Formation of the First Injectable Poly(vinyl alcohol) Hydrogel by Mixing of Functional PVA Precursors

Dmitri A. Ossipov,[1] Karin Brännvall,[2] Karin Forsberg-Nilsson,[2] Jöns Hilborn[1]

[1]Polymer Chemistry, Material Chemistry Department, Uppsala University, S-75121 Uppsala, Sweden
[2]Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden

ABSTRACT: In this study we describe the development of an injectable, *in situ* chemical hydrogel forming system. The gelation occurs under neutral pH and at room temperature immediately upon mixing of the two aqueous poly (vinyl alcohol) components specifically derivatized through carbamate linkages with aldehyde (PVA-AL) and hydrazide (PVA-HY) functional groups, respectively. Aldehyde and hydrazide pendant groups were incorporated with a low degree of substitution (DS) into the PVA backbone to keep PVA structural homogeneity minimally altered. As a result, the hydrazone crosslinks are formed rapidly between aldehyde and hydrazide pendant groups when the correspondingly modified PVA components are brought in contact as water solutions. To assess *in situ* hydrazone crosslinks formation for *in vitro* cytocompatibility, murine neuroblastoma N2a cells were suspended in cell culture medium with the dissolved PVA-HY prior to addition to the PVA-AL aqueous solution. Thus, the cells were chemically encapsulated in a polymer network that was formed by mixing of the corresponding aqueous solutions of PVA functional precursors. Biochemical analysis revealed that cells survived chemical crosslinking and remained viable in the hydrogel for 4 days of culture. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 60–70, 2007

Key words: poly(vinyl alcohol); injectable hydrogels; hydrazone crosslinking; cell encapsulation

INTRODUCTION

Hydrogels have been well established as scaffolds for tissue engineering which is generally based on seeding cells on a three-dimensional polymer matrix for incubation *in vitro* or *in vivo*. Depending on the cell type, cells can proliferate, produce its own extracellular matrix (ECM), and stimulate vascularization and can thus regenerate the lost tissue.[1] The requirement for surgical implantation of the scaffold is an obvious major drawback of the present technology. Another limitation of this approach is the difficulty of molding the scaffold to the shape of the injured part. Recently, injectable, *in situ* gel-forming systems have attracted increasing interest, since they minimize the invasiveness of the procedure, ensure moldability to fill irregular-shaped defects, and provide means for the incorporation of bioactive factors.[2] Schematically, the cells are suspended in hydrophilic polymer solution, which forms a hydrogel at the site of administration in response to some stimulus provided at that time. The mechanism that may be involved in the *in situ* gel formation depends on the nature of forces interconnecting polymer chains in the 3D polymer network. Physical crosslinking may result from van der Waals interactions, ionic or hydrogen bonding, and may be a consequence of prevalent conditions in physiological environment, such as temperature, pH, or ionic strength. The most common physical gel that has been used for more than two decades for encapsulation purposes is sodium alginate.[3,4] This physical gel belongs to the so-called "ionotropic" hydrogels, which are formed through the ionic interactions between the oppositely charged groups, those between carboxylic acid groups of alginate and bivalent cations Ca^{2+}. However, ionic interactions, as other physical crosslinking mechanisms, have been difficult to control, and the poor mechanical stability of physically crosslinked gels has significant limitations on biomedical applications. In contrast, chemical crosslinking results in a covalently crosslinked network, thereby providing mechanical integrity to the *in situ* forming polymeric scaffold. Chemical crosslinking can be initiated either by exposure to light of the polymers reacting with free radicals or by mixing of two or more precursor components that react to each other under physiological conditions. Cell encapsulation within chemically crosslinked materials has been mainly accomplished via free radical polymerization of vinyl derivatized water-soluble polymers including diacrylate derivatives of poly(ethylene glycol) (PEG),[5–7] poly(propylene

fumarate-co-ethylene glycol),[8] poly(vinyl alcohol) (PVA) grafted with different acryloyl-containing groups,[9–11] or natural hyaluronic acid (HA) modified with glycidyl methacrylate.[12] Photopolymerization generally requires introduction of a photoinitiator, which along with highly reactive free radicals may be toxic to cells and tissue. Moreover, photoirradiation is perceived as more invasive than a simple injection especially if high light intensities are required that produce adverse exothermal effect.

Gelation upon simple injection of the reactive polymer components would be highly desirable for *in situ* cell implantation. Development of such injectable systems that form a gel upon mixing of polymeric reactants solutions represents, however, a considerable challenge. Clearly, gelation in a physiological environment imposes several strict demands to the chemical crosslinking reaction: (i) it should be performed in aqueous media within a narrow range of physiologically acceptable temperatures and pH; (ii) such a reaction should preferably be an addition reaction or substitution reaction with no release of harmful by-product; (iii) crosslinking must be chemoselective and bio-orthogonal with narrow distribution of reagents reactivity; (iv) finally, gelation must occur at a sufficiently rapid rate for clinical use, yet allowing adequate time for proper mixing and injection of cell–polymer solutions. Highly selective chemical reactions executable within the complex environment of living organism have found numerous biological applications, such as functional assembly of complex biostructures and chemical targeting of biomolecules, including living cells. Some chemoselective crosslinking reactions initiated by mixing of aqueous polymer solutions have been applied for the formation of hydrogels, including coupling between an aldehyde and a hydrazide group,[13–15] reaction of aldehydes with cysteine β-aminothiol group,[16] and Michael addition of thiols to acrylates[17,18] or vinyl sulfones.[19,20] Despite the potential of these reactions to form hydrogels *in situ* under physiological conditions, little information has been acquired about cell viability during chemical crosslinking. Recently, Prestwich's group explored Michael-type addition of thiols to acrylates for *in situ* fibroblasts encapsulation in HA-PEG hybrid hydrogel.[21] Can a covalent chemical reaction achieve a level of selectivity comparable to that of specific noncovalent associations between biomolecules and, hence, be nontoxic to living cells? To answer this question, one needs first to explore the biocompatibility of each potentially perspective crosslinking reaction. Clearly, the search for biocompatible reactions can be greatly simplified by the development and use of a model synthetic polymer that can be easily derivatized with a broad range of chemical functionalities.
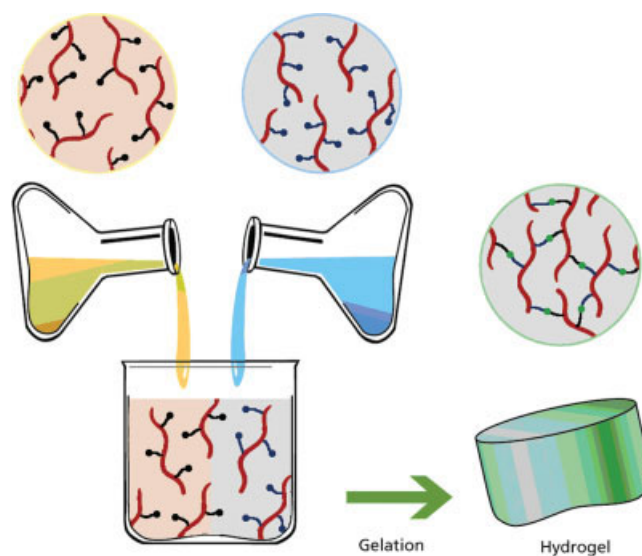

