Michael-Type Addition Reactions for the In Situ Formation of Poly(vinyl alcohol)-Based Hydrogels

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Michael-type addition reactions offer the possibility to obtain in situ formation of polymeric hydrogels in the absence of a radical mechanism for the networking process. We explored such a synthetic route for obtaining a poly(vinyl alcohol) (PVA)-based hydrogel as a potential biomaterial for applications in vitro-retinal replacement surgery. The presence of radicals in the reaction medium can represent a risk for in situ surgical treatment. To circumvent this problem we have applied nucleophilic addition to ad hoc modified PVA macromers. The gel formation has been studied with respect to the timing required in this surgery and in terms of the structural characteristics of the obtained network.

Introduction

The formulation of new biomaterials displaying tailored features is the main issue in the present scenario of drug delivery, tissue engineering, and substitution.[1] The properties of a polymeric biomaterial can be addressed by suitable chemical modifications of the starting polymer structure and by the choice of the most convenient type of cross-linking reaction.

Our activity is focused on the formulation and characterization of new polymeric materials for vitreous replacement, a major demand of vitro-retinal surgery.[2] The requirements of an ideal material for vitreous substitution are dictated not only by the tamponade and shock absorption functions of the material but also by its capability to sustain the delivery of metabolites and drugs. The vitreous body structure is complex despite the fact that most of its weight is made of water. The main polymeric components, collagen and hyaluronate, are responsible for the confinement of water and for the gel-like properties of vitreous by forming a double network[3] of randomly distributed collagen fibers and random-coiled high molecular weight hyaluronate, as demonstrated by transmission electron microscopy. The interaction pattern occurring in this multicomponent scaffold, not yet completely disclosed, is made of ionic bridges and entanglements although the presence of chemical cross-links has been hypothesized.

Vitreous replacement has been used as a treatment in tissue reconstructive surgery for several retinal pathologies, systemic diseases, degenerative processes, and trauma caused by mechanical, chemical, and thermal injuries. Several kinds of materials have been used for the posterior segment of eye replacement including gases, silicone oil, biopolymers, and synthetic polymers, the selection rule being the requirements of transparency, biocompatibility, and permeability to gases and metabolites to assure retina functions.

In the design of a vitreous substitute a benchmark is certainly a satisfactory simulation of the viscoelastic behavior of the biomaterial. The viscoelastic properties of the vitreous are a direct consequence of the dual nature of the polymeric constituents as the collagen fiber scaffold provides the elastic behavior and the hyaluronate moiety is the damping element for the vitreous shock-absorbing function.[4]

The determination of the rheological properties of the vitreous is difficult due to its structural complexity and its deterioration in the preparation of the experiment and during the measurement. Experiments on intact bovine vitreous were carried out by measuring the dynamic compression moduli,[4] revealing different viscoelastic behaviors depending on the probed vitreal region. Modern vitro-retinal surgery is oriented toward the formulation of vitreous substitution treatments with reduced invasiveness where the replacement of the vitreous is carried out by injecting the material in the posterior of the eye and in situ triggering of the process leading to the shaping of the mechanical and chemical functionalities of the material.

Biopolymers are a natural choice for the design of a vitreous substitute.[5−7] However, they are subjected to chemical and enzymatic hydrolysis with a fast deterioration of the gel properties. Modified biopolymers and synthetic biocompatible polymers have been used in the past. As far as PVA is concerned, in vivo viability tests showed that PVA cross-linked by γ-irradiation in dilute aqueous solution and injected in the vitreal cavity was a suitable material for vitreous substitution.[8] A toxic response was observed with commercial PVA obtained by methanolysis.[9]

In the context we have recently formulated a hydrogel based on the in situ cross-linking of methacryloyl-PVA upon irradiation that can be envisaged as a potential surgical treatment for vitreous replacement. A possible bias to this synthetic route is a transient and low concentration of free radicals triggered by the photoinitiation of the cross-linking reaction that may damage the vitreal cavity tissues. To circumvent this difficulty, we have devised a synthetic route based on the Michael addition reaction and applied by the Hubbell group to several molecules and polymer functionalities.[10] This approach allows the in situ formation of a PVA-based network, avoiding the drawbacks of the photopolymerization of the methacryloyl-PVA system.

Scheme 1 depicts the synthetic route followed for obtaining the polymer network by mixing two macromolecular components, and it is based on the coupling of (i) an end-capped thiol-PVA macromer with (ii) a methacryloyl derivative of PVA.

