Poly(vinyl alcohol) Cross-Linkers for in Vivo Injectable Hydrogels

Dmitri A. Ossipov,* Sonya Piskounova, and Jöns Hilborn

Polymer Chemistry, Material Chemistry Department, Uppsala University, S-75121 Uppsala, Sweden

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ABSTRACT: New poly(vinyl alcohol) (PVA) derivatives containing different pendant chemoselective functionalities have been prepared for the in situ formation of hydrogels at physiological conditions. Particularly, incorporations of thiol, cysteine 1,2-aminothiol, and aminooxy side chains to PVA were performed for the first time by direct coupling of the protected nucleophilic functionalities to PVA's hydroxyl groups via carbamate linkages followed by acidic deprotection. In the second approach, PVA was first derivatized to a low degree (3%) with amino groups which were used to quantitatively react with different *N*-hydroxysuccinimide esters of carboxylic acids containing maleimide, α-iodoacetyl, or acrylate thiophilic functionalities. The utility of the amino-derivatized PVA was also demonstrated for further functionalization with semicarbazide terminated side chains. The ability of the new PVA derivatives to act as multifunctional cross-linking agents was examined in the course of in situ cross-linking reactions with hyaluronic acid carrying aldehyde groups. Use of multifunctional PVA cross-linkers was shown to give short gelation times, i.e., within half a minute, which is critical for clinical applications. The hyaluronan hydrogels were enzymatically degradable as evidenced by the results of in vitro degradation by hyaluronidase. Moreover, these hydrogels were found to be nontoxic to human dermal fibroblasts. Hence, PVA-based multifunctional cross-linkers can extend the scope of in situ preparation and properties of hydrogel-based synthetic mimics of extracellular matrix as compared with well established bifunctional poly(ethylene glycol) analogs.

Introduction

Poly(vinyl alcohol) is a hydrophilic polymer and can be used for the preparation of biocompatible hydrogels.[1] Mechanically robust PVA hydrogels with high water content have been used in several biomedical applications including drug delivery,[2] contact lenses,[3] and for tendon[4] and cartilage[5] repair. Recently, hydrogels formed in situ under physiological conditions have received much attention because they are amenable to minimally invasive surgical technique, allow preparation of complex shapes, and permit homogeneous loading of bioactive compounds. In vivo cross-linking reactions are, however, especially demanding, requiring tolerance to water, gelation within a minute for clinical applicability, formation of nontoxic gels with no formation of low molecular weight side products, and storage stability of the gel-forming substances prior transformation to gel in vivo. Anseth et al. prepared in situ forming degradable PVA hydrogels by photopolymerization of PVA equipped with acrylate side chains on hydrolytically cleavable ester groups.[6–9] When loaded with chondrocytes, these gels were shown to enhance cartilage formation in vitro.[9] Photocross-linked PVA hydrogels carrying cell adhesive (RGD) peptide side chains were prepared by West at al. and shown to support spreading of human dermal fibroblasts on the surface of these gels.[10] Despite the promising technology, there are, however, inherent limitations associated with photocuring process such as use of potentially toxic photoinitiators, generation of highly reactive radicals in vivo, limited accessibility of light, and eventual exothermic effect of photo reactions. In many cases, photopolymerization has not yet been proven suitable for injectable in vivo use, despite the development of a transdermal photoactivation methodology.[11]

Chemoselective covalent cross-linking procedures were recently proposed for production of injectable synthetic matrices to controllably deliver growth factors while simultaneously gradually giving way to permit tissue regeneration. Two classes of in vivo resorbable materials have been developed: (1) completely synthetic scaffolds that comprise either hydrolytically labile or enzymatically degradable peptide cross-linkages, and (2) naturally derived components of extracellular matrix (ECM) chemically premodified with cross-linkable functionalities and degrading in vivo by virtue of endogenous enzymes. For example, Michael addition between thiols and either acrylates or vinyl sulfones was utilized for in situ formation of poly(ethylene glycol) (PEG)-based hydrogels composed of cleavable ester linkages[12] or bis-cysteine peptides with sequences that are sensitive to matrix metalloproteases (MMPs).[13] Hydrogel prepared from thiol-modified dextran and PEG tetraacrylate was designed to degrade through hydrolysis of the ester bond between the dextran thioether and PEG.[14] Hydrogels containing hydrolytically cleavable ester linkages were also prepared from dextrans that were variously functionalized with vinyl sulfone pendant groups and subsequently cross-linked with the help of linear or four-arm mercaptopoly(ethylene glycol).[15,16] Prestwich et al. pioneered modification of hyaluronic acid (HA),[17] a major nonsulfated glucosaminoglycan constituent of ECM, by HA thiolation making it capable of cross-linking with PEG diacrylate[18] or PEG bis-maleimide[19] for in situ production of hydrogel scaffolds that are biodegradable by hyaluronidase. Gelation of thiolated HA with RGD-peptide containing triblock acrylate−PEG−CC(PEG-acrylate)RGDS cross-linker[20] or combination of thiol-modified HA with thiol-modified gelatin[21,22] allowed to construct cytoadherent synthetic ECM mimics for tissue engineering. An alternative functionalization, when electrophilic acrylate groups are localized on HA, whereas the sulfhydryl groups are on PEG[23,24] or bis-cysteine peptide cross-linker,[25] was also examined for hydrogel preparation by Michael addition. Formation of reversible thiozolidine cross-linkages between cysteine 1,2-aminothiol terminated dendron and PEG dialdehyde was used to render a hydrogel that stays intact for approximately 1 week after preparation.[26] Finally, hydrazone formation is among other few cross-linking chemistries that were suggested for in vivo production of hydrogel materials.[27–29] Thus, both hydrolytically (due to hydrolysis of hydrazone linkages) and enzymatically degradable hydrogels were prepared from hy-

* Corresponding author. E-mail: dmitri.ossipov@mkem.uu.se. Fax: +46-18-4713477. Telephone: +46-18-4715836.

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drazide-modified HA and PEG dialdehyde[27,28] and used for wound healing.[29]

For practical applicability in the surgical setting, the liquid-to-gel transformation upon injection of liquid formulation must be rapid (within a minute). A limited number of cytocompatible cross-linkers with suitable reaction kinetics are available for preparation of injectable hydrogels. This issue was addressed by changing the reactivity of thiophilic bis-functional cross-linkers.[19] Another approach to optimally decrease gelation time and exclude the risk that the hydrogel may not be formed, displaced, or diluted at the injection/deposition site is the use of multifunctional cross-linkers. This approach was very recently realized for HA by its modification into two HA derivatives comprising side chains terminated with electrophilic aldehyde and nucleophilic hydrazide chemoselective functionalities respectively.[30–32] Additionally, formation of hybrid hydrogels can be achieved by multiple placing mutually reactive nucleophile−electrophile pairs on different polymers with different chemical structure of their backbones which would allow for tuning degradation rate in vivo.

In this report, we describe a straightforward approach for incorporation of different types of the orthogonal chemoselective functionalities into PVA with low degree of substitution, thus providing a plethora of multifunctional PVA-based cross-linkers as alternative to well established bis-functional PEG analogs. Compared to PEG, PVA cross-linkers can provide gels from HA with lower degrees of functionalization and shorten the time for gel formation. The HA/PVA hybrid hydrogels are also advantageous in terms of mechanical properties, because multifunctional PVA provides higher portion of elastically active intermolecular cross-links than PEG does. In the latter case intermolecular cross-linking is substantially accompanied by the formation of intramolecular loops that do not take part in network formation. Additionally, the amount of "clicking" functionalities can be widely varied, permitting greater manipulation of the mechanical properties of the hydrogel. Moreover, diverse functionalization of relatively low reactive PVA hydroxyl groups provides many more available sites for attachment of additional bioactive molecules before cross-linking procedure.

Experimental Part

General. 1,1′-Carbonyldiimidazole (CDI), trifluoroacetic acid (TFA), acrylic acid *N*-hydroxysuccinimide ester, DL-dithiothreitol (DTT), and *N*-(methoxycarbonyl)maleimide were purchased from Aldrich Chemical Co. (*N*-*tert*-Butyloxycarbonyl)-*S*-tritylcysteine (Boc-Cys(Trt)-OH), 3-maleimidopropionic acid *N*-hydroxysuccinimide ester, and PVA with weight average molecular weight (M_w) 16 000 g/mol (degree of deacetylation 98.0−98.8%) were purchased from Fluka. The reagents were used as received. 2-(2-Pyridyldithio)-ethylamine hydrochloride,[33] *N*-Boc-*S*-trityl-*N*-2-aminoethyl-L-cysteinamide,[34] *tert*-butyl 3-aminopropoxycarbamate,[35] (*N*-*tert*-butyloxycarbonyl)ethylene diamine,[36] 2-maleimidoacetic acid *N*-hydroxysuccinimide ester,[37] 2-iodoacetic acid *N*-hydroxysuccinimide ester,[38] and *tert*-butyl phenyl hydrazodiformate[39] were synthesized according to literature. Aldehyde-modified PVA **14** and hydrazide-modified PVA **15** were prepared as previously described.[40] Hyaluronic acid sodium salt from *Streptococcus equi* with nominal molecular weight (M_w) 1.3 MDa was purchased from Fluka. All solvents were of analytical quality (p.a.) and were dried over 4Å molecular sieves. Dialysis membranes Spectra/Por 6 (1000, 3500, and 25 000 g/mol cutoff) 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. Human dermal fibroblasts (hDFn) were purchased from European Collection of Cell Cultures (ECACC).

Synthesis of Thiolated PVA 1. PVA (200 mg, 0.0125 mmol, 4.5 mmol of OH groups) was dissolved in dry DMSO (4 mL) under heating and the solution was dried by addition of dry toluene (∼0.5 mL) followed by its azeotropic distillation at atmospheric pressure using a Dean−Stark trap before starting the reaction. CDI (365 mg, 2.25 mmol) was added in one portion to the magnetically stirred PVA solution under argon atmosphere at room temperature. The reaction mixture was then stirred under argon at room temperature for another 2.5 h. 2-(2-Pyridyldithio)ethylamine hydrochloride[33] (100.2 mg, 0.45 mmol) followed by triethylamine (63 μL, 0.45 mmol) was added to the reaction solution. Stirring was continued at room temperature and under argon atmosphere overnight. Afterward, 2 mL of concentrated aqueous NH_3 was added, and the mixture was stirred for 45 min at room temperature. Finally, the reaction mixture was diluted with 10 mL of water, filtered until clear, and reduced to ∼4 mL volume by rotary evaporation. The residual gel material swollen in DMSO was treated with DTT (694 mg, 4.5 mmol) under exclusion of oxygen which led to gel degradation within 10 min. Clear DMSO solution was stirred with DTT under argon for 48 h more. The solution was diluted with dilute HCl (pH 3) and transferred to 1000 M_w cutoff dialysis tubing. Dialysis was performed twice against dilute HCl (pH 3). The dialyzed solution was subsequently freeze-dried to give white solid product, 4.5% PVA−(OCONHCH$_2$CH$_2$SH)$_n$ (**1**). DS: 0.045; M_w ∼ 17670; ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of the modified unit), 4.00−3.70 (1H, m, polymer backbone CH of the unmodified unit), 3.23−3.14 ((DS × 2)H, m, −C*H*$_2$CH$_2$SH), 2.59−2.49 ((DS × 2)H, m, −CH$_2$C*H*$_2$SH), 2.00−1.35 (2H, m, polymer backbone CH$_2$). Yield: 211.0 mg (0.0119 mmol, 95.5%).