Figure 1 *In situ* gelation of differently modified polymer components after their injection and mixing as aqueous solutions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Recently, we have developed a new approach to the preparation of chemical hydrogels by mixing two polymer components' aqueous solutions, each containing its own type of functional groups capable to react to each other chemoselectively and rapidly under physiological conditions.[22] According to this approach (Fig. 1), a hydrophilic polymer is chemically modified to a low extent by two synthetic routes giving two derivatives, A and B, grafted with more than two pendant functional groups of complementary reactivity. For example, component A could be functionalized with nucleophilic groups of type (Nu), while component B is functionalized with electrophilic groups of type (E) that is chemospecific to (Nu). Particularly, azide and alkyne pendant groups were incorporated into poly(vinyl alcohol), and the ability of the prepared functional PVAs to form hydrogel by "click chemistry"[23] has been demonstrated. However, the limitation of "click chemistry" to *in vivo* application is the necessity to use the toxic Cu^+ as a catalyst.

Herein, we expand the scope of chemical functionalities introduced to PVA by the previously elaborated method.[22] We describe the preparation of PVAs bearing a spacer with terminal hydrazido (PVA-HY) or aldehyde (PVA-AL) groups. The formation of hydrazone crosslinks between the PVA-HY and the PVA-AL polymers occurs quickly at room temperature when aqueous solutions of the corresponding functional PVA precursors are mixed, giving rise to a novel chemical PVA hydrogel. Thus, the gelation is performed in a fashion suitable for both cell encapsulation and *in vivo* injection.

To examine the biocompatibility of the hydrazone crosslinking chemistry, murine neuroblastoma N2a cells were encapsulated during mixing of the PVA-HY and PVA-AL solutions and cultured for up to 1 week. The cells in the resulting polymer scaffold were analyzed histologically to examine cell morphology. We found that the employed crosslinking chemistry is well tolerated by the cells, thus demonstrating its potential for use with cells and tissues. The ease with which different electrophilic and nucleophilic groups can be introduced into PVA makes it a robust model synthetic polymer for evaluation of the biocompatibility of different crosslinking chemistries.

EXPERIMENTAL

General

1,1'-carbonyldiimidazole, 3-amino-1,2-propanediol, glycine ethyl ester hydrochloride, and PEG methyl ether of molecular weight 2000 g/mol were purchased from Aldrich Chemical, and was used as received. PVA with average molecular weight 16,000 g/mol (degree of deacetylation, 98.0%–98.8%) was from Fluka. All solvents were of analytical quality (p.a) and were dried over 4-Å molecular sieves. Dialysis membranes Spectra/Por® 6 (cut-off 500, 1000, and 25,000 g/mol) 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.

Synthesis of 3-amino-1,2-propanediol-modified PVA 1

PVA (400 mg, 9.0 mmol of hydroxyl groups) was dissolved in dry DMSO (8 mL) and the solution was dried by addition of some amount of dry toluene followed by its azeotropic distillation using a Dean Stark trap before starting the reaction. CDI (730 mg, 4.5 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 3 h. A solution of 3-amino-1,2-propanediol (0.9 mmol, 0.1 equiv.) in DMSO (1 mL) was then added, followed by continued stirring at room temperature for $\sim$ 20 h under argon atmosphere. Afterward 4 mL of concentrated aqueous NH_3 was added and the mixture was stirred for 1 h at room temperature. Finally, the reaction mixture was diluted with 50 mL of water, filtered until clear, and reduced to $\sim$ 8 mL volume by rotary evaporation. The substituted polymer was precipitated from the residual DMSO solution by adding a 10-fold excess of an 80/20 mixture of diethyl ether and ethanol. The precipitated polymer was redissolved in small amount of water and dialyzed against water for 24 h in 1000 g/mol cut-off membrane. The dialyzed solution was subsequently freeze-dried to give 379 mg of white solid. DS: 0.048 (48%); ^{1}H-NMR (D_2O): 4.90 ((DS ×1)H, m partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.00–3.60 ((1 + DS)H, m, polymer backbone CH of unmodified unit + —CH_2CH(OH)CH_2—), 3.52, 3.40 ((DS ×2)H, 2×m, —CH_2CH(OH)CH_2OH), 3.20–2.98 ((DS ×2)H, 2×m, —CH_2CH(OH)CH_2OH), 2.00–1.35 (2H, m, polymer backbone CH_2).

Synthesis of acetaldehyde-modified PVA (PVA-AL)

3-Amino-1,2-propanediol-modified PVA **1** (377 mg, 0.5 mmol of 1,2-diol groups) was dissolved in 10 mL water, and the solution was cooled to 0°C. Twenty milliliters of 0.31M aqueous solution of $NaIO_4$ (12.5 equiv. per 1,2-diol group) was added to the cooled polymer solution and the mixture was stirred in the dark at 0°C for 30 min. The reaction solution was then dialyzed against water overnight in 1000 g/mol cut-off membrane. The dialyzed solution was freeze-dried to give 313 mg of white solid. DS: 0.048 (100%); ^{1}H-NMR (D_2O): 4.95 ((DS ×1)H, s, —CH_2CH(OH)$_2$), 4.90 ((DS ×1)H, m partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.00–3.60 (1H, m, polymer backbone CH of unmodified unit), 3.14–3.05 ((DS ×2)H, m, —CH_2CH(OH)$_2$), 2.00–1.35 (2H, m, polymer backbone CH_2).