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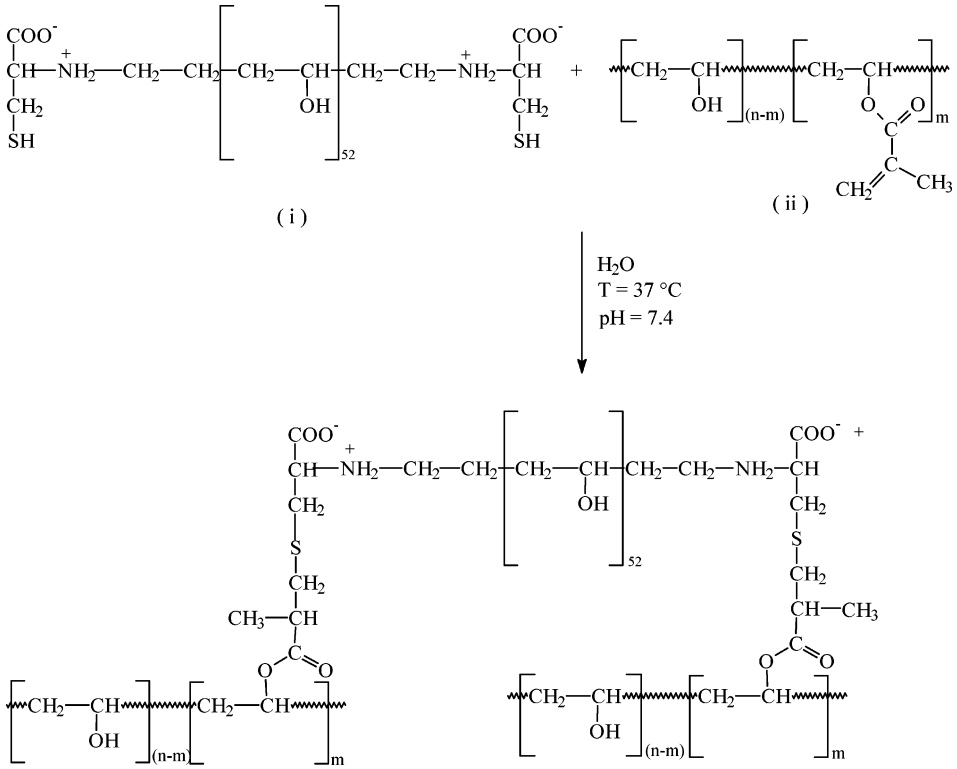
Scheme 1. Michael-Type Reaction for the Formation of a PVA-Based Polymer Network



Molecule (i) is obtained by the selective oxidation of the head-to-head sequence of PVA chains by metaperiodate followed by the conjugation of the amine group of cysteine by reductive amination. Molecule (ii) is the trans-esterification product of the reaction between glycidyl methacrylate and the hydroxyl moiety of PVA. The mechanism of this reaction was first elucidated by Hennink et al.[11,12] in the synthesis of glycidyl-methacrylate-derivatized dextran.

The end-capped thiol-PVA (i) is then used as cross-linker for networking the methacryloyl-substituted PVA (ii) chains by Michael-type addition in the absence of radicals. Recently, Anseth et al. suggested an analogous strategy based on a Michael-type addition reaction with the use of multifunctional thiols and linear PEG-PLA terminating with acrylate ends.[13] Along this line, chemical cross-links were introduced in a physical polysaccharide−peptide network by a Michael-type addition reaction, yielding environmentally sensitive biomimetic matrixes.[14]

The formation of the polymer network is established on the availability of methacrylated PVA (PVA-MA) and of thiol end-capped PVA (PVA-SH). The synthesis of PVA-MA was already described in a former paper from our group.[2] For thiol end-capped PVA we devise a synthetic route starting from the selective splitting of the end-to-end sequence of PVA.[15,16] The obtainment of functionalized PVA chains with aldehydic terminal groups allows the reductive amination with any molecules carrying amine moiety. In this work we coupled PVA with cysteine to obtain thiol end-capped PVA chains, as described in Scheme 2, starting from the splitting of vicinal diols occurring in the PVA commercial sample to an extent of approximately 1.5−2%. The metaperiodate oxidation will produce PVA chains bearing two terminal aldehydes and two PVA chains for each starting polymer chain bearing only one aldehyde terminal group as a result of the splitting of the outermost diols present in the PVA chains. The mono-aldehyde PVA will be subjected to coupling with cysteine and eventually incorporated in the network as a dangling chain and without contributing to the mechanical properties of the hydrogel.