Synthesis of Cysteine-Modified PVA 3. PVA (200 mg, 0.0125 mmol, 4.5 mmol of OH groups) was dissolved in dry DMSO (4 mL) under heating, and the solution was dried as described before. CDI (365 mg, 2.25 mmol) was added in one portion to the magnetically stirred PVA solution under argon atmosphere at room temperature. The reaction mixture was then stirred under argon at room temperature for another 2.5 h. *N*-Boc-*S*-trityl-*N*-2-aminoethyl-L-cysteinamide[34] (227.5 mg, 0.45 mmol) was added to the reaction solution, and the resulting mixture was stirred overnight at room temperature. Afterward, 2 mL of concentrated aqueous NH_3 was added, and the mixture was stirred for 45 min at room temperature. Finally, the reaction mixture was diluted with 10 mL of water, stirred for 1.5 h more, and reduced to ∼4 mL volume by rotary evaporation. The residual DMSO solution that contained the product with protected amino and thiol groups was precipitated by addition of 50 mL of water. The precipitate was filtered off and treated with TFA (4 mL) for 30 min which led to complete solubilization of the material. TFA was then evaporated, and the residue was triturated with diethyl ether to give the intermediate PVA derivative **2**, 5.1% PVA−(OCONHCH$_2$CH$_2$NHCOCH(NH$_2$)CH$_2$STr)$_n$, as a white solid. DS: 0.051; M_w ∼ 23750; ^{1}H NMR (D_2O): 7.50−6.70 ((DS × 15)H, m, Tr aromatic protons), 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of the modified unit), 4.10−3.65 ((1 + DS × 1)H, m, polymer backbone CH of the unmodified unit + −COC*H*(NH$_2$)CH$_2$STr), 3.65−3.30 ((DS × 4)H, m, −NHC*H*$_2$ C*H*$_2$NH−), 2.75−2.50 ((DS × 2)H, m, −C*H*$_2$STr), 2.10−1.35 (2H, m, polymer backbone CH$_2$). Yield: 222.0 mg (0.0094 mmol, 80.0%).

The solid PVA **2** (222 mg, 0.0094mmol, 0.173 mmol of STr groups) was dissolved in TFA/water (5 mL, 4:1) and dichloromethane (1 mL) followed by triisopropylsilane (75 μL) was added to the solution. The two-phase mixture was stirred vigorously for 15 min at room temperature. The mixture was then evaporated, coevaporated with water, and redissolved in 30 mL of water again, and the insoluble material was filtered off. The filtrate was dialyzed against dilute HCl (pH 3.0) two times. The dialyzed solution was subsequently freeze-dried to give the white solid product, 5.1% PVA−(OCONHCH$_2$CH$_2$NHCOCH(NH$_2$)CH$_2$SH)$_n$ (**3**). DS: 0.051; M_w ∼ 19450; ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of the modified unit), 4.07−4.02 ((DS × 1)H, m, −C*H*(NH$_2$)CH$_2$SH), 3.99−3.58 (1H, m, polymer backbone CH of the unmodified unit), 3.40−3.03 ((DS × 4)H, m, −NHC*H*$_2$ C*H*$_2$NH−), 2.96−2.89 ((DS × 2)H, m,

−C*H*$_2$SH), 2.02−1.35 (2H, m, polymer backbone CH$_2$). Yield: 156.0 mg (0.008 mmol, 85.3%).

Synthesis of Aminooxy-Modified PVA 4. PVA (207 mg, 4.66 mmol of hydroxyl groups) was dissolved in dry DMSO (4 mL), and the solution was dried as described before. CDI (378 mg, 2.33 mmol) was added in one portion to the magnetically stirred PVA solution under argon atmosphere at room temperature. The reaction mixture was then stirred for another 2.5 h. *tert*-Butyl 3-aminopropoxycarbamate[35] (89 mg, 0.46 mmol) in 2 mL of DMSO was added to the reaction solution, and the resulting mixture was stirred overnight at room temperature. Afterward, 2 mL of concentrated aqueous NH_3 was added, and the mixture was stirred for 45 min at room temperature. Finally, the reaction mixture was diluted with 25 mL water, stirred for 2 h, and reduced to ∼6 mL volume by rotary evaporation. The substituted polymer was precipitated from the residual DMSO solution by adding 10-fold excess of an 80/20 (v/v) mixture of diethyl ether/ethanol. The precipitated polymer was then directly deprotected by treatment with TFA/water (8 mL, 95:5) for 1 h at room temperature. The reaction solution was evaporated to dryness and 20 mL of water was added to the remaining residue. The pH was adjusted to 10 which caused complete solubilization of the material (it was poorly soluble at acidic pH). The solution was filtered, and the filtrate was dialyzed 1000 M_w cutoff tubing against water two times, and finally freeze-dried to give 4.6% PVA−(OCONHCH$_2$CH$_2$CH$_2$ONH$_2$)$_n$ (**4**). DS: 0.046 M_w ∼ 17920; ^{1}H NMR (D$_2$O): 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.00−3.60 (1H, m, polymer backbone CH of the unmodified unit), 3.65 ((DS × 2)H, t, H$_2$NOC*H*$_2$−), 3.11−3.03 ((DS × 2)H, m, H$_2$NOCH$_2$CH$_2$C*H*$_2$−), 2.00−1.35 ((2 + DS × 2)H, m, polymer backbone CH$_2$ + H$_2$NOCH$_2$C*H*$_2$CH$_2$−). Yield: 124.0 mg (0.007 mmol, 53.6%).

Synthesis of Amino-Modified PVA 6. PVA (800 mg, 0.05 mmol, 18 mmol of OH groups) was dissolved in dry DMSO (16 mL) under heating and the solution was dried as described before. CDI (1.56 g, 9 mmol) was added in one portion to the magnetically stirred PVA solution under argon atmosphere at room temperature. The reaction mixture was then stirred under argon at room temperature for another 2.5 h. (*N*-*tert*-Butyloxycarbonyl)ethylenediamine[36] (177.6 mg, 1.11 mmol) in 6 mL of DMSO was added to the reaction solution, and the resulting mixture was stirred overnight at room temperature. Afterward, 8 mL of concentrated aqueous NH_3 was added, and the mixture was stirred for 45 min at room temperature. Finally, the reaction mixture was diluted with 50 mL water, stirred for 2 h, and reduced to ∼16 mL volume by rotary evaporation. The substituted polymer was precipitated from the residual DMSO solution by adding 10-fold excess of an 80/20 (v/v) mixture of diethyl ether/ethanol. The precipitated polymer was redissolved in a small amount of water and dialyzed twice against water in 1000 M_w cutoff tubing. The dialyzed solution was subsequently freeze-dried to give the white solid intermediate PVA derivative **5**, 3.2% PVA-(OCONHCH$_2$CH$_2$NHBoc)$_n$. DS: 0.032; M_w ∼ 18 175; ^{1}H NMR (D$_2$O): 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.00−3.60 (1H, m, polymer backbone CH of the unmodified unit), 3.19−3.09 ((DS × 4)H, m, −NHC*H*$_2$C*H*$_2$NH−), 2.00−1.35 (2H, m, polymer backbone CH$_2$), 1.4 ((DS × 9)H, m, Boc). Yield: 727.0 mg (0.04 mmol, 80.0%).

To the solid 3.2% PVA−(OCONHCH$_2$CH$_2$NHBoc)$_n$ (**5**) (720 mg, 0.0396 mmol, 0.464 mmol of NHBoc groups) was added TFA/water (17.5 mL, 95:5), and the mixture was shaken for 1 h at room temperature. After 1 h the reaction solution was evaporated to dryness and coevaporated with water. The residue was redissolved in water and the pH was adjusted to 10 which caused complete solubilization of the material (it was poorly soluble at acidic pH). The solution was then dialyzed twice against water in 1000 M_w cutoff tubing, filtered, and finally freeze-dried to give 3.0% PVA−(OCONHCH$_2$CH$_2$NH$_2$)$_n$ (**6**). DS: 0.03 M_w ∼ 16930; ^{1}H NMR (D$_2$O): 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.08−3.66 (1H, m, polymer backbone CH of the unmodified unit), 3.20−3.10 ((DS × 2)H, m, −CONHC*H*$_2$CH$_2$NH$_2$), 2.80−2.66 ((DS × 2)H, m, −CONHCH$_2$C*H*$_2$NH$_2$), 2.00−1.35 (2H, m, polymer backbone CH$_2$). Yield: 421.0 mg (0.025 mmol, 62.9%).

General Procedure for Acylation of Amino-Modified PVA 6. 3.0% PVA−(OCONHCH$_2$CH$_2$NH$_2$)$_n$ (**6**) (62.4 mg, 0.0037 mmol, 0.04 mmol of NH$_2$ groups) was dissolved in 12 mL of 10 mM $Na_2B_4O_7$ buffer (pH 8.5), while the corresponding carboxylic acid NHS ester (0.4 mmol) was dissolved in 1.5 mL of acetonitrile. These solutions were mixed by adding the reagent/acetonitrile solution to the aqueous solution of amino-modified PVA **6**. The mixture was stirred overnight at room temperature. The mixture was then filtered and the filtrate was dialyzed twice against water in 1000 M_w cutoff tubing, and finally freeze-dried.

*Maleimidoacetyl-Derivatized PVA **7***. Amino-modified PVA **6** (135.6 mg, 0.0078 mmol, 0.123 mmol of NH$_2$ groups), and 2-maleimidoacetic acid *N*-hydroxysuccinimide ester[37] (310.2 mg, 1.23 mmol) were used in the synthesis to give the PVA derivative **7**. DS: 0.044 M_w ∼ 19500; ^{1}H NMR (D$_2$O): 6.83 ((DS × 2)H, s, maleimide), 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.12 ((DS × 2)H, s, NC*H*$_2$CO), 4.00−3.57 (1H, m, polymer backbone CH of the unmodified unit), 3.25−3.02 ((DS × 4)H, 2 × m, −NHC*H*$_2$C*H*$_2$NH−), 2.00−1.36 (2H, m, polymer backbone CH$_2$). Yield: 126.0 mg (0.00645 mmol, 82.6%).