Synthesis of hydrazide-modified PVA (PVA-HY)

PVA (400 mg, 9.0 mmol of hydroxyl groups) was dissolved in dry DMSO (8 mL) and the solution was dried as described before. CDI (730 mg, 4.5 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 3 h. Glycine ethyl ester hydrochloride (126 mg, 0.9 mmol) followed by triethylamine (125 μL, 0.9 mmol) were added to the reaction mixture. Stirring was continued at room temperature and argon atmosphere overnight. Hydrazine (3 mL) was at last added to the reaction mixture and the mixture was stirred for another 24 h at room temperature, and then diluted with 50 mL of water. The product was isolated in the same way as it was done for PVA derivative **1**. DS: 0.075 (75%); ^{1}H NMR (D_2O): 4.90 ((DS ×1)H, m partially overlapped with H_2O signal, polymer backbone CH of modified unit), 3.96–3.50 ((1 + DS ×2)H, m, polymer backbone CH of unmodified unit + —CH_2CONHNH_2), 2.00–1.35 (2H, m, polymer backbone CH_2).

The content of hydrazide groups in PVA-HY was quantified by adding trinitrobenzene sulfonic acid (TNBS) and the resulting trinitrophenyl derivative was measured spectrophotometrically at 510 nm. Typically, to the solution of PVA-HY (1 mL, 0.001% w/v) in 0.1M borate buffer pH 9.3 was added 25 μL of 0.03M TNBS solution. The last solution was prepared extemporaneously from a commercial 5% (w/v) aqueous TNBS solution by dilution with 0.1M borate buffer pH 9.3. The PVA-HY solution mixed with TNBS was transferred into a cuvette, agitated to ensure complete mixing, and allowed to stand for 30 min at room temperature.[24] Absorbance was read at 510 nm, using a solution containing 1 mL of 0.1M borate buffer and 25 μL of 0.03M TNBS solution as a blank reference. Solutions of adipic dihydrazide (ADH) in 0.1M borate buffer were used as standards to obtain a calibration curve for determining the hydrazide groups in PVA-HY sample.

N-[2,3-Dihydroxypropyl]carbamoyl-PEG methyl ether 3

After it was dehydrated by coevaporation with dry pyridine, PEG methyl ether with average M_W 2000 g/mol (400 mg, 0.2 mmol) was dissolved in dry DMSO (4 mL). CDI (97 mg, 0.6 mmol) was added to the solution and the mixture was stirred for 2.5 h at room temperature. 3-Amino-1,2-propanediol (64 mg, 0.7 mmol) in DMSO (1 mL) was then added to the reaction mixture and stirring was continued at room temperature for another 17 h under argon atmosphere. The reaction mixture was diluted with water (50 mL) and the solution was extracted with dichloromethane (50 mL, twice). The combined organic layers were evaporated to a volume of approximately 3 mL and poured into diethyl ether ($\sim$ 150 mL) to precipitate the polymeric product. The precipitate was collected by filtration, redissolved in water, and the aqueous solution was dialyzed against water overnight in a 500 g/mol cut-off membrane followed by freeze-drying to give finally 336 mg of white solid. Yield: 79%. ^{1}H NMR (D$_2$O): 4.18–4.13 (2H, m, —CH_2OCONH—), 3.82–3.42 ($\sim$ 181H, m, —CH$_2$O(CH$_2$CH$_2$O)$_{44}$— + —CH$_2$CH(OH)CH$_2$OH), 3.30 (3H, s, CH$_3$), 3.21, 3.08 (2H, 2×dd, —CH_2CH(OH)CH$_2$OH, J_{gem} = 14.2 Hz, J = 5.8 Hz). ^{1}H NMR (CDCl$_3$): 5.51 (1H, m, NH), 4.19–4.15 (2H, m, —CH_2OCONH—), 3.77–3.38 ($\sim$ 181H, m, —CH$_2$O(CH$_2$CH$_2$O)$_{44}$— + —CH$_2$CH(OH)CH$_2$OH), 3.31 (3H, s, CH$_3$), 3.30–3.15 (2H, m, —CH_2CH(OH)CH$_2$OH), 2.85 (1H, m, OH).

N-[[Formyl]methyl]carbamoyl-PEG methyl ether 4

Compound 3 (332 mg, 0.157 mmol) was dissolved in 5 mL of water and the solution was cooled to 0°C.

To this solution was added 10 mL of 0.2M aqueous solution of NaIO$_4$ (12.5 equiv. per 1,2-diol group) and the mixture was stirred in the dark at 0°C for 30 min and for another 30 min at room temperature. The reaction solution was then dialyzed against water overnight in 500 g/mol cut-off membrane followed by freeze-drying. Crude product was further purified by partitioning between dichloromethane and water. The organic phase was separated, redissolved in water, and finally freeze-dried to give 293 mg of white solid. Yield: 90%. ^{1}H NMR (D$_2$O): 8.04 (0.5 × 1H, s, (HO)CH= of enol form), 4.94 (0.5 × 1H, t, (HO)$_2$CH— of 1,1-diol form, J = 5.1 Hz), 4.25–4.22 (2H, m, —CH_2OCONH—), 4.12–4.08 (1H, m), 3.77–3.37 ($\sim$178H, m, —CH$_2$O(CH$_2$CH$_2$O)$_{44}$—), 3.25 (3H, s, CH$_3$), 3.10 (0.5 × 2H, d, (HO)$_2$CHCH_2— of 1,1-diol form, J = 4.8 Hz). [1]H NMR (CDCl$_3$): 9.59 (0.5 × 1H, s, O=CH— of aldehyde form), 8.02 (0.5 × 1H, s, (HO)CH= of enol form), 5.58 (0.5 × 1H, m, (HO)CH=CH— of enol form), 4.25, 4.19 (2H, 2×t, —CH_2OCONH—, J = 4.7 Hz), 4.04 (0.5 × 2H, d, —CH_2CHO of aldehyde form, J = 5.1 Hz), 3.77–3.38 ($\sim$178H, m, —CH$_2$O(CH$_2$CH$_2$O)$_{44}$—), 3.31 (3H, s, CH$_3$).

