allowed to warm up to room temperature for another 17 h. Pyridine was evaporated, and the residue was coevaporated with toluene two times. The residue was redissolved in a minimal amount of methanol and the concentrated methanolic solution was poured into 200 mL of DCM, stirred for 15 min, and then kept for 1 h at −20 °C. The insoluble waxy product was collected by decantation and dried under vacuo overnight. Yield: 2.11 g (3.43 mmol, 73%). ^{1}H NMR (CD_3OD): 4.29 (4H, m, 2 × -$OC\underline{H}_2CH_2S$-), 4.02 (4H, m, 2 × -$C\underline{H}_2OC(O)NHNH_2$), 2.96 (4H, m, 2 × -$OCH_2C\underline{H}_2S$-), 2.21 (4H, m, 2 × -$C\underline{H}_2C(O)$-, J = 7.3 Hz), 1.64 (8H, m, 2 × -$C\underline{H}_2CH_2CH_2$-), 1.42 (4H, m, 2 × -$CH_2C\underline{H}_2CH_2$-). ^{13}C NMR: 174.6, 174.3, 156.9, 64.8, 63.2, 37.1, 33.2, 28.5, 25.1, 24.8.

Disulfanediylbis(ethane-2,1-diyl) Bis(2-hydroxyethoxycarbamate) 7. The procedure was analogous to that for the preparation of **6**. CDI (712 mg, 4.4 mmol) was used for the activation of 2,2′-dithiobisethanol **1** (322 mg, 2.1 mmol) in 6 mL of DCM. 2-Aminooxyethanol **4** (339 mg, 4.4 mmol) and DMAP (25.5 mg, 0.21 mmol) in 12 mL of DMF containing pyridine (0.36 mL, 4.4 mmol) were added to the solution of activated **1**. After 20 h, DMF was evaporated under vacuo. The concentrate was coevaporated with toluene and the residue was triturated with a mixture of DCM (200 mL) and diethyl ether (85 mL). The precipitate (217 mg) was collected by filtration and analyzed by ^{1}H NMR. It consisted of the product **7** in a mixture with imidazole side product. The filtrate was evaporated to dryness and the residue was suspended in 20 mL of DCM. This gave first a turbid solution from which the pure product **8** was then separated as a small liquid phase. The small phase was isolated from the bulk DCM phase yielding 156.4 mg of pure **7** (0.43 mmol, 20.7%). ^{1}H NMR (CD_3OD): 4.36 (4H, t, 2 × -$OC\underline{H}_2CH_2S$-, J = 6.4 Hz), 3.88 (4H, t, 2 × -$NHOC\underline{H}_2$-, J = 4.8 Hz), 3.70 (4H, t, 2 × -$C\underline{H}_2OH$, J = 4.8 Hz), 2.97 (4H, t, 2 × -$C\underline{H}_2S$-, J = 6.4 Hz).

5,14-Dioxo-3,6,13,16-tetraoxa-9,10-dithia-4,15-diazaoctadecane-1,18-diyl Bis(hydrazinecarboxylate) 12. The procedure was analogous to that for the preparation of **11**. The compound **7** (160 mg, 0.44 mmol) was activated with CDI (173 mg, 1.07 mmol) in dry pyridine (3 mL) for 2 h. Hydrazine (65 μL, 1.33 mmol) was added to the activated **7**. After 21 h, pyridine was evaporated and the residue was coevaporated with toluene two times. The residue was redissolved in a minimal amount of methanol and the concentrated methanolic solution was poured into 130 mL of DCM, stirred for 5 min, and then kept for 1.5 h at −20 °C. The precipitate was collected by filtration and dried under vacuo overnight. Yield: 78.5 mg (0.17 mmol, 38.6%). ^{1}H NMR (D_2O): 4.43−4.22 (8H, m, 2 × -$OC\underline{H}_2CH_2S$- and 2 × -$NHOC\underline{H}_2$-), 4.12−3.96 (4H, m, 2 × -$C\underline{H}_2OCONHNH_2$), 3.01−2.90 (4H, t, 2 × -$C\underline{H}_2S$-). ^{13}C NMR: 169.7, 169.5, 74.7, 74.2, 63.1, 37.0.