*Maleimidopropionyl-Derivatized PVA **8***. Amino-modified PVA **6** (62.4 mg, 0.0037 mmol, 0.04 mmol of NH$_2$ groups) and 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (106.5 mg, 0.4 mmol) were used in the synthesis to give the PVA derivative **8**. DS: 0.03 M_w ∼ 18550; ^{1}H NMR (D$_2$O): 6.85 ((DS × 2)H, s, maleimide), 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.08−3.70 ((1 + DS × 2)H, m, polymer backbone CH of unmodified unit + NC*H*$_2$CH$_2$CO), 3.26−3.16 ((DS × 4)H, m, −NHC*H*$_2$C*H*$_2$NH−), 2.53−2.47 ((DS × 2)H, m, NCH$_2$C*H*$_2$CO), 2.04−1.47 (2H, m, polymer backbone CH$_2$). Yield: 53.0 mg (0.00286 mmol, 77.5%).

*Acrylamide-Derivatized PVA **9***. Amino-modified PVA **6** (62.4 mg, 0.0037 mmol, 0.04 mmol of NH$_2$ groups) and acrylic acid *N*-hydroxysuccinimide ester (67.7 mg, 0.4 mmol) were used in the synthesis to give the PVA derivative **9**. DS: 0.03 M_w ∼ 17550; ^{1}H NMR (D$_2$O): 6.27−6.19 ((DS × 2)H, m, C*H*$_2$=CH−), 5.78 ((DS × 1)H, d, CH$_2$=C−, J = Hz), 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of the modified unit), 4.09−3.70 (1H, m, polymer backbone CH of unmodified unit), 3.40−3.32, 3.32−3.24 ((DS × 4)H, 2 × m, −NHC*H*$_2$C*H*$_2$-NH−), 2.04−1.48 (2H, m, polymer backbone CH$_2$). Yield: 53.3 mg (0.00304 mmol, 82.5%).

*2-Iodoacetamide-Derivatized PVA **10***. Amino-modified PVA **6** (62.4 mg, 0.0037 mmol, 0.04 mmol of NH$_2$ groups) and 2-iodoacetic acid *N*-hydroxysuccinimide ester[38] (113.5 mg, 0.4 mmol) were used in the synthesis to give the PVA derivative **10**. DS: 0.03 M_w ∼ 18740; ^{1}H NMR (D$_2$O): 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal, polymer backbone CH of modified unit), 3.97−3.60 (1H, m, polymer backbone CH of the unmodified unit), 3.64 ((DS × 1)H, s, −COC*H*$_2$I), 3.24−3.11 ((DS × 4)H, 2 × m, NHC*H*$_2$C*H*$_2$NH−), 2.04−1.48 (2H, m, polymer backbone CH$_2$). Yield: 55.0 mg (0.00294 mmol, 79.6%).

Synthesis of Maleimide-Modified PVA 11. Amino-modified PVA **6** (61 mg, 0.0036 mmol, 0.039 mmol of NH$_2$ groups) was dissolved in 5 mL of 1 M $NaHCO_3$, and the solution was treated with *N*-methoxycarbonylmaleimide (60.5 mg, 0.39 mmol) at 0 °C for 5 min. After 5 min the mixture was diluted with mixture of water and acetonitrile (5 mL, 1:1), and then stirred for another 1 h at room temperature. The mixture was neutralized with diluted HCl to pH 6.0 and then dialyzed against water in 1000 M_w cutoff tubing. Freeze-drying of the dialyzed solution afforded the mixture of maleimide-modified PVA **11** and the imide−amide intermediate (see Results and Discussion for details). DS = DS$_{maleimide}$ + DS$_{intermediate}$ = 0.012 + 0.018 M_w ∼ 17170; ^{1}H NMR (D$_2$O): 6.80 ((DS$_{maleimide}$ × 2)H, s, maleimide), 6.41 and 5.92 ((DS$_{intermediate}$ × 1)H, 2 × d, −CH=), 6.22 and 5.84 ((DS$_{intermediate}$ × 1)H, 2 × d, −CH=), 4.90 ((DS × 1)H, m partially overlapped with H$_2$O signal,

polymer backbone CH of the modified unit), 4.00−3.50 ((1 + DS × 2 + $DS_{intermediate}$ × 3)H, m, polymer backbone CH of the unmodified unit + NCH_2CH_2NH- + OCH_3), 3.28−3.11 ((DS × 2)H, m, NCH_2CH_2NH-), 2.04−1.35 (2H, m, polymer backbone CH_2). Yield: 54.8 mg (0.00319 mmol, 88.6%).

Synthesis of Semicarbazide-Modified PVA 13. Amino-modified PVA **6** (57.7 mg, 0.0034 mmol, 0.037 mmol of NH_2 groups) was dissolved in 12 mL of 10 mM $Na_2B_4O_7$ buffer (pH 8.5), while *tert*-butyl phenyl hydrazodiformate[39] (506.5 mg, 2.0 mmol) was dissolved in 2.5 mL of DMSO. These solutions were mixed by adding the reagent/DMSO solution to the aqueous solution of amino-modified PVA **6**. Five mL of acetonitrile was then added to the mixture in order to solubilize it. The mixture was stirred overnight at room temperature. It was then filtered, and the filtrate was dialyzed twice against water in 1000 M_w cutoff tubing, and finally freeze-dried to give the white solid Boc-protected semicarbazide PVA **12**, 3.0% PVA−$(OCONHCH_2CH_2NHCONHNHBoc)_n$. DS: 0.03; $M_w \sim 18\,640$; ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of the modified unit), 4.00−3.60 (1H, m, polymer backbone CH of the unmodified unit), 3.22−3.07 ((DS × 4)H, m, $-NHCH_2CH_2$-NH−), 2.00−1.30 (2H, m, polymer backbone CH_2), 1.39 ((DS × 9)H, m, Boc). Yield: 42.2 mg (0.00226 mmol, 66.7%).

To the solid Boc-protected semicarbazide PVA **12** (42 mg, 0.00225 mmol, 0.024 mmol of NHBoc groups) was added TFA/water (10 mL, 95:5), and the mixture was shaken for 1 h at room temperature. After 1 h the reaction solution was evaporated to dryness and coevaporated with water. The residue was redissolved in water, and the pH was adjusted to 9 which caused complete solubilization of the material (it was poorly soluble at acidic pH). The solution was then dialyzed twice against water in 1000 M_w cutoff tubing, filtered, and finally freeze-dried to give 3.0% PVA−$(-(OCONHCH_2CH_2NHCONHNH_2)_n$ **13**. DS: 0.03 $M_w \sim$ 17250; ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of the modified unit), 4.06−3.60 (1H, m, polymer backbone CH of unmodified unit), 3.18−3.06 ((DS × 4)H, m, $-NHCH_2CH_2NH-$), 2.00−1.35 (2H, m, polymer backbone CH_2). Yield: 33.5 mg (0.0021 mmol, 91.5%).

Synthesis of Aldehyde-Modified HA 16. Chemical modification of HA was carried out in aqueous conditions following previously described procedure.[32] Hyaluronic acid (500 mg, 1.34 mmol of disaccharide repeating units) was dissolved in deionized water (100 mL), 0.5 M aqueous solution of sodium periodate (2.7 mL corresponding to 0.5 of periodate per HA repeating unit) was added dropwise, and the mixture was stirred for 2 h at room temperature in the dark. Ethylene glycol (4 equiv) was then added to inactivate any unreacted periodate. The reaction was stirred for 1 h at ambient temperature, and the solution was purified by dialysis in 25 000 MW cutoff tubing against water twice. The dry product was obtained by freeze-drying.

The weight average molecular weight (M_w) of aldehyde-modified HA **16** was measured using static light scattering on a Hamamatsu photon-counting device with a 3 mW He−Ne laser. Toluene was used as a reference (Rayleigh ratio, $R_{tol} = 13.59 \times 10^{-6}$ cm^{-1} at 633 nm. The optical constant for vertically polarized light is $K = 4\pi^2 n_0^2 (dn/dc)^2 / N_A \lambda^4$, where N_A is the Avogadro constant, λ is the wavelength, n_0 is the refractive index of the solvent, and (dn/dc) is the refractive index increment measured in a differential refractometer with Rayleigh optics at 25 °C. The Rayleigh ratio at the angle 90°, R_{90} was determined from $[(I - I_0)/I_{tol}]R_{tol}(n_s/n_{tol})^2$, where n_s is the refractive index of the solvent and n_{tol} that of toluene, I is the measured intensity of the solution, I_0 is the intensity of the solvent, and I_{tol} that of toluene. The values of Kc/R_{90} were then plotted versus the concentration, c, and the M_w value was obtained from the intercept.

The amount of aldehyde groups was obtained by reaction with *tert*-butyl carbazate (TBC) followed by reduction with $NaBH_3CN$. Briefly, periodate-oxidized HA (∼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 to prepare periodate-oxidized HA). The mixture was stirred for 1 h at room temperature after which 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 polymer was recovered by dialysis in 25 000 MW cutoff tubing against water twice.

Cell Culture. Human dermal fibroblasts (hDFn) 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, 5% CO_2 and used at passages 11 and 12.

Cytotoxicity of the Individual Gel Components. hDFn were seeded in 96-well plates at a 2.5×10^4 cells/mL density and cultured in 200 μL/well of complete DME/F12 medium at 37 °C, 5% CO_2. After 24 h the old medium was removed and replaced with fresh medium containing 1.7 mg/mL of the PVA derivatives **4**, **13**, or **15**, or 13 mg/mL of the aldehyde-modified HA **16**. The material was dissolved directly in cell culture medium and then passed through 0.25 μm sterile filter before being added to the cells. Fibroblasts were allowed to grow with the material at 37 °C, 5% CO_2. Cells grown in plain cell culture medium were used as a negative control. After 0, 24, and 48 h the MTT assay was performed to evaluate the cytotoxicity of the individual components. At each time point a 5 mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) in PBS was prepared and passed through a 0.22 μm sterile filter. Thereafter, 20 μL of the MTT solution was added to each well, and the plate was incubated at 37 °C, 5% CO_2 for 3 h. Later on, the medium was carefully removed and the dark blue crystals were dissolved in 200 μL 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.