MeO-PEG-*graft*-PVA copolymer 5

PVA-HY (5 mg, 0.25 μmol of hydrazide groups) and compound 4 (52 mg, 0.025 mmol) were dissolved separately in 1 mL of deionized water each. The solutions were mixed and stirred for 19 h. The solution was then dialyzed twice against water first in 3500 g/mol cut-off membrane and then in 25,000 g/mol cut-off membrane. Yield: 28 mg.

Hydrogel formation and characterization

The PVA components were separately dissolved in H$_2$O at concentrations 100, 50, 25, or 12.5 mg/mL to give solutions A and B. Equal volumes of solutions A and B of the same concentration were added to a glass vial to give equimolar equivalents of aldehyde and hydrazide groups, and the solution was mixed with swirling. The mixtures were agitated for another 24 h to obtain uniform hydrogels. The prepared gel after crosslinking reaction was swollen for 48 h in deionized water during which the nonincorporated network fraction (= sol fraction) was extracted. The extracted gel was freeze-dried and the mass of the freeze-dried network W_p was determined. 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 crosslinking reaction W_0 and W_p, $W_{sol} = W_0 - W_p$. The dried gel was again reswollen for 24 h in water and weighed in air.

The mechanical properties of the swollen gel were measured on the AR2000 rheometer (TA Instruments, UK) with an aluminum parallel plate geometry of 8 mm diameter. Storage and loss moduli (G' and G'') were obtained from a frequency sweep (from 0.01 to 10 Hz) performed at the normal force of $\sim$ 150 mN and 25°C. Data are reported at a frequency of 0.5 Hz.

Cell encapsulation in PVA-HY/PVA-AL hydrogel

Murine neuroblastoma N2a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, La Jolla, CA), containing L-glutamine (Life Technologies), penicillin-streptamycin (1000 U/mL; Life Technologies), and 10% fetal bovine serum. Twenty-five milligrams of each component, PVA-AL and PVA-HY, were dissolved separately in deionized water (in 0.5 mL for PVA-AL and in 1 mL for PVA-HY, respectively) by heating. Both solutions were allowed to cool down, and 400,000 N2a cells in 0.5 mL of cell culture medium were added to the solution of PVA-HY. The components were then mixed by addition of PVA-HY-cells suspension to PVA-AL solution. The formed gel was cut into pieces and placed in wells of a 24-well plate and covered with 1 mL cell culture medium. The encapsulated cells were pulsed for 12 h with 5-bromodeoxyuridine (BrdU, 10 μM, Sigma) to monitor proliferation and fixed for 15 min at 37°C using 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 at different time points (0, 1, 2, 4, 5 days), quickly frozen on dry ice, and cryo-sectioned (10–20 μm).

Immunocytochemistry

Cryo-sectioned N2a-matrix on microscope slides or N2a cells grown on glass cover slips were blocked to avoid unspecific binding of antibodies for 30 min with 2% normal goat serum (NGS) in PBS with 0.05% TritonX-100 (PBS-T) at room temperature. Primary mouse anti β-III-tubulin (BabCO, Berkeley, CA; 1 : 500) was added overnight in 2% NGS-PBS-T at 4°C. Cells were washed 3 × 10 min in PBS, and incubated for 40 min at room temperature with secondary N,N'-dipropyltetramethylindocarbocyanine (Cy3)-conjugated goat anti-mouse antibody (Jackson, West Grove, PA; 1 : 200), washed and mounted with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Pictures were taken with a 20× objective on a Zeiss Axioplan2 fluorescent microscope. For BrdU staining, sectioned matrix (and positive control) was treated with 2M HCl for 15 min at room temperature to denature the DNA, washed in PBS, followed by incubation with primary biotinylated mouse anti-BrdU antibody (Zymed laboratories;1:100) during 90 min at RT. Cells were washed in PBS, and incubated for 30 min with avidin-horseradish peroxidase conjugate (1 : 100; A+B kit, Dako AS), and developed using the peroxidase substrate, 3',3'-diaminobenzidine, resulting in a dark brown nucleus in BrdU immunoreactive cells.

RESULTS AND DISCUSSION

Synthesis and characterization of PVA-AL and PVA-HY

The chemical versatility of PVA is mainly provided by the presence of pendant hydroxyl groups in the backbone, which is amenable to chemical modifications such as grafting and crosslinking. Built up from exclusively stable C—C bonds, PVA can tolerate a wide range of chemical modification conditions without decomposition, which along with its solubility in water makes it a logical choice for incorporation of a variety of substituents. We have recently described the introduction of azide and alkyne functional groups into PVA by first activation of PVA hydroxyl groups with 1,1'-carbonyldiimidazole (CDI) followed by reaction of O-(imidazol-1-ylcarbonyl)-activated PVA with the appropriate amine.[22] This approach involves formation of a carbamate linkage and utilizes reagents that contain an amino group and a functional group of interest separated by a linker. The degree of substitution can be controlled by the feeding amount of amine. In this paper, we demonstrate for the first time the introduction of hydrazide and aldehyde groups into the PVA backbone. Generally, a functional group of interest have to be protected or used in the form of its nonreactive precursor to exclude possible interference with the amino group and/or CDI in the course of carbamate coupling. Thus, the synthesis of functional PVA derivatives by chemical modification of hydroxyl groups was carried out in two steps (Scheme 1). The first step is the carbamate linking of a PVA hydroxyl group to an amine reagent containing functional group of interest in its masked form. The second step consists of conversion of the functional precursor into its reactive form.