Experimental Section

Materials. Poly(vinyl alcohol) with a molecular weight of 37 000 g/mol, L-cysteine, and 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) were Sigma products. Glycidyl methacrylate (GMA) and 4-(dimethylamino) pyridine (DMAP) were purchased from Fluka. Sodium metaperiodate, sodium cyanoborohydrate, barium chloride, HCl, dimethylsulfoxide (DMSO), and acetone were purchased from Carlo Erba. All products were used without further purification.

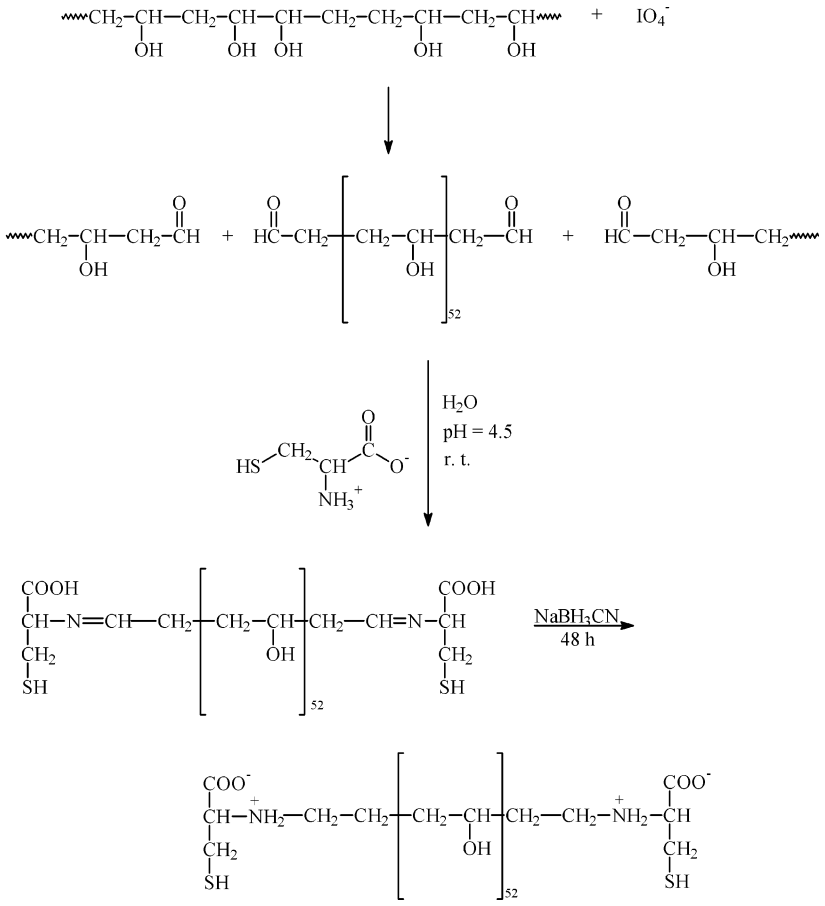
The degree of deacetylation of PVA, determined by ^{1}H NMR, was 0.98.

Purified, deionized water (Milli-Q purity grade with a conductivity of 18.2 MΩ cm) was produced with a purification apparatus (PureLab).

Methods. *Synthesis of Methacryloyl-PVA.* Approximately 10 g of PVA and 250 mL of DMSO were placed in a 500 mL two-necked flask. The suspension was stirred at 80 °C. After dissolution of PVA, the system reaction was allowed to cool at room temperature, and the solution was fluxed with nitrogen. Then, 5.0 g of DMAP was added under nitrogen atmosphere. Finally, 0.64 g of GMA was added to the solution. The reaction was carried out for 48 h at room temperature and stopped by adding 8 mL of 6 M HCl, an equimolar amount to GMA. The neutralized solution was transferred into dialysis tubing (molecular weight cutoff of 12 000−14 000 g/mol) to replace DMSO with water and to eliminate the low molecular weight reagents and dialyzed exhaustively against Milli-Q quality water. The dialyzed aqueous solution was concentrated to 125 mL and was poured dropwise into 250 mL of acetone. The white precipitate was collected by filtration and dried. The degree of substitution of methacryloyl-PVA was determined by ^{1}H NMR.