Hydrogel Formation and Characterization. Hydrogels were prepared from two complementary reactive PVA derivatives by dissolving each derivative in 250 μL of DMSO and subsequently mixing them in a glass vial. The concentration of polymer components in 500 μL of gel-forming mixture was 4%, and the ratio of cross-linkable groups was 1:1 for all gels. The gels began to form within half a minute after mixing. They were kept in capped glass vials overnight and then transferred for repeated (3×) swelling in refreshed water during which the fractions nonincorporated into network (= sol fractions) were extracted and DMSO solvent was substituted with water to obtain PVA hydrogels with weight W_s. After swelling the storage and loss moduli (G' and G'') of hydrogels were obtained on an AR2000 rheometer (TA Instruments Inc., UK). After triple solvent exchange the hydrogels were also freeze-dried and the mass of the freeze-dried network W_{gel} was determined. The swelling ratio, Q, was calculated as $Q = W_s/W_{gel}$.

To form the HA hydrogels cross-linked with aldehyde-reactive PVA derivatives, the solutions of aldehyde-modified HA **16** (degree of modification 4%) and corresponding PVA component were prepared in water at concentrations to give equimolar equivalents of aldehyde and hydrazide (or aminooxy, or semicarbazide) functionalities (around 25 mg/mL for HA **16** and 5 mg/mL for PVA derivative). Equal volumes of these solutions were injected using a double-barreled syringe to obtain the hydrogels with the concentration of solid contents 1.5%. Disk-shaped gels were prepared by injection of the solutions into a rubber mold sandwiched between two slide glasses. Gel formation occurred within 30 s. The obtained hydrogels were kept in the mold for 2 h to ensure complete cross-linking. The diameter and thickness of hydrogels were 20 and 3.2 mm (∼1 mL).

Gel/sol fraction of the prepared disk gels were determined by swelling in deionized water for 24 h during which the nonincorporated network fraction (= sol fraction) was extracted. The extracted gels were stored in an open dish overnight at room temperature and then under vacuo at 37 °C to allow water evaporation and thus provide a hydratable HA hydrogel film with the mass W_{gel}. The dried gel was again reswollen for 24 h in PBS buffer (pH 7.4) and weighed in air. The amount of polymer components in the soluble fraction of the gel, W_{sol}, was defined as the difference in weight between the initial mass of both polymer components taken for the cross-linking reaction W_0 and W_{gel}, $W_{sol} = W_0 - W_{gel}$. The swelling ratio, Q, was calculated as $Q = W_s/$

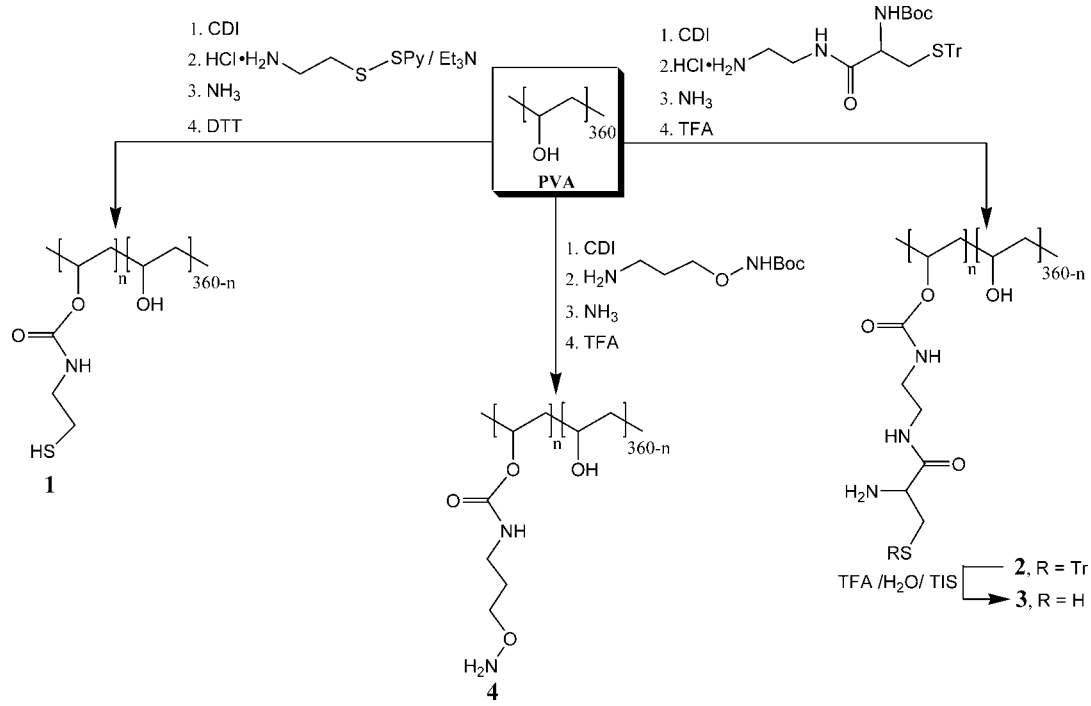


Figure 1. Incorporation of nucleophilic functionalities to PVA via direct coupling to PVA hydroxyl groups.

W_{gel}, where W_s is the weight of hydrogel after swelling in PBS buffer (pH 7.4).

Storage and loss moduli (G' and G'') of the prepared disk gels were measured with an AR2000 rheometer (TA Instruments Inc., UK). The disks gels were immersed in PBS for 24 h and allowed to swell to equilibrium. The swollen in PBS disk gels were placed on an aluminum plate of 20 mm diameter with parallel geometry. Storage and loss moduli (G' and G'') were obtained from a stress sweep (from 10 to 250 Pa) performed at the normal force of ca. 150 mN at 25 °C. Data are reported at a frequency of 1.0 Hz.

Hydrogel Degradation. Enzymatic degradation of the gels was explored. After the hydrogels were dried, they 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.

Cytotoxicity of in Situ Formed Hyaluronan-Based Hydrogels. Hydrogels were prepared by injection of filter-sterilized (0.2 μm filter) aqueous solutions (100 μL of each component, and concentrations of components in solutions were made to give equimolar equivalents of reactive functionalities) through a double-barreled syringe into a round-bottom 5 mL polystyrene tubes giving a final solid concentration of 1.5% (w/v). Mixed solutions were allowed to gel at room temperature for 1 h. Once polymerized, each gel (0.2 mL) was covered with 4 mL of complete DME/F12 medium and incubated at 37 °C for 1 week. On day 6, hDFn were seeded in 96-well plates at a 2.5×10^4 cells/mL density and cultured in 200 μL/well of complete DME/F12 medium at 37 °C, 5% CO_2 for 24 h. On day 7, the old medium was removed and 200 μL of the medium used for gel incubation was added to the wells. The cells were allowed to grow at 37 °C, 5% CO_2 for 0, 24 and 48 h before performing the MTT assay according to the earlier described procedure. Fibroblasts grown in regular cell culture medium were used as a control in this study.

Results and Discussion

Synthesis and Characterization of PVAs Derivatized with Chemoselective Functionalities. *A. Incorporation of Nucleophilic Functionalities via Direct Coupling to PVA Hydroxyl Groups.* We have previously reported on the derivatization of PVA with different functional groups by first activation of PVA hydroxyl groups with 1,1′-carbonyldiimidazole (CDI) followed by reaction of *O*-imidazolylcarbonyl-activated PVA with the appropriate amine.[40,41] In this approach, the functional group of interest forms part of a reagent that contains also an amino group for coupling to PVA via carbamate linkage. While incorporation of some groups was shown can be accomplished directly, as in the case of azide or alkyne,[41] generally, nucleophilic groups require protection in order to exclude possible interference with the amino group in the course of carbamate coupling, as in the case of hydrazide.[40] The degree of substitution is controlled by the feeding amount of amine, and it does not normally exceed 5% to maintain water solubility of the parent PVA. In this work, we extend the range of nucleophilic groups incorporated into PVA macromolecule and include incorporation of thiol, cysteine β-aminothiol, and aminooxy groups.

Thiolated poly(vinyl alcohol) was previously obtained by esterifying PVA with 3-mercaptopropionic acid in a HCl solution[42] or with 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester.[43] In our version of PVA thiolation, we present here the attachment of the primary amine, 2-(2-pyridyldithio-)ethylamine (PDA), via carbamate linkage. In our first attempt, the synthesized PDA hydrochloride salt[33] was converted to the free base by passing the PDA·HCl through a silica gel column eluted with ammonium hydroxide/MeOH/DCM (2/10/90). However, this preparation led to substantial breakdown of PDA, as noted previously.[44] 10% of the free base could only be recovered in this way and used immediately for coupling with PVA. Recently, alternative procedure involving phase separation of liquid PDA from sodium hydroxide solution was reported.[45] However, the product contained residual water as a contaminant that could not be removed due to the instability of the free base form. Therefore, in our second trial, we used PDA·HCl salt in combination with triethylamine in order to generate free amine in situ, in the reaction mixture, where it was immediately reacted with *O*-imidazolylcarbonyl-activated PVA (Figure 1).

Irrespective of how the free base form of PDA was obtained and used in the reaction with PVA, in both cases quenching of the reaction mixture with aqueous concentrated ammonia followed by evaporation of water solvent (see Experimental Part

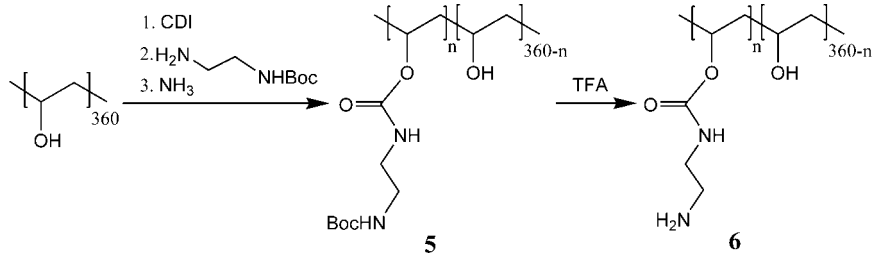


Figure 2. Synthesis of amino-derivatized PVA.

for details) gave a material which was not soluble in any solvents even at elevated temperatures. We hypothesized that the residual gel-like material is actually a disulfide cross-linked PVA that was formed upon exposure of −S−S−Py appended groups to ammonia. The cross-linking of 2-(2-pyridyldithio)ethylamine-derivatized PVA most probably occurs through disproportionation reaction, similar to other structurally related unsymmetrical disulfides.[46] Through this exchange pathway, the unsymmetrical 2-(2-pyridyldithio)ethylamine-modified PVA, PVA−(OCONH-CH_2CH_2−S−S−Py)$_n$, is partially converted into its symmetrical counterparts, namely 2,2′-dipyridyl disulfide $Py_2S_2$and cross-linked PVA−(OCO*NHCH*$_2$*CH*$_2$−*S*−*S*-*CH*$_2$*CH*$_2$*NH*COO)$_n$−PVA. The proposed cystamine bridges in such cross-linked PVA can, however, be cleaved by reaction with excess of dithiothreitol (DTT), as was actually verified by complete dissolution of the in-DMSO swollen gel species after their treatment with DTT under exclusion of oxygen. This DMSO solution was then diluted with 0.3 M HCl (pH 3.0) and was dialyzed in 1000 MW cutoff membranes against 0.3 M HCl two times. After freeze-drying of the dialyzed solution the pure thiolated PVA derivative **1** (Figure 1) was obtained.