According to Scheme 1, the synthesis of aldehyde-modified PVA (PVA-AL) was performed by carbamate linking of a hydroxyl group in PVA to 3-amino-1,2-propanediol yielding 1,2-diol functionalized PVA **1** which was then treated with NaIO$_4$ to generate the aldehyde groups in the final PVA-AL derivative. Oxidation of the 1,2-diol group was carried under mild conditions, namely a 10-fold excess of NaIO$_4$ per each 1,2-diol group incorporated in PVA **1** at 0°C for 30 min. Precautions were undertaken to avoid possible PVA fragmentation of the head-to-head sequences, which amount is much
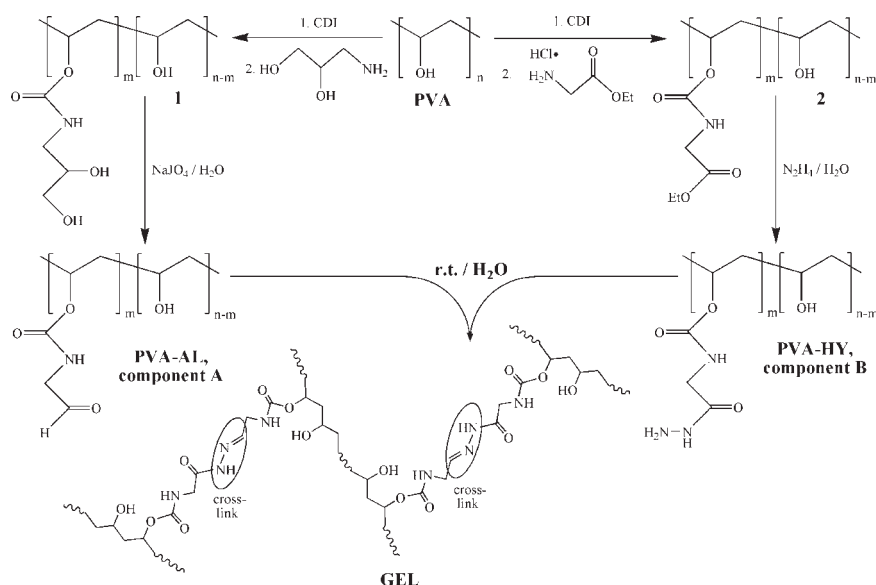
Scheme 1 Synthesis of PVA-AL and PVA-HY and their crosslinking reaction leading to gel formation.

smaller than the head-to-head arrangements but still is detectable in atactic commercial PVA samples.[25]

To synthesize the PVA-HY component containing hydrazide groups, we used glycine ethyl ester as a hydrazide building block. Treatment of PVA with this reagent afforded ester containing PVA intermediate **2**, which was then converted without isolation to the corresponding hydrazide-modified PVA-HY. The instability of the ester group toward hydrolysis, normally applied in our procedure[22] after carbamate coupling, dictated to change the work-up strategy. Upon completion of the carbamate coupling in which glycine ethyl ester is employed, hydrazine was added into the reaction mixture in anhydrous DMSO to convert the esters to hydrazides. Moreover, after completion of conversion to hydrazides and addition of water, hydrazine acts as a deprotecting agent hydrolyzing the excess of hydroxyls activated by CDI. This *in situ* generation of hydrazides from esters is a versatile approach for introduction

of pendant hydrazide groups into polymers that are stable toward hydrazinolysis.

Introduction of aldehyde pendant groups into the PVA core was confirmed by applying the same synthetic strategy to poly(ethylene glycol) methyl ether, following the same experimental conditions that were used for PVA modification (Scheme 2). Comparing ^{1}H NMR spectra of the corresponding PVA and PEG derivatives, characteristic signals of the appended aldehyde and hydrazide groups were obtained. The amphiphilic nature of PEG methyl ethers allowed us to analyze them both in D_2O and $CDCl_3$, and thus to detect those protons that are not visible in ^{1}H NMR spectra recorded in D_2O due to their exchange with water protons. The inspection of spectra of MeO-PEG-aldehyde derivative **4** in $CDCl_3$, shown in Figure 2(A), revealed the aldehyde proton at 9.59 ppm and an additional peak at 8.03 ppm which can be attributed to the corresponding proton of enol tautomeric form. Surprisingly, enol tautomer
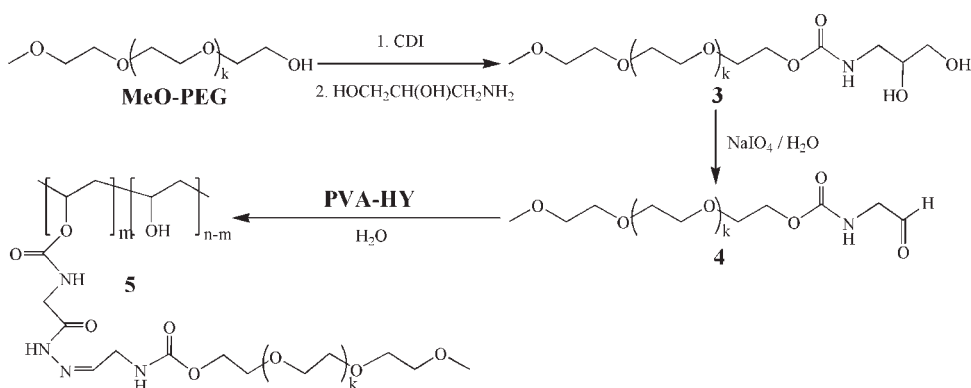
Scheme 2 Synthesis of PEG methyl ethers **4** functionalized with aldehyde group and hydrazone crosslinking model reactions between MeO-PEG-aldehyde derivative **4** and PVA-HY.
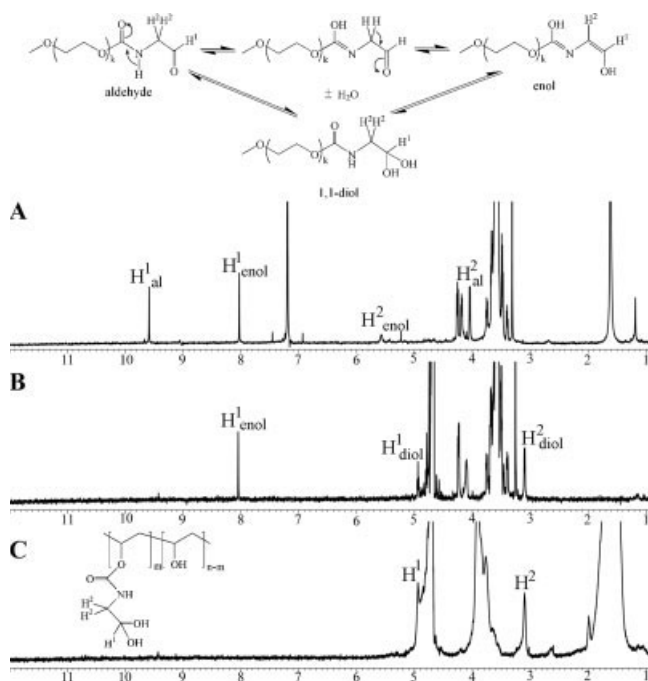
Figure 2 NMR spectra of (A) MeO-PEG-aldehyde **4** in CDCl$_3$, (B) MeO-PEG-aldehyde **4** in D$_2$O, and (C) PVA-AL **7** in D$_2$O.