Synthesis of Thiol End-Capped Telechelic PVA. In a 500 mL two-necked flask, 10.0 g of PVA and 100 mL of water were added. The suspension was stirred at 80 °C to allow PVA dissolution. Approximately 0.98 g of sodium metaperiodate was added to the solution, and the system was stirred at 80 °C. After 30 min, the solution was

Scheme 2. Synthesis of Thiol End-Capped PVA Chains



cooled, and 1.12 g of barium chloride was added to precipitate the iodate produced during the head-to-head sequence splitting. The formed white powder was filtered off. Approximately 15.8 g of cysteine was dissolved in 100 mL of acetate buffered at pH = 4.5 and added to the PVA solution, adjusting the pH to 4.5 by means of 5 M NaOH. PVA and cysteine solutions were fluxed with nitrogen for 1 h and then mixed and stirred at room temperature for 30 min. Finally, 1.26 g of sodium cyanoborohydride were added, and the mixture was reacted for 48 h. The solution was transferred into dialysis bags with a molecular weight cutoff of 1000 g/mol and dialyzed exhaustively against dilute HCl at pH 3.5. After concentration of the solution to 100 mL, it was poured dropwise into 200 mL of acetone. The white powder was collected by filtration and dried.

PVA-MA and Thiol End-Capped PVA Cross-Linked Hydrogels. PVA-MA and thiol end-capped PVA were dissolved in a phosphate buffer at pH 7.4 under nitrogen protection to give a total concentration of 6% (w/v) with a 1:1 molar ratio of the reacting groups. The gel point was determined by the test tube inversion assay. After 60 min of exposure to air at 37 °C, the polymer-containing test tubes were turned upside down without observing any fluidity, thus concluding that a gel phase was formed.

Characterization Methods. ^{1}H NMR spectra were recorded at 40 °C in D_2O or in DMSO-d_6 with a Bruker 400 MHz at a proton resonance frequency of 100 MHz.

Thiol content was determined according to the Ellman method[17] with a JASCO spectrometer model 7850 UV/vis using a extinction coefficient ϵ at 412 nm of 13 250 M^{-1} cm^{-1} for the product of the reaction of DTNB in thiol-containing solutions.

Viscosimetric measurements were carried out in a suspended flow viscosimeter immersed in a water bath at 30 °C. Total polymer concentrations ranged from 4% to 8% (w/v). Flow times were measured at different reaction times.

Compression modulus measurements were performed by dynamic mechanical analysis (DMA) as described by Meyvis et al.[18] to evaluate the cross-linking density of the hydrogels. PVA-MA/PVASH networks were compressed in the linear range with a DMA-7 dynamic mechanical analyzer (Perkin-Elmer) equipped with a parallel plate accessory with a diameter of 20 mm. Disks with the same diameter and thickness of 2−4 mm were cut from hydrogel slabs and fully immersed in a water bath. The experiments were performed at room temperature by applying a static stress between 30 and 300 mN to allow a good contact between the gel and the rheometer plates. A dynamic strain of 0.2% with 1 Hz periodic oscillation was applied with three measurement replicas for each sample.

Equilibrium swelling measurements were carried out by weighing hydrogel cylindrical slabs (W_0). Slabs with the same dimensions used for DMA analysis were immersed in a large volume of water, and at predetermined time intervals, the wet disks were weighed (W_t) immediately after the removal of surface water by blotting.

Results and Discussion

Thiol End-Capped PVA. The synthesis of PVA chains having two aldehydes as terminal groups has been already reported.[15,16] The number average molecular weight, M_n, depends on the relative molar amounts of head-to-head sequences and splitting agent. In the present work an M_n value of 2400 g/mol was achieved by complete oxidation of the vicinal diols in the PVA backbone.

The conjugation of thiol groups with PVA aldehydes is achieved by a Schiff reaction with the amine group of cysteine followed by a reductive amination with cyanoborohydrate of the corresponding imines with a yield of 75%. Scheme 2 summarizes the steps of this one-pot reaction in an aqueous medium.

The reaction product was exhaustively dialyzed against a dilute solution of HCl at pH 3 to prevent thiol oxidation.

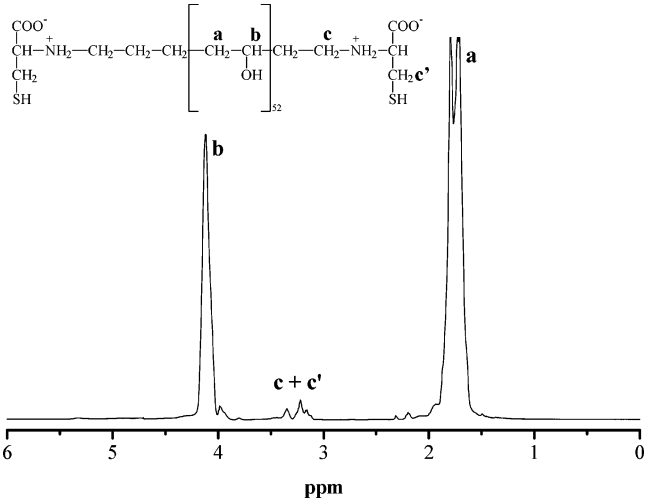


Figure 1. ^{1}H NMR spectrum of dithiol PVA in D_2O at 40 °C.