In the search for suitable conjugation/cross-linking chemistry for in vivo gelation of multivalent polymer−(ligand)$_n$ conjugates, a method of "native ligation" of unprotected peptide fragments, one containing C-terminal thioester and the other an N-terminal cysteine, came to our attention. This ligation has been elaborated as a general synthetic route for semisynthesis of native proteins.[47] Recently, this technique was also used to render a cross-linked hydrogel.[26,48] However, no suitable route for multiple incorporation of cysteine β-aminothiol groups into PVA and other linear polymers has been reported.

Therefore, to enable preparation of PVA derivative possessing several carboxyl-linked cysteine residues, we have chosen a protected cysteine derivative, *N*-Boc-*S*-Trityl-*N*-2-aminoethyl-L-cysteinamide,[34] which has an amino-terminated arm suitable for reaction with *O*-imidazolylcarbonyl-activated PVA (Figure 1). This reaction afforded a PVA intermediate derivative with appended and fully protected cysteine moieties. Because of the low water solubility of this intermediate, it was precipitated during treatment with aqueous ammonia, the postsynthetic step required to hydrolyze the excess of CDI.[41] That is why the intermediate product was isolated by filtration and directly subjected to a removal of Boc-protective group by treatment with trifluoroacetic acid which afforded PVA derivative **2** with trityl-protected thiol groups. It appeared that thiol groups remained protected in neat TFA which was confirmed by NMR analysis. This is due to the reverse reaction of trityl trifluoroacetate with free thiols. Therefore, the trityl group was subsequently removed in a dichloromethane−water two-phase system upon action of TFA in the presence of triisopropylsilane scavenger to yield the cysteine-modified PVA **3**. To the best of our knowledge, this is the first example of multiple introducing of cysteine side groups into PVA and linear polymers in general.

To exploit the potential of oxime cross-linkage formation for production of hydrogel in situ, we prepared aminooxy-derivatized PVA **4** (Figure 1). As with hydrazides, the aminooxy -ONH_2 group retains its nucleophilicity in acidic aqueous media, thereby permitting the coupling with aldehydes to occur quickly at room temperature and physiological pH. Surprisingly, this chemistry has not been yet investigated for in situ preparation of hydrogel materials. Thus, in order to attach side chains terminated with aminooxy functionality, the CDI-activated PVA was reacted with *N*-Boc protected aminooxypropylamine[35] following the elaborated coupling protocol and deprotection with TFA in a one pot procedure.

B. Incorporation of Electrophilic Functionalities by Acylation of Amino-Derivatized PVA. Despite that most of the appropriately protected nucleophilic functionalities can be directly introduced into PVA following the procedure presented above, coupling of thiophilic groups (maleimide, acrylamide, α-haloacetyl derivatives, or thioesters) is not compatible with the coupling conditions applied (ammonia treatment). This obstacle can, however, be overcome by attachment of the amino-functionalized side groups to some of the PVAs hydroxyls to increase coupling potential of PVA and perform its functionalization under much milder conditions. In this case, coupling via acylation of an amino group with *N*-hydroxysuccinimide ester of suitable carboxylic acid containing thiophilic group can be performed in aqueous solution without any harsh postsynthetic treatment.

In order to incorporate amino groups into parent PVA, it was first derivatized with the monoprotected diamine following the same protocol that was used for PVA modification via carbamate linkages. Hydrophilic spacer's arms are preferable for this purpose in order to keep hydrophilicity of PVA unaltered. Thus, *N*-Boc-protected ethylene diamine was linked to some portion of PVA hydroxyls (3.0%) (Figure 2, PVA derivative **5)** followed by deprotection with trifluoroacetic acid which afforded amino-PVA **6** (Figure 2). The reaction of the free amino groups with *N*-hydroxysuccinimide ester of several acids (Figure 3) proceeded smoothly in aqueous sodium tetraborate buffer (pH 8.5) to afford derivatives with the following thiophilic functionalities: maleimidoacetyl (PVA derivative **7**), maleimidopropionyl (PVA derivative **8**), acrylamide (PVA derivative **9**), and α-iodoacetyl (PVA derivative **10**). The acylation of amino groups in amino-PVA **6** was quantitative in all cases when a 5- to 10-fold excess of NHS−ester reagent was applied for these amide coupling reactions.

The maleimido-substituted PVAs have previously been prepared with carboxylic ester linkages.[43] However, partial hydrolysis of these carboxylic ester linkages cannot be avoided when reaction is performed in aqueous buffer, leading to unpredictable and variable amounts of esterification of PVA hydroxyl groups. Conversely, our approach ensures the control over the content of introduced chemoselective functionalities which is predetermined by the amount of amino groups in amino-PVA **6**. Higher reactivity of amino groups compared with hydroxyl groups as well as the stability of the amide linkages toward hydrolysis allows to use a minimal excess of the

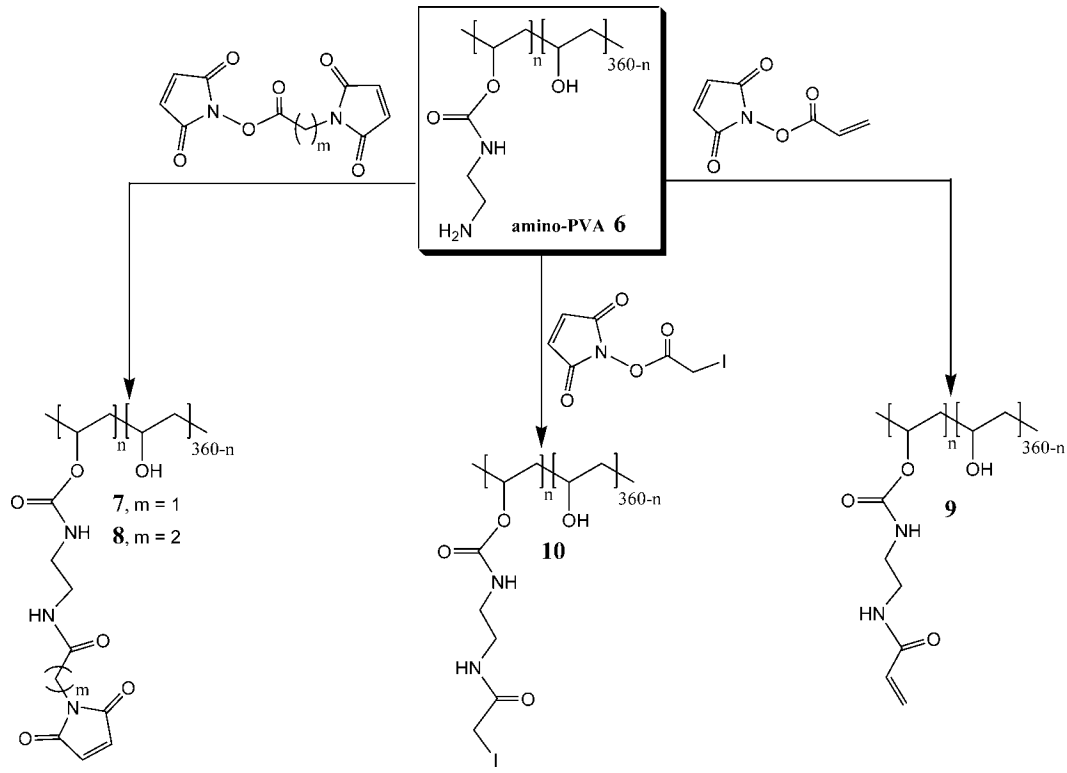


Figure 3. Incorporation of electrophilic functionalities into PVA by acylation of amino-derivatized PVA.

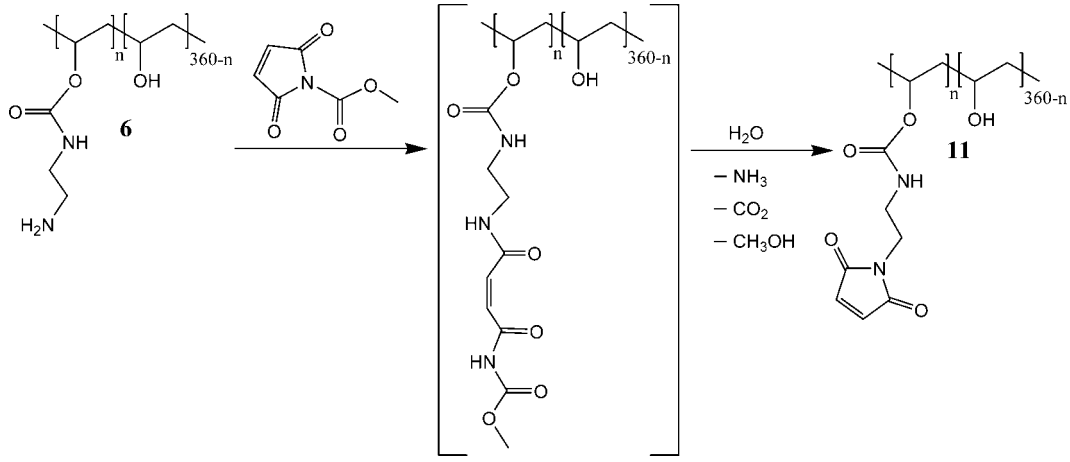


Figure 4. Incorporation of maleimide group by condensation of amino-derivatized PVA with *N*-methoxycarbonyl maleimide.

NHS−ester reagent over amount of amino groups for complete acylation.