is present in sufficient amount probably due to its stabilization through additional delocalization of an electron pair centered on carbamate nitrogen. The CO—NH bond has partially double character and tends to move into conjugation with the aldehyde group. It is interesting that dissolving polymer **4** in D$_2$O leads to the disappearance of the aldehyde signal at 9.59 ppm but appearance of another triplet at 4.94 ppm [Fig. 2(B)]. This indicates that aldehyde functionality is hydrated in D$_2$O giving 1,1-diol which is thus in equilibrium with the enol form. The integration of these peaks shows that both 1,1-diol and enol forms of **4** are present in approximately equal amounts in water. Chemical shift of the adjacent α-methylene protons is also affected by aldehyde hydration that differs for aldehyde [4.09 ppm, Fig. 2(A)] and 1,1-diol forms [3.10 ppm, Fig. 2(B)]. In contrast, dissolution of polymer PVA-AL in water causes an almost complete hydration of aldehyde functionality as it is seen from ^{1}H NMR spectrum of PVA-AL [Fig. 2(C)]. The signal at 3.10 ppm was assigned as α-CH$_2$ protons adjacent to hydrated aldehyde group by comparison with NMR signal corresponding to analogous group of MeO-PEG-aldehyde derivative **4** in D$_2$O [Fig. 2(B)]. Also, very weak aldehyde resonance is detected at 9.43 ppm, while the dominating CH(OH)$_2$ signal at 4.95 ppm is clearly visible despite of its partial overlapping with HDO. The extent of incorporated aldehyde group was determined by comparison of the integration of

the α-methylene protons of CH$_2$CH(OH)$_2$ pendant groups of PVA-AL with the CH or CH$_2$ of the PVA backbone appearing at 3.6–4.0 ppm and 1.3–2.0 ppm, respectively. 4.8% incorporation of aldehyde moiety per PVA monomer unit was obtained in a two-step synthesis when feed molar ratio [3-amino-1,2-propanediol]/[—OH]$_0$ = 0.1 has been used, indicating at least 48% incorporation efficiency during the first step. Thus, the reaction of PVA with 0.5M equivalents of CDI followed by addition of 0.1M equivalents of 3-amino-1,2-propanediol allowed us to obtain a 1,2-diol functionalized PVA which upon oxidation with NaIO$_4$ gave highly pure PVA-AL derivative with a low degree of substitution (0.01 < DS < 0.05).

The presence of hydrazide functionality in PVA-HY could not be shown as clearly as it was done for aldehyde group in PVA-AL and MeO-PEG **4**. The α-methylene protons of CH$_2$CONHNH$_2$ do not show clearly distinct ^{1}H NMR signals due to their overlapping with the backbone protons of PVA-HY. That is why the formation of hydrazide derivatives was demonstrated by their reactivity toward aldehydes. We first quantified the number of hydrazide groups by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay. TNBS is known to react with hydrazides, and the decrease in its content was used for the analysis. The molar fraction of hydrazide groups obtained in this way was 0.075.

The reaction of PVA-HY with 10M excess of 2-fur-aldehyde in water at room temperature gave a material insoluble in water (data not shown) which prompted us to explore functionalization of PVA-HY with MeO-PEG-aldehyde derivative **4**. In our experiment, this was accomplished by addition of two-fold excess of **4** to each hydrazide group of PVA-HY in water at room temperature for 18 h. The expected MeO-PEG-*graft*-PVA copolymer was recovered by dialysis against water twice (first in 3500 g/mol cut-off membrane and then in 25,000 g/mol cut-off membrane) and then characterized by NMR spectroscopy (Fig. 3). In the ^{1}H NMR spectrum of PVA-
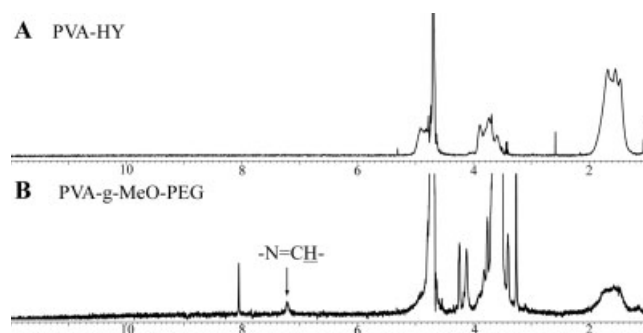

Figure 3 NMR spectra in D$_2$O of (A) PVA-HY and (B) MeO-PEG-*graft*-PVA copolymer **5**.

TABLE I
Macroscopic gel properties of PVA hydrogels

No.	Polymer conc.[a]	Functional group conc.[b] (mM)	Initial polymer mass, W_0 (mg)	Gel fraction, W_P (mg) (%)	Mass of swollen gel, W_s (mg)	W_s/W_P	G' (Pa)	G'' (Pa)
1	5.0%	24.7	50.0	32.6 (55.2%)	258.6	7.93	6437	1325
2	2.5%	12.3	50.0	2.1 (4.2%)	–	–	–	–

[a] Overall concentration of polymer components in the final cross-linking mixture.
[b] Concentration of aldehyde groups (=hydrazide groups) in the final cross-linking mixture.