Dimethyl 2,15-Bis(hydroxymethyl)-4,13-dioxo-5,12-dioxa-8,9-dithia-3,14-diazahexadecane-1,16-dioate 8. 2,2′-Dithiobisethanol **1** (500 mg, 3.24 mmol) was placed in a round-bottom flask that was degassed and then filled with argon. A total of 16 mL of DCM was added to the flask followed by CDI (1.31 g, 8.1 mmol) under stirring. Stirring was continued for another 2 h under argon at room temperature. Serine methyl ester hydrochloride **5** (1.26 g, 8.1 mmol) was added to the reaction mixture followed by triethylamine (1.36 mL, 9.72 mmol). The mixture was stirred for 18 h and evaporated to dryness. The crude material was purified by silica gel flush column chromatography, eluting with 0−6% ethanol in DCM. The product was eluted from the column with 6% ethanol/DCM. Yield: 534 mg (1.2 mmol, 37.1%) for two isolated stereoisomers. ^{1}H NMR ($CDCl_3$): 6.07 and 5.77 (2H, two broad

singlets, 2 × -NH-), 4.43 (4H, t, 4 × -$SCH_2C\underline{H_2}O$-, J = 6.2 Hz), 4.43−4.28 (2H, m, 2 × -C<u>H</u><), 4.04−3.90 (4H, m, 2 × -$C\underline{H_2}OH$), 3.79 and 3.78 (4H, two singlets, 2 × -$OC\underline{H_3}$), 2.97 (4H, t, 2 × -$C\underline{H_2}S$-, J = 6.2 Hz). ^{13}C NMR: 171.1, 156.1, 63.3, 63.2, 56.2, 52.9, 37.9.

Disulfanediylbis(ethane-2,1-diyl) Bis((1-hydrazinyl-3-hydroxy-1-oxopropan-2-yl)carbamate) 13. Compound **8** (400 mg, 0.9 mmol) was dissolved in methanol (9 mL). Hydrazine (135 μL, 2.7 mmol) was added to the mixture, which was then stirred for 2 days at room temperature. During the reaction, the product was precipitated, which was collected by filtration. The solid white material was dried under vacuum. Yield: 191 mg (0.43 mmol, 47.8%). ^{1}H NMR (D_2O): 4.33 (4H, t, 2 × -$SCH_2C\underline{H_2}O$-, J = 5.5 Hz), 4.19 (2H, t, 2 × -C<u>H</u><, J = 5.1 Hz), 3.79 (4H, d, 2 × -$C\underline{H_2}OH$, J = 5.1 Hz), 2.97 (4H, t, 2 × -$C\underline{H_2}S$-, J = 5.5 Hz). ^{13}C NMR: 171.3, 157.9, 63.4, 61.4, 56.0, 37.1.

2-Bis(2-((1,3-dioxoisoindolin-2-yl)oxy)ethyl) O′1,O1-(Disulfanediylbis(ethane-2,1-diyl)) Bis(hydrazine-1,2-dicarboxylate) 10. *N*-(2-Hydroxyethyloxyphthalimide) **9** (904 mg, 4.36 mmol) was placed in a round-bottom flask that was degassed and then filled with argon. A total of 13 mL of DCM was added to the flask followed by CDI (745 g, 4.59 mmol) under stirring. Stirring was continued for another 3 h under argon at room temperature. Dithiobis(ethanol *O*-hydrazinocarbonyl) **2** (512 mg, 1.9 mmol) was dissolved in hot DMF (13 mL) in a separate flask. After cooling to room temperature, DMAP (61 mg, 0.5 mmol) followed by pyridine (0.81 mL, 10 mmol) were added to the DMF solution. The combined solution of **2** and DMAP in DMF−pyridine was added dropwise to the solution of CDI-activated **9** over a period of 10 min at room temperature. The mixture was stirred for 20 h and DMF was evaporated under vacuo. The concentrate was coevaporated with toluene and the residue was worked-up with ethyl acetate−water. The organic phase was separated, washed with water, and finally dried over $MgSO_4$. The solvent was evaporated, yielding yellow oily crude material, which was purified by silica gel flush column chromatography eluting with ethyl acetate−hexane 1:1 (v/v) mixture followed by pure ethyl acetate. Yield: 387 mg (0.53 mmol, 53%). ^{1}H NMR ($CDCl_3$): 8.00−7.73 (8H, m, arom.), 4.59−4.36 (12H, m, 4 × -$C\underline{H_2}OCONH$- and 2 × >$NOC\underline{H_2}$-), 3.08−2.95 (4H, t, 2 × -$C\underline{H_2}S$-).

2-Bis(2-(aminooxy)ethyl) O′1,O1-(Disulfanediylbis(ethane-2,1-diyl)) Bis(hydrazine-1,2-dicarboxylate) 14. Compound **10** (385 mg, 0.52 mmol) was dissolved in a mixture of ethanol and DCM (8 mL, 3:1 v/v). Hydrazine (150 μL, 3.14 mmol) was added to the mixture, which was then stirred for 2 days at room temperature. The formed precipitate was filtered off and the filtrate was evaporated to dryness. After subsequent drying under vacuum, 113 mg of **12** was obtained as a white solid. Yield: 0.24 mmol (45.4%). ^{1}H NMR (D_2O): 4.43−4.18 (8H, m, 4 × -$C\underline{H_2}OCONH$-), 3.96−3.80 (4H, m, 2 × -$C\underline{H_2}ONH_2$), 3.05−2.89 (4H, t, 2 × -$C\underline{H_2}S$-).