Alternatively, incorporation of maleimide side chains to give maleimide-appended PVA **11** (Figure 4) has been also accomplished by method of Keller and Rudinger[49] which makes use of *N*-(methoxycarbonyl)maleimide. This reaction is very dependent on pH, temperature, and time. At pH 8.5, in the presence of bicarbonate ions which catalyze cyclization of the imide−amide intermediate into maleimide **11**, NMR analysis of the reaction mixture showed the presence of the reaction intermediate together with its cyclized maleimide product (Figure 5). ^{1}H NMR analysis reveals the maleimide singlet of **11** at 6.8 ppm (Figure 5B) by comparison with the spectrum for the maleimide-functionalized PVA **8** (Figure 5C) which was obtained via acylation with NHS−ester reagent. Additionally, ^{1}H NMR spectrum taken from the reaction mixture after neutralization and dialysis contained doublets of −CH=CH− protons of the imide−amide derivative of maleamic acid at 6.41, 6.22, 5.91, and 5.83 ppm, as well as a singlet peak for the three methoxy protons at 3.68 ppm. Integration of peaks for the double bond protons revealed that the ratio of the intermediate to the maleimide **11** was approximately 3:2. No optimization of the reaction conditions has been done at this stage in order to shift the ratio toward the formation of exclusively maleimide−PVA product **11**.

C. Use of Amino-Derivatized PVA 6 for Incorporation of Nucleophilic Functionalities. The usefulness of the amino-modified PVA **6** has also been demonstrated for incorporation of the protected nucleophilic functional groups that cannot be introduced directly from PVA. Thus, condensation between *tert*-butyl carbazate and phenyl chloroformate gave *tert*-butyl phenyl hydrazodiformate[39] which underwent a smooth displacement reaction with amino-groups of PVA **6** to provide Boc-protected semicarbazide−PVA **12** (Figure 6). The last compound was then treated with TFA to afford the required semicarbazide−PVA derivative **13**. The structures of PVA derivatives **6**, **12**, and **13** were confirmed by ^{1}H NMR in D_2O. The spectrum of the amino-modified PVA **6** (Figure 7A) is characterized by resonances at

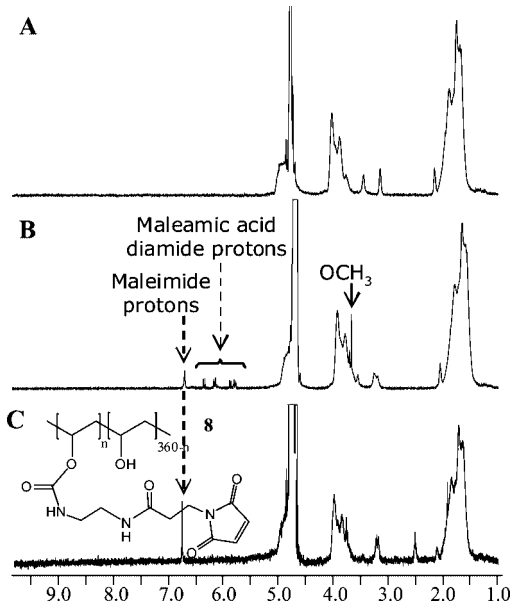


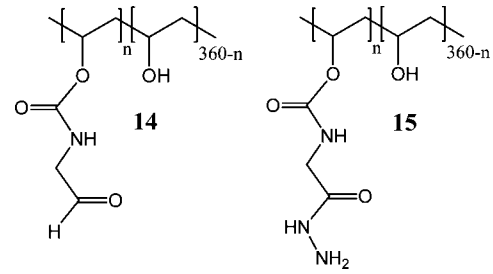
Figure 5. ^{1}H NMR spectra in D_2O. (A) Amino-modified PVA **6**. (B) The product of treatment of amino-modified PVA **6** with *N*-(methoxycarbonyl)maleimide. (C) Maleimide-modified PVA **8** obtained by acylation of **6** with 3-maleimidopropionic acid NHS−ester.

2.74 ppm and 3.15 ppm corresponding to the methylene protons of the ethylene diamine spacer having one free amino group and the other linked to the PVA backbone via carbamate linkage. By attaching *tert*-butyl carbazate to PVA **6** via urea linkage, the parent resonance at 2.74 ppm is shifted downfield to 3.16 ppm merging with signal at 3.15 ppm (Figure 7B). The Boc protection in PVA derivative **12** is shown up by the singlet resonance of *tert*-butyl at 1.39 ppm (Figure 7B) which then disappeared upon conversion to the free semicarbazide derivative **13** (Figure 7C).

This scheme which makes use of amino-modified PVA **6** represents an alternative synthetic pathway to the direct functionalization of PVA. Moreover, the new semicarbazide−PVA derivative **13** along with the aminooxy analog **4** greatly expands the repertoire of multifunctional nucleophilic cross-linkers for selective cross-linking of polymers carrying appended aldehyde/ketone groups. Despite the utility of hydrazone formation to render a hydrogel material in situ,[27–32] no oxime, semicarbazone, or thiosemicarbazone cross-linking has earlier been demonstrated to spontaneously afford a hydrogel within a minute at room temperature and at neutral pH.

Hydrogels Prepared with PVAs Derivatized with Chemoselective Functionalities. *A. Hydrogels Formed by Mixing of the PVA Derivatives Modified with Complementary Reactive Groups.* We have been interested in cross-linking approaches which afford spontaneous hydrogel formation after mixing of two aqueous solutions at room temperature and neutral pH, and at a suitable rate for the creation of a localized depot in vivo at the site of injection. Polymers that are modified to a low extent with chemoselective functionalities have the following advantages for practical in vivo gelation: (i) hydrogels are formed upon mixing of appropriately functionalized polymeric components which eliminates the use of a potentially toxic low molecular weight cross-linker, (ii) cross-linking multivalency of such polymeric components provide high cross-linking efficiency and short gelation times at low polymer concentration, which allows hydrogel formation promptly following injection of low viscosity aqueous solutions, and (iii) the use of both multifunctional cross-linking components provides hydrogels with higher portion of elastically active intermolecular cross-links compared to cross-linking with bifunctional cross-linkers. We have shown previously,[41] for example, that under the same concentration of cross-linkable functional groups, the gel formed from both multifunctional PVA components were characterized by shorter gelation time, higher gel fraction, and higher elastic modulus than the gel formed from multifunctional PVA and bifunctional PEG cross-linker. In the last case, cross-linking was substantially accompanied by the formation of intramolecular cross-links that do not take part in network formation. Finally, the choice of cross-linking chemistry is of particular importance since it should take place within the complex environment of a living organism.

The ability to form gel materials by interaction between two complementary reactive PVA derivatives has been consequently examined and compared with our previous results for aldehyde-modified PVA **14** and hydrazide-modified PVA **15**.[40] As



expected, gels were formed from the two types of PVA derivatives containing suitable pairs of chemoselective cross-linking groups in 1:1 stoichiometry in DMSO solution at 4% concentration (Figure 8). Mixing of complementary reactive PVA components has been done in DMSO because of limited solubility of the modified PVAs in water. DMSO was then substituted to water during repeated aqueous extractions. Because linkages used for cross-linking are susceptible to hydrolysis, the gels that were initially formed in DMSO can potentially be degraded upon repeated swelling in water. This was indicated by substantially reduced gel fraction ranging from 34% for the least stable thiazolidine cross-linked PVA hydrogel to 89% for hydrogel that was made by Michael addition of cysteine-modified PVA **3** to maleimide-modified PVA **11**. Substantially lower appearing gel fraction for hydrogels formed from hydrazide- and aminooxy-modified PVAs compared to those formed by Michael addition reactions can be explained by the well-known hydrolytic lability of acyl hydrazone and oxime linkages at acidic pH. It should be noted that thiazolidine cross-linked hydrogel appeared to be not stable at all, slowly disintegrating while keeping it in water overnight. It was, however, completely intact while stored as it was formed from DMSO solutions. This fact clearly indicates the hydrolytic instability of thiozolidine cross-links in water. Mechanical properties of the obtained hydrogels are determined by both the intrinsic stability of their cross-linkages and the density of cross-links which in turn can be dependent on cross-linking chemistry. Thus, hydrogels formed in the course of Michael addition of thiol-containing PVAs **1** and **3** to maleimide-derivatized PVA **8** and **11** as well as hydrazone cross-linked hydrogel were characterized by much higher elastic moduli and lower swelling ratio than the oxime cross-linked analog.

B. HA Hydrogels Cross-Linked with PVA Derivatives Modified with Chemoselective Reactive Groups. Figure 8 illustrates the preparation of nondegradable or hydrolytically degradable gels based exclusively on two PVA derivatives with "built-in" chemoselective functional groups, one electrophilic and one nucleophilic. For many therapeutic applications, synthetic biomaterials mimicking natural ECMs must be fully degradable dictated by cellular activity, which can be attained either by incorporation of proteolytically degradable sites into cross-

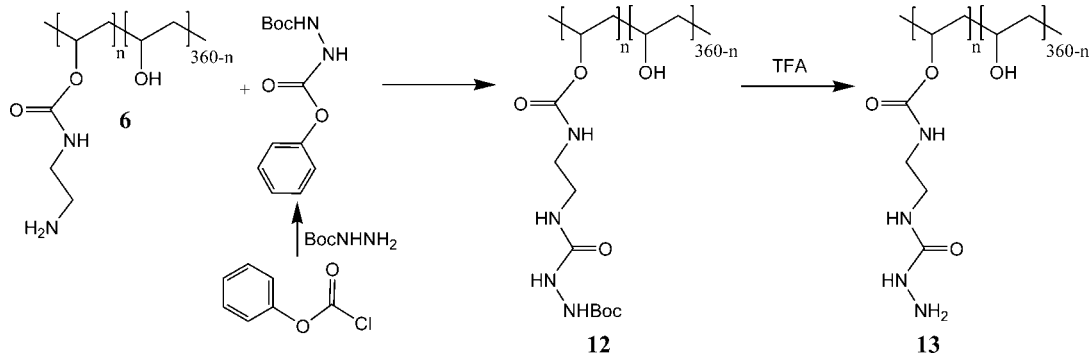


Figure 6. Synthesis of semicarbazide-derivatized PVA from amino-derivatized PVA precursor.

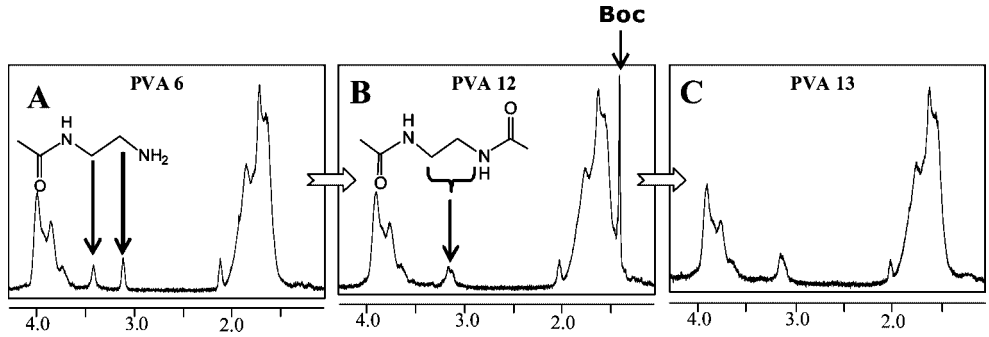


Figure 7. ^{1}H NMR spectra in D_2O of (A) amino-modified PVA **6**, (B) *N*-Boc protected semicarabazide-modified PVA **12**, and (C) semicarabazide-modified PVA **13**.