HY [Fig. 3(A)], the signal between δ 1.3 and 1.9 ppm is assigned to methylene protons of the PVA chain, while the peaks between δ 3.5 and 3.9 ppm are attributed to the PVA backbone CH protons that overlap with the α-methylene protons from $CH_2CONHNH_2$ grafted units. The success of grafting of MeO-PEG-aldehyde derivative 4 via hydrazone linkages was confirmed by the appearance of a signal at 7.20 ppm that is assigned to the —N=CH— proton attached to the carbon that was modified with PVA-HY hydrazide group [Fig. 3(B)]. Methylene groups of the PVA stem in MeO-PEG-*graft*-PVA copolymer were found in the range of 1.4–1.9 ppm while signal for the corresponding PVA methine groups are overlapped with that of PEG chains in the range of 3.4–4.0 ppm. By comparison of the signal intensities of the hydrazone —NH—N=CH— links and PVA backbone —CH_2— groups, the degree of MeO-PEG grafting to PVA-HY was calculated to be 5.8% of PVA monomer units. It was observed, however, that the intensity of the singlet corresponding to methoxy groups of PEG was 1.6 times higher than what would be expected for MeO-PEG-*graft*[5.8%]-PVA copolymer. This implies that free MeO-PEG-aldehyde 4 remains in the mixture even after double dialysis, which may reflect either the inefficiency of purification by dialysis or possible hydrolysis of hydrazone linkages.[26] In fact, slow hydrolysis was evident with acylhydrazones even at neutral pH, which means that they always exist in equilibrium with starting aldehyde and hydrazide species.

By grafting long MeO-PEG chains onto hydrazide-functionalized PVA-HY, the novel material 7 was obtained in which the PVA serves as a skeleton, and the flexible PEG chains serve as branches giving rise to brush-like molecules. Its properties differ from PVA. Thus, unlike hydrophilic PVA that is soluble in only highly polar solvents (H_2O, DMSO, NMP), copolymer 7 is soluble in pure water as well as in CH_2Cl_2 due to the amphiphilic nature of the grafted MeO-PEG. The solution of PEG-*g*-PVA in water was opaque. This indicated phase separation of the grafted PEG chains from the PVA backbone and also agglomeration to create domains large enough to scatter light in the same manner as was shown previously for PEG-*graft*-hyaluronic acid solutions.[27]

In situ formation of PVA hydrogels

In this study, the hydrazone formation of PVA-HY with MeO-PEG-aldehyde 4 thereby served as a model for crosslinking reaction between PVA-HY and PVA-AL (Scheme 1). Since the degree of substitution (DS) with functional groups was kept low in the generated PVA derivatives, they were soluble in water thus allowing hydrogel formation under physiological conditions (in fact, PVA-HY appeared to be more easily soluble in water than PVA-AL, which could be dissolved only by heating). Indeed, mixing of water solutions of PVA-HY and PVA-AL yielded hydrogels with mechanical properties that should be dependent on the PVA concentration in the final mixture. Two gels were prepared from 5% and 2.5% solutions (Table I). The gelation occurred immediately after mixing of 5% solution (Fig. 4), while the gelation time for 2.5% solution was extended to 10 min and no gel was actually formed from 1.25%

Figure 4 Photographs of (A) PVA-HY and PVA-AL solutions in water before mixing and (B) 1 min after their mixing. Both solutions contain 5 wt % of the PVA component. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
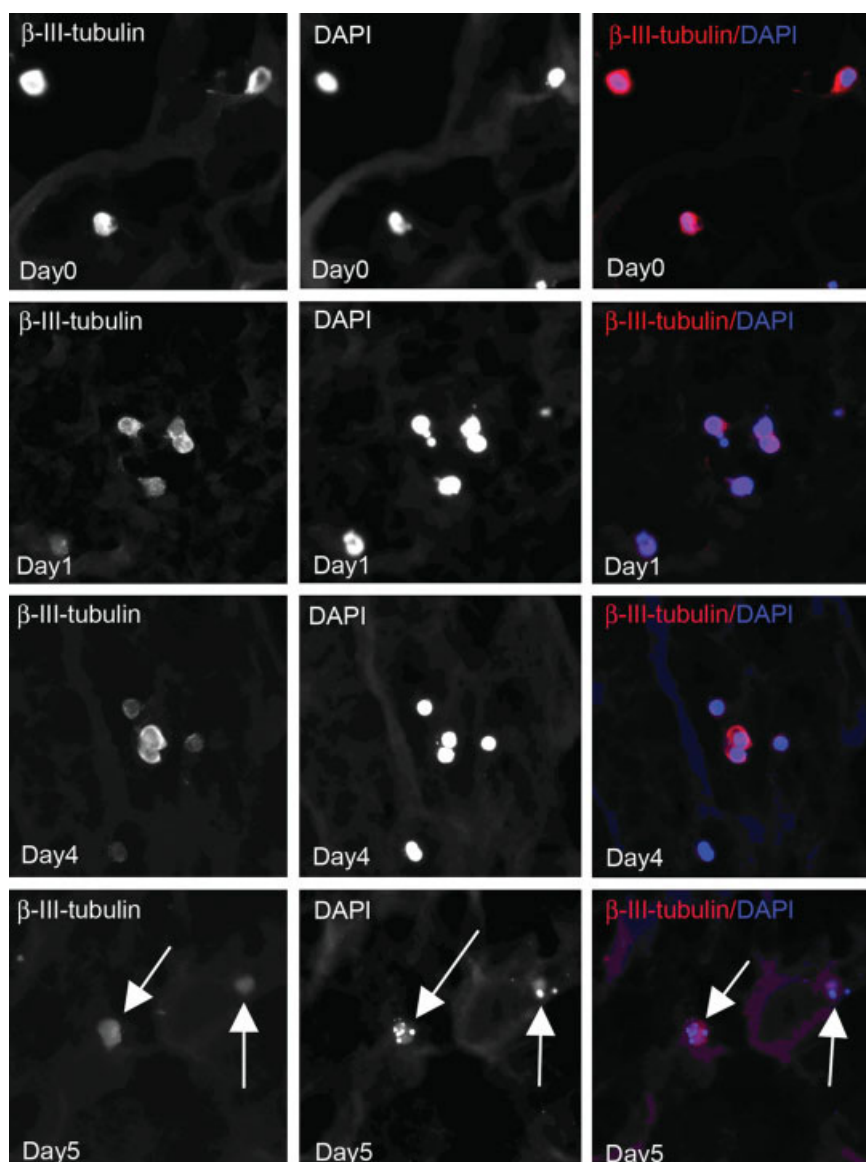
Figure 5 Histological analysis of N2a cells encapsulated in chemically crosslinked PVA-HY/PVA-AL hydrogel. β-III-tubulin was used to visualize the cell cytoplasm, while 4′,6-diamidino-2-phenylindole (DAPI) was used to study the nuclei morphology. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