Modification of HA with Side Chains Terminated with Chemoselective Nucleophilic Groups. Hyaluronan was dissolved in deionized water at concentration 8 mg/mL. The reagent (**11**, **12**, **13**, or **14**) was added to the HA solution at the reagent/HA disaccharide molar ratio specified in Table 1, and the obtained mixture was stirred until complete dissolution of the reagent. *N*-Hydroxybenzotriazole (HOBt) was separately dissolved in a 1:1 (v/v) mixture of acetonitrile−water at concentration 0.2 M and added to the solution of HA. Molar ratio of HOBt to HA disaccharide was 1 (see Table 1). The pH of the resultant solution was adjusted to 4.7 after which the coupling reaction was initiated by addition of solid EDC (see Table 1 for EDC/HA disaccharide feeding ratio) to the reaction mixture. The mixture was stirred overnight. The pH of the reaction solution was slightly increasing during this time which evidenced the coupling of the reagents to HA carboxylates. The reaction solution was basified to 8.5 with 1 M NaOH and DTT was added to the solution. A 5-fold molar excess of DTT was used relative to the applied reagent (**11**, **12**, **13**, or **14**) to ensure the cleavage of disulfide bond of the reagent. The mixture was again stirred overnight, after which the solution was acidified to 3.5 with 1 M HCl and transferred to a dialysis tube (M_w cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) containing 0.1 M NaCl, followed by dialysis against dilute HCl, pH 3.5 two times, the solution was lyophilized to give the corresponding nucleophile-modified HA **15**, **16**, **17**, or **18**. The incorporation of nucleophile-terminated side chains was verified by ^{1}H NMR. Specifically, the peaks corresponding to the native HA protons, such as acetamide protons at 1.9 ppm, 2′-, 3′-, 4′-, 5′-, and 6′-protons of HA disaccharide unit at 3.2−4.0 ppm, as well as anomeric 1′-protons at 4.4 ppm, were compared with newly appeared peaks of the grafted side chains. Additionally, the presence of free nucleophile-terminated side chains was confirmed spectrophotometrically by reaction with trinitrobenzene sulfonic acid (TNBS).[38]

Synthesis of Aldehyde-Modified HA 20. HA-serine derivative **17** (370 mg, 0.96 mmol of disaccharide units) was dissolved in 45 mL of deionized water. A 0.15 M aqueous solution of sodium periodate (5.8 mL, 0.96 mmol) was added to the HA-serine solution, which caused formation of a gel. The gel was diluted with 60 mL of water, which led to the partial dissolution of the gel. After 5 min, ethylene glycol (0.5 mL, 9.6 mmol) was added to the mixture. The mixture was stirred in the dark overnight during which the gel was completely dissolved. The reaction solution was dialyzed against pure water two times (M_w cutoff = 3500) and finally lyophilized. Yield: −347 mg (94.4%). The amount of aldehyde groups was obtained by reaction with *tert*-butyl carbazate (TBC) followed by reduction with $NaBH_3CN$. Briefly, HA-aldehyde (∼20 mg) was dissolved in 2 mL of water and to this solution was added the 0.5 M aqueous solution of TBC (10-fold excess per molar amount of sodium periodate that was used in the preparation of HA-aldehyde derivative). The mixture was stirred for 1 h at room temperature after which a 0.5 M aqueous solution of $NaBH_3CN$ (equimolar amount to that of TBC) was added to the mixture. The mixture was allowed to react for 24 h at room temperature. The TBC-modified HA was recovered by dialysis in 3500 MW cut off tubing against water twice. ^{1}H NMR of the obtained product was examined and the peak corresponding to the *tert*-butyl substituent (($C\underline{H_3})_3$COCONHNH-, δ = 1.38 ppm) was compared with the peak of HA acetamide protons at 1.9 ppm. Degree of aldehyde functionalization in **20** (∼5%) was calculated from the amount of reacted TBC reagent.

Hydrogel Formation and Characterization. HA hydrogels were prepared in cylindrical glass vials (10 mm in diameter) from 2% w/v precursor solutions listed in Table 2. In a typical gelation experiment, HA-aldehyde and HA-nucleophile derivatives were dissolved separately in water and then mixed by addition of the HA-nucleophile solution (0.5 mL) to the HA-aldehyde solution (0.5 mL), followed by brief vortexing for several seconds. Gelation time was determined by a flow test as the point at which the mixture would no longer flow under the force of gravity. The gels were left in the capped vials for another 4 h to complete cross-linking reactions. The cylindrically shaped hydrogels were then carefully transferred from the vials and applied to the bottom plate of AR2000 rheometer (TA Instruments, Inc., U.K.). The mechanical properties were measured at a frequency of 0.1 to 10 Hz at 25 °C using an 8 mm aluminum plate geometry. The gap was adjusted starting from the original sample height and compressing the sample to reach a normal force of about 50 mN (gap sizes were between 7 and 8 mm).