Nu / E = 1:1

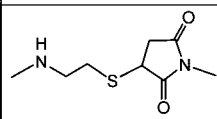
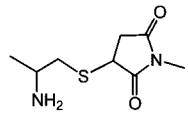
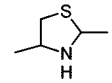
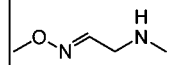
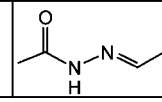
	Maleimide-modified PVA 8 & 11				Aldehyde-modified PVA 14*			
	cross-link	gel fraction, W_{gel}, mg (%)	swelling ratio, Q	G′, Pa	cross-link	gel fraction, W_{gel}, mg (%)	swelling ratio, Q	G′, Pa
Thiol-modified PVA 1		**1**+ **8**: 13.8 (69%) **1**+ **11**: 15.2 (76%)	5.0 11.5	8375 5470				
Cysteine-modified PVA 3		**3**+ **8**: 17.5 (89%) **3**+ **11**: 14.2 (71%)	3.8 3.6	6080 6200		6.8 (34%)	51.3	420
Aminooxy-modified PVA 4						9.9 (50%)	44.2	1230
Hydrazide-modified PVA 15*						11.5 (58%)	6.0	10680

Figure 8. Schematic presentation of PVA-based gels. The chemical drawings represent the structures of cross-linkages for matrices prepared from corresponding PVA components. *Hydrazide and aldehyde (see structure in text) were prepared as described earlier.[40]

linkages or by involvement of naturally derived biopolymers in construction of 3-D networks. Hyaluronic acid (HA), an abundant glycosaminoglycan component of ECM and the leading candidate for numerous biomedical applications, thus has been proposed as another injectable component that is degraded by endogenous hyaluronidase (HAse). For this purpose, HA has been functionalized by periodate treatment that is well-known to oxidize proximal hydroxyl groups at C2 and C3 carbons of glucuronic acid moiety to aldehydes[30] with the degree of aldehyde modification of 4%. Keeping the stoichiometry of chemoselective cross-linking groups 1:1, the mass ratio for HA/PVA components in hydrogels was actually around 5:1 due to substantial differences in the molar masses for repeating units of hyaluronic acid and PVA, respectively. Hence, HA is a major component by mass in presented here HA/PVA heterocomponent hydrogels which allowed to prepare them in pure water. Thus, the sol-to-gel transformation of the aldehyde-modified HA **16** was readily triggered by addition of the aldehyde-reactive PVAs modified with hydrazide (PVA **15**),[40] semicarbazide, or aminooxy groups due to hydrazone, semi-

Nu / E = 1:1

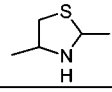
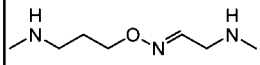
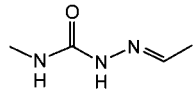
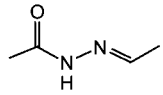
	cross-link	concentration	gel fraction, W_{gel}, mg (%)	swelling ratio, Q	G′, Pa	G″, Pa
Thiol-modified PVA 1	gel was not formed					
Cysteine-modified PVA 3		1.5% in H_2O	Not stable after swelling in PBS buffer			
Aminooxy-modified PVA 4		1.5% in H_2O	15.0 (100%)	33.5	950	270
Semicarbazide-modified PVA 13		1.5% in H_2O	5.8 (39%)	24.4	Not determined	Not determined
Hydrazide-modified PVA 15*		1.5% in H_2O	13.4 (90%)	30.3	4850	620

Figure 9. Schematic presentation of HA/PVA-based hybrid hydrogels. The chemical drawings represent the structures of cross-linkages for matrices prepared from corresponding HA and PVA components. *Hydrazide and aldehyde (see structure in text) were prepared as described earlier.[40]

carbazone, or oxime cross-links formation (Figure 9). As expected, thiol-containing PVA **1** did not afford any gel with aldehyde-modified HA (Figure 9) but it was selectively reactive toward the maleimide-modified PVA **8** (Figure 8), while cysteine-modified PVA **3** acted as a cross-linker for both aldehyde-modified HA (Figure 9) and maleimide-modified PVA **8** (Figure 8) due to thiozolidine cyclization and Michael addition reactions, respectively.

Cross-linking of the aldehyde-modified HA with different PVA derivatives gave mechanically robust hydrogels with physicochemical properties ranging from a very soft pliable material to more elastic networks. Analogously to thiazolidine cross-linked PVA hydrogel, the gel formed from aldehyde−HA **16** and cysteine-appended PVA **3** was gradually decomposing back to a clear solution in 7 days after formation and keeping it in a capped glass vial without additional swelling. However, it was disintegrated to small gelatinous pieces when after formation it was placed to swell in PBS (pH 7.4) buffer overnight. Given that thiazolidine formation is reversible,[48] the sulfhydryl groups can form disulfide bridges between the same or different molecules of cysteine-appended PVA **3** at this pH, thus liberating the HA component from the HA/PVA network. Hydrazone cross-linked HA/PVA hydrogel showed the highest storage and loss moduli (G' and G'') among all HA hydrogels that were cross-linked by aldehyde-reactive PVA derivatives. The gel fraction W_{gel} for HA cross-linked with hydrazide- and aminooxy-modified PVAs were 90% and 100%, respectively, which was almost twice higher than the values for gel fraction for corresponding PVA/PVA homocomponent hydrogels. One reason for that is the significantly higher molecular weight of the hyaluronic acid component in the HA/PVA hydrogels. Another contributing factor could also be that swelling experiments for HA/PVA hydrogels were performed in PBS buffer at pH 7.4 where the acyl hydrazone and oxime cross-linkages are reasonably stable.

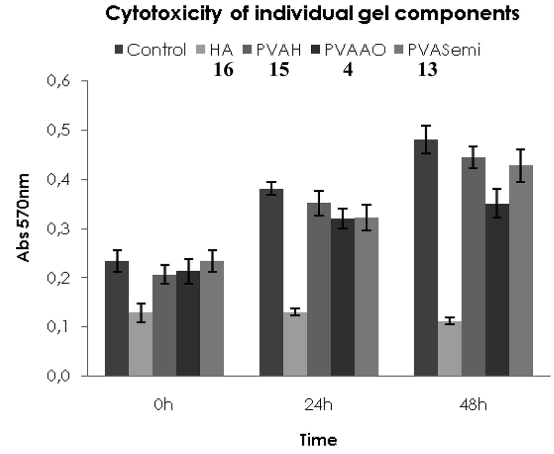


Figure 10. Human dermal fibroblasts were cultured with 13 mg/mL HA **16**, 1.7 mg/mL of PVAs **15**, **4**, or **13** for 0, 24, and 48 h. Decreased cell viablity in fibroblasts cultured with aldehyde-modified HA **16** could be seen already after 0 h. The aminooxy derivatized PVA **4** showed minor cytotoxicity after 24 and 48 h, whereas hydrazide and semicarbazide derivatized PVAs **15** and **13** had no significant effect on the cell viability.

Cytotoxicity Studies on New Multifunctional PVA Cross-Linkers. Human dermal fibroblasts were used to evaluate the cytotoxicity of the new PVA-based cross-linkers **4**, **13**, and **15**. The cysteine-modified PVA **3** could not be used in this study because it readily precipitates due to disulfide cross-linking while taken in cell culture medium (pH 7.4). The PVA cross-linkers' concentration was chosen to be the same typically used to cross-link the aldehyde-modified HA. Figure 10 illustrates decreased cell viability of fibroblasts incubated with the aldehyde-modified HA **16**, while no significant cytotoxic effect of the PVA derivatives could be detected, apart from the minor decrease in cell viability in fibroblasts cultured with aminooxy-modified PVA **4**. The concentration of cross-linkable functionalities

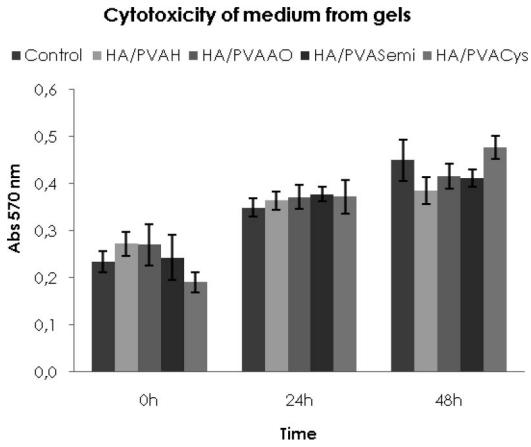


Figure 11. Cell culture medium was placed on preformed gels for 1 week at 37 °C and then used to culture human dermal fibroblasts, in order to demostrate that no toxic substances leak out of the gel after cross-linking. The HA/PVAH, HA/PVAAO, HA/PVASemi, and HA/PVACys preformed gels showed no significant cytotoxicity.

(either aldehyde or hydrazide/semicarbazide/aminooxy), however, were the same (2.8 mM) in the cytotoxicity tests of aldehyde-modified HA **16** and PVA cross-linkers. It can be concluded that the maximum concentrations of these PVA components present in a gel are not cytotoxic even in the non-cross-linked form.

Cytotoxicity of in Situ Formed Hyaluronic Acid−Based Hydrogels. Hydrogels formed from aldehyde-modified HA **16** and aldehyde-reactive PVA cross-linkers **15**, **4**, **13**, or **3** (HA/PVAH, HA/PVAAO, HA/PVASemi, HA/PVACys, respectively) were covered with cell culture medium and incubated at 37 °C for 1 week in order to mimic their behavior inside living tissue. The medium was then used to culture hDFn cells. In order to determine the release of potentially cytotoxic molecules from gels, the cell viability was evaluated with an MTT assay after 0, 24, and 48 h. The results presented in Figure 11 show that none of the four gels showed any significant cytotoxicity or effect on cell viability. It is noteworthy that the potentially toxic aldehyde−HA component is efficiently cross-linked in the obtained hydrogels so that it does not present reactive aldehyde functionalities freely exposed to the cellular environment.