solution of the mixed components A and B. Already, the 2.5% solution gave a gel with only 4.2% of gel fraction precluding its proper mechanical characterization. Unfortunately, the limited solubility of PVA derivatives in water did not allow to prepare more than 5% concentrated solutions to investigate the effect of PVA precursor concentration on macroscopic network properties.

The hydrazides remain reactive at neutral to slightly acidic conditions due to their lower pK_a of typically 3–4 compared with that of primary amines ($pK_a > 8.5$). Hydrazide amino groups in PVA-HY are thus not protonated at pH > 5.0 and could be crosslinked with aldehyde group of PVA-AL in pure water. On the other hand, the hydrazone crosslin-

kages are less easily hydrolyzed than Schiff's bases, thus allowing crosslinking reactions without reduction of the products. Only under acidic conditions (at pH 4 or below) or at high temperatures do hydrolysis and transimination reach significant rates.[28,29] Furthermore, hydrazone formation is "traceless" crosslinking, in which no side product or potentially toxic reagents are involved. Therefore, hydrazone crosslinking can be pursued *in vivo*.

Cell encapsulation studies

The cytocompatibility of hydrazone crosslinking was investigated by encapsulation of the murine neuroblastoma N2a cells upon mixing of PVA-HY and

PVA-AL solutions. N2a cells are a tumor cell line with some neuronal characteristics. These cells have a high proliferative capacity and thus constituted a good model system for cell encapsulation in this particular PVA matrix. The cells seeded in the hydrogel were examined following 0, 1, 4, and 5 days in culture. The histological analysis of N2a cells encapsulated in PVA-HY/PVA-AL hydrogel formed from 5% solutions is shown in Figure 5. N2a cells, normally expressing the cytoskeletal protein β-III-tubulin, were stained using a specific antibody against β-III-tubulin at different time points in culture as described in the experimental part (Fig. 5, left column). To visualize the cell nuclei, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Fig. 5, middle column), which is known to form fluorescent complexes with natural double-stranded DNA. The nuclei are considered to have a normal morphology when their membranes are intact, with a round or oval shape, and the DAPI is distributed homogenously over the nucleus. Apoptotic nuclei can be identified by fragmented DNA, which is seen in the microscope after DAPI incubation as small round dots. Viable cells, expressing β-III-tubulin and having intact round or oval nuclei, as shown by the DAPI staining, were observed during the first 4 days of culture in the PVA matrix (Fig. 5, right column shows also overlapping of images from both analyses). In addition, staining with DAPI revealed homogenous distribution of cells in the matrix. These results demonstrate that cells can be homogeneously incorporated into new PVA hydrogel scaffold during chemical crosslinking between hydrazide and aldehyde reactive groups without a loss in cell viability. The cell-polymer suspensions could therefore be mixed while being injected through the syringe having two barrels attached to a "Y" connection that is fitted to a mixer nozzle. The cell–polymer suspension is chemically crosslinked as soon as it is delivered *ex vivo* or *in situ* and can fill the cavities at the site of injection.

However, the encapsulated cells did not proliferate in the present matrix and died on the fifth day, which is seen in Figure 5 by the appearance of fragmented DNA in cells nuclei visualized by staining with DAPI (arrows). Cell proliferation was studied using 5-bromo-deoxyuridine (BrdU), which is incorporated into replicated DNA during the course of replication. The cells did not incorporate BrdU, indicating thus no cells proliferation, as was shown by immunostaining with an antibody that is specific against BrdU base analog (data not shown). A possible reason for the lack of cell proliferation could be the inability of the formed network to degrade the scaffold, a property that is critical for tissue regeneration. It has been demonstrated that tissue formation is favorable in degradable materials, presumably since cells can degrade surrounding hydrogel material to provide space for additional cells and deposition of a new ECM to replace the scaffold.[5]

CONCLUSIONS

In this study, we have prepared the first *in situ* crosslinkable PVA hydrogel that is formed by mixing of aqueous solutions of two PVA components derivatized with hydrazide and aldehyde pendant groups. In contrast to commonly used *in situ* approaches, which utilize photopolymerization of PVA derivatives with attached vinyl groups, here hydrogel formation is based on the chemoselective crosslinking reaction. Particularly, addition of hydrazides to aldehydes occurs in aqueous media at room temperature with no need for a catalyst and at a sufficient rate to be used for *in situ* hydrogel formation. The employed hydrazone crosslinking between hydrazide and aldehyde groups was also shown to be tolerated by the cells, demonstrating the potential of the aldehyde-hydrazide crosslinking strategy in the design of injectable biodegradable materials. The lack of degradable linkages in the present system, however, could lead to trapping of the cells with no evolution of void space around them, thus, physically preventing their proliferation. The incorporation of cleavable linkages into linkers, connecting functional groups with polymer backbone, can be a solution for making PVA-based degradable systems. Another option to apply hydrazone crosslinking strategy for production of tissue engineering scaffolds *in situ* is the use of the appropriately modified natural polymers which are intrinsically biodegradable. However, the incorporation of different chemical functionalities into natural polysaccharides could be a considerable challenge. The desired lability of polysaccharides glucosidic linkages, on the other hand, limits the range of chemical transformations that could be applied for incorporation of the required functional groups into complex polysaccharide structure. Taking into account the ease, with which different functional groups can be attached to PVA hydroxyl groups, PVA could serve as a good starting model polymer to assess the biocompatibility of crosslinking chemistry of interest before planning to use it in more sophisticated chemical gelling systems.

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