Enzymatic Hydrogel Degradation. In 4 h after mixing of the corresponding components, the hydrogels were swollen in PBS for 24 h and the swelling buffer then was diluted with HAse solution such that the final concentration of hyaluronidase was 500 U/mL. The hydrogels were then stored in PBS buffer containing HAse at room temperature for several days.

Cell Culture. Human dermal fibroblasts (hDFns) were cultured in complete DME/F12 medium (Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham with L-glutamine, 15 mM HEPES, and sodium bicarbonate (DME/F12) supplemented with 10% fetal bovine serum). Cells were maintained at 37 °C in 5% CO_2 and used at passages 4 and 5.

Cytotoxicity of the Individual Gel Components. hDFns were seeded in 24-well plates at concentration 5 × 10^4 cells/well in 1 mL of complete medium and grown at 37 °C, 5% CO_2. After 24 h, the old medium was replaced with fresh medium containing 0.1 or 0.6% of the HA derivatives. The HA derivatives were dissolved directly in cell culture

medium and then passed through 0.8 μm sterile filter before being added to the cells. Fibroblasts grown in complete cell culture medium were used as a control. Cells grown in plain cell culture medium were used as a positive control. After 48 h of culturing, cytotoxicity of the individual components was examined by performing thiazolyl blue tetrazolium bromide (MTT) assay. MTT dye was dissolved in PBS at a concentration of 5 mg/mL and passed through a 0.22 μm sterile filter. A total of 100 μL of the sterile MTT solution was added to each well and the plate was incubated at 37 °C, 5% CO_2. After 4 h the medium was carefully removed and the dark blue crystals were dissolved in 1 mL of DMSO. The absorbance was measured at 570 nm, and the results were compared with that of the control wells to determine relative cell viability.

Cytotoxicity of the Gels. The 2% w/v solutions of the corresponding gel components were placed in two separate 1 mL syringes, the tips of which were subsequently interconnected by means of a luer-lock adapter. The solutions were mixed by passing the contents of the syringes from one to another. After 20 passages, the hydrogels were formed and extruded from the syringes into cell culture inserts (Falcon, 1.0 μm pore size). hDFns were seeded in 24-well plates at concentration of 50000 cells/well in 1 mL complete medium and grown at 37 °C, 5% CO_2. After 24 h, the medium was changed and the inserts with 0.2 mL of the hydrogels were added in contact with the medium. After 3 days of culturing, the cytotoxicity of hydrogels was evaluated by performing an MTT assay analogously to the cytotoxicity analysis of the individual gel components.

Conclusion

We elaborated a method for mild and highly controlled functionalization of HA with chemoselective nucleophilic functionalities. The method is based on the development of novel divalent symmetric protecting group, cleavage of the central disulfide bond of which causes the formation of the unstable 2-thioethyl carbamate under basic conditions. The internal nucleophilic attack of the generated thiolate anion on the α-carbon atom of the 2-thioethyl moiety leads to the loss of 1 equiv of ethylene episulfide and 1 equiv of gaseous carbon dioxide, thus, liberating the free amine-type group. We established a convenient synthesis of several symmetric dimeric reagents with a central disulfide-based divalent protecting group. Using low submolar amounts of the above modifying reagents and condensing agents, we obtained novel HA derivatives that were quantitatively functionalized with chemoselective nucleophilic functionalities, such as hydrazide, aminooxy, and carbazate. To the best of our knowledge, this work is the first attempt to graft free functional aminooxy and carbazate groups to HA. We also converted the appended serine side chains of the newly synthesized HA-serine derivative into corresponding aldehyde groups using the conditions that do not affect the structure of the HA backbone. This procedure, based on very fast and selective periodate oxidation of the 2-aminoalcohol side groups, is a convenient alternative to the existing method of glucosaminoglycan (GAG) functionalization with aldehyde groups. It is not destructive to GAGs in terms of molecular weight and provides the reactive 2-oxoaldehyde group on HA. Upon reaction with different amine-type functionalities, the 2-oxoaldehyde group forms the resonance stabilized -C═N- cross-linkages, thus, preventing hydrolytic degradation of the corresponding hydrogels. These hydrogels, however, remain completely degradable upon action of HA degrading enzyme, hyaluronidase. The hydrogels described in this study showed no apparent toxic effect and therefore can be considered as potential candidates in various biomedical applications.

Supporting Information Available. ^{1}H NMR spectra and structures of the reagents **12** and **14** (Figure S1) and swelling ratios of hydrogels as a function of incubation time in PBS buffer (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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