Degradation of Hyaluronic Acid−Based Hydrogels. To estimate whether the heterogeneous HA/PVA hydrogels are degradable or not, they were incubated in PBS at pH 7.4 in the presence of hyaluronidase (500 U/mL). By the second day the oxime cross-linked hydrogel was totally degraded, while the hydrazone cross-linked hydrogel appeared totally degraded on the third day. These experiment clearly demonstrated that HAse can recognize the HA that is cross-linked with multifunctional PVA cross-linkers proposing that the prepared gels would be fully resorbable in vivo.

Conclusion

New PVAs derivatized with nucleophilic chemoselective functionalities (aminooxy, thiol, cysteine) have been synthesized directly from parent PVA by a two-step procedure while incorporation of amino groups into parent PVA allowed easy derivatization of PVA with electrophilic chemoselective functionalities (maleimide, acrylamide, 2-haloacetyl) as well as with nucleophilic groups that cannot be introduced directly to the commercial PVA (semicarbazide). A series of new "click" hydrogel materials has been prepared by simple mixing of the corresponding complementary reactive PVA derivatives as well as by mixing of aldehyde-reactive PVAs with aldehyde-modified hyaluronic acid. It is worth mentioning that hyaluronic acid was for the first time cross-linked with the help of another polymeric multifunctional cross-linker such as PVA, as well as new cross-linking chemistries yielding formation of oxime, semicarbazone, thiazolidine cross-links were invented for the production of hyaluronan-based hydrogel materials. Furthermore, maleimide-modified PVAs may be useful in cross-linking of thiolated hyaluronic acid, the only chemically modified HA that provided an injectable cell-seeded hydrogel for 3-D culture.[21] The synthesized PVA-based multifunctional cross-linkers showed fairly good cytocompatibility, exhibiting no signs of cytotoxicity after incubation for up to 48 h at the concentration normally used to cross-link aldehyde-modified hyaluronic acid (maximal possible concentration of a free gel forming PVA component that one could expect in case of no gel formation at all). Importantly, even the cytotoxic aldehyde-modified HA component can be rapidly and effectively cross-linked using those PVA components forming the HA/PVA hydrogels that showed no apparent cytotoxicity. Finally, the prepared HA/PVA hydrogels span a wide range of mechanical properties and are formed in less than a minute at pH 7.4 and 37 °C which may provide in vivo injectable materials for different biomedical applications.

References and Notes

(1) Peppas, N. A., Ed.; *Hydrogels in Medicine and Pharmacy*; CRC Press: Boca Raton, FL, 1986; Vol. *1*.
(2) Uhlich, T.; Ulbricht, M.; Tomaschewski, G. *Enzyme Microbiol. Technol.* **1996**, *19*, 124–131.
(3) Bühler, N.; Haerri, H. P.; Hofmann, M.; Irrgang, C.; Muehlebach, A.; Mueller, B.; Stockinger, F. *Chimia* **1999**, *53*, 269–274.
(4) Kobayashi, M.; Toguchida, J.; Masanori, O. *J. Biomed. Mater. Res.* **2001**, *58* (B), 344–351.
(5) Yusong, P.; Dangsheng, X.; Xiaolin, C. *J. Mater. Sci.* **2007**, *42*, 5129–5134.
(6) Martens, P.; Holland, T.; J. H.; Anseth, K. S. *Polymer* **2002**, *43*, 6093–6100.
(7) Nuttelman, C. R.; Henry, S. M.; Anseth, K. S. *Biomaterials* **2002**, *23*, 3617–3626.
(8) Anseth, K. S.; Metters, A. T.; Bryant, S. J.; Martens, P. J.; Elisseeff, J. H.; Bowman, C. N. *J. Controlled Release* **2002**, *78*, 199–209.
(9) Martens, P. J.; Bryant, S. J.; Anseth, K. S. *Biomacromolecules* **2003**, *4*, 283–292.
(10) Schmedlen, R. H.; Masters, K. S.; West, J. K. *Biomaterials* **2002**, *23*, 4325–4332.
(11) Elisseeff, J.; Anseth, K.; Sims, D.; McIntosh, W.; Randolph, M.; Langer, R. *Proc. Natl. Acad. Sci.* **1999**, *96*, 3104–3107.
(12) Elbert, D. L.; Pratt, A. B.; Lutolf, M. P.; Halstenberg, S.; Hubbell, J. A. *J. Controlled Release* **2001**, *76*, 11–25.
(13) Lutolf, M. P.; Hubbell, J. A. *Biomacromolecules* **2003**, *4*, 713–722.
(14) Hiemstra, C.; van der Aa, L. J.; Zhong, Z.; Dijkstra, P. J.; Feijen, J. *Biomacromolecules* **2007**, *8*, 1548–1556.
(15) Hiemstra, C.; van der Aa, L. J.; Zhong, Z.; Dijkstra, P. J.; Feijen, J. *Macromolecules* **2006**, *40*, 1165–1173.
(16) Hiemstra, C.; Zhong, Z.; van Steenbergen, M. J.; Hennink, W. E.; Feijen, J. *J. Controlled Release* **2007**, *122*, 71–78.
(17) Shu, X. Z.; Liu, Y.; Luo, Y.; Roberts, M. C.; Prestwich, G. D. *Biomacromolecules* **2002**, *3*, 1304–1311.
(18) Shu, X. Z.; Liu, Y.; Palumbo, F. S.; Luo, Y.; Prestwich, G. D. *Biomaterials* **2004**, *25*, 1339–1348.
(19) Vanderhooft, J. L.; Mann, B. K.; Prestwich, G. D. *Biomacromolecules* **2007**, *8*, 2883–2889.
(20) Shu, X. Z.; Ghosh, K.; Liu, Y.; Palumbo, F. S.; Luo, Y.; Clark, R. A.; Prestwich, G. D. *J. Biomed. Mater. Res* **2004**, *68* (A), 365–375.
(21) Shu, X. Z.; Shama, A.; Liu, Y.; Prestwich, G. D. *J. Biomed. Mater. Res* **2006**, *79* (A), 902–912.
(22) Prestwich, G. D.; Liu, Y.; Yu, B.; Shu, X. Z.; Scott, A. *Advan. Enzyme Regul.* **2007**, *47*, 196–207.
(23) Kim, G. W.; Choi, Y. J.; Park, Y.; Lee, K. B.; Kim, I. S.; Hwang, S. J.; Noh, I. *Curr. Appl. Phys.* **2007**, *7* (S1), e28−e32.
(24) Kim, J.; Kim, I. S.; Cho, T. H.; Lee, K. B.; Hwang, S. J.; Tae, G.; Noh, I.; Lee, S. H.; Park, Y.; Sun, K. *Biomaterials* **2007**, *28*, 1830–1837.
(25) Hahn, S. K.; Oh, E. J.; Miyamoto, H.; Shimobouji, T. *Int. J. Pharm.* **2006**, *322*, 44–51.
(26) Wathier, M.; Jung, P. J.; Carnahan, M. A.; Kim, T.; Grinstaff, M. W. *J. Am. Chem. Soc.* **2004**, *126*, 12744–12745.

(27) Luo, Y.; Kirker, K. R.; Prestwich, G. D. *J. Controlled Release* **2000**, *69*, 169–184.
(28) Kirker, K. R.; Prestwich, G. D. *J. Polym. Sci. Part B* **2004**, *42*, 4344–4356.
(29) Kirker, K. R.; Luo, Y.; Nielson, J. H.; Shelby, J.; Prestwich, G. D. *Biomaterials* **2002**, *23*, 3661–3671.
(30) Bulpitt, P.; Aeschlimann, D. *J. Biomed. Mater. Res.* **1999**, *47*, 152–169.
(31) Jia, X.; Colombo, G.; Padera, R.; Langer, R.; Kohane, D. S. *Biomaterials* **2004**, *25*, 4797–4804.
(32) Jia, X.; Yeo, Y.; Clifton, R. J.; Jiao, T.; Kohane, D. S.; Kobler, J. B.; Zeitels, S. M.; Langer, R. *Biomacromolecules* **2006**, *7*, 3336–3344.
(33) Ebright, Y. W.; Chen, Y.; Pendergrast, S.; Ebright, R. H. *Biochemistry* **1992**, *31*, 10664–10670.
(34) Wood, R. J.; Pascoe, D. D.; Brown, Z. K.; Medlicott, E. M.; Kriek, M.; Neylon, C.; Roach, P. L. *Bioconjugate Chem.* **2004**, *15*, 366–372.
(35) Salisbury, C. M.; Maly, D. J.; Ellman, J. A. *J. Am. Chem. Soc.* **2002**, *124*, 14868–14870.
(36) Xu, P.; Zhang, T.; Wang, W.; Zou, X.; Zhang, X.; Fu, Y. *Synthesis* **2003**, *8*, 1171–1176.
(37) Kitagawa, T.; Shimozono, T.; Aikawa, T.; Yoshida, T.; Nishimura, H. *Chem. Pharm. Bull.* **1981**, *29*, 1130–1135.
(38) Krutzsch, H. C.; Inman, J. K. *Anal. Biochem.* **1993**, *209*, 109–116.
(39) Gray, C. J.; Ireson, J. C.; Parker, R. C. *Tetrahedron* **1977**, *33*, 739–743.
(40) Ossipov, D. A.; Brännvall, K.; Forsberg-Nilsson, K.; Hilborn, J. *J. Appl. Polym. Sci.* **2007**, *106*, 60–70.
(41) Ossipov, D. A.; Hilborn, J. *Macromolecules* **2006**, *39*, 1709–1718.
(42) Dicharry, R. M.; Ye, P.; Saha, G.; Waxman, E.; Asandei, A. D.; Parnas, R. S. *Biomacromolecules* **2006**, *7*, 2837–2844.
(43) Sharma, S. D.; Granberry, M. E.; Jiang, J.; Leong, S. P. L.; Hadley, M. E.; Hruby, V. J. *Bioconjugate Chem* **1994**, *5*, 591–601.
(44) Sigurdsson, S. T.; Seeger, B.; Kutzke, U.; Eckstein, F. *J. Org. Chem.* **1996**, *61*, 3883–3884.
(45) Zugates, G. T.; Anderson, D. G.; Little, S. R.; Lawhorn, I. E. B.; Langer, R. *J. Am. Chem. Soc.* **2006**, *128*, 12726–12734.
(46) Bellas, M.; Tuleen, D. L.; Field, L. *J. Org. Chem.* **1967**, *32*, 2591–2595.
(47) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.
(48) Wathier, M.; Johnson, C. S.; Kim, T.; Grinstaff, M. W. *Bioconjugate Chem.* **2006**, *17*, 873–876.
(49) Keller, O. *Rudinger, Helv. Chim. Acta* **1975**, *58*, 531–541.

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