From the ^{1}H NMR spectrum in D_2O, Figure 1, the multiplets in the range of 3.0−3.5 ppm were assigned to the terminal methylenes of PVA, indicated with c and c′, respectively.

The amount of thiol groups per gram of PVA as a function of the molar ratio of aldehyde/cysteine was determined by the Ellman method[17] by titrating the thiols of cysteine end-capped telechelic PVA present in solution after purification. The degree of substitution resulted in a leveling off to a value of 0.38 mmol/g of PVA at a molar ratio of aldehyde/cysteine of 1:15. A further increase of cysteine to a ratio of 1:50 did not yield a PVA with a higher thiol content as available reactive aldehydes were a fraction of the total at the pH condition used for avoiding thiol oxidation. Nitrogen purging of oxygen to limit the oxidation of thiols to −S−S− bridges had no effect on the degree of substitution of PVA.

The methacryloyl derivative of PVA (PVA-MA) has been obtained in our laboratory[2] according to the description of a similar reaction provided by Hennink et al.[11,12] for the synthesis of the methacryloyl derivative of dextran.

PVA-MA with increasing substitution degrees, from 1% to 10%, was insoluble for degree of substitution (DS) values higher than 3%. A PVA sample with a DS value of approximately 2% has been used throughout this study for the coupling with thiol end-capped PVA.

Our focus is the formation of hydrogels in the vitreous cavity, avoiding the use of compounds, such as photo-cross-linkers usually characterized by limited biocompatibility or even toxicity depending on the concentrations used. According to the work of Hubbell and Tirelli,[19] Michael-type addition of thiols to unsaturated esters offers an interesting route for networking functionalized polymer chains without using a photoreticulation process. In this context, we have studied the kinetics of the addition of thiol end-capped PVA chains to grafted PVA-MA. The reaction was studied by monitoring the time decrease of the ^{1}H NMR peaks of the vinyl protons ($[H_{vin}]_t$) at 5.75 and 6.2 ppm, respectively, with a total polymer concentration of 6% (w/v) at 40 °C (Figure 2).

Although in similar conditions the gelation time was 60 min, the segmental polymer dynamics is not influenced by the sol-to-gel transition with the ^{1}H NMR relaxation times of the vinyl protons close to the solution state values. In this case it has been possible to follow the kinetics of the coupling via the decrease of the peak area of the vinyl protons well beyond the macroscopic gelation time.

Determination of the order and of the rate constant of the addition reaction was carried out by plotting the left-hand side of eq 1 (M^{-1}) versus time, as reported in Figure 3.

$$\frac{1}{[\text{vinyl}]_0 - [\text{thiol}]_0} \ln \frac{[\text{vinyl}]_t[\text{thiol}]_0}{[\text{vinyl}]_0[\text{thiol}]_t} = kt \qquad (1)$$

Linearity was maintained beyond two half-life times, and the value of 0.2 ± 0.01 M^{-1} min^{-1} of the observed second-order rate constant was determined.

The true kinetic constant is evaluated by taking into account that the effective reactive specie participating in the addition reaction is the thiolate, i.e., S^-. This is accomplished by dividing the observed rate constant by the factor $K_a/(K_a + [H^+])$, i.e., the degree of ionization of the terminal thiols. In buffered solutions at pH 7.4 and assuming for K_a the dissociation constant value of cysteine ($pK_a = 8.2$), approximately 16% of the total concentration of thiols determined by the Ellman assay[17] is dissociated, and the value of the effective rate constant of the reaction studied in this work is estimated to be 1.25 M^{-1} min^{-1}.

Comparison with the reactivity of charged oligopeptides containing cysteinyl groups on poly(ethylene glycol) diacrylate indicates that thiols anchored to PVA are remarkably less reactive than oligopeptides containing five amino acid residues.[19] This effect can be explained by the limited diffusivity of the macromolecular chains involved in the addition reaction. However, a similar rate constant for the addition of β,β-methylethylcysteine to acrylonitrile, i.e., 0.26 M^{-1} min^{-1} has been reported in the literature.[20] In the kinetic experiments, the gel point was reached after 60 min, indicating that the minimum number of chemical junctions required for the percolation of the system corresponds to a consumption of 20% of the total PVA terminal thiols.

Gel formation was monitored by viscosimetry at $T = 30$ °C with a total concentration ranging from 4% to 8% (w/v). In this concentration range the gel point is achieved in suitable times for vitrectomy, and the gel phase shows a good transparency.

As shown in Figure 4, the increase of the relative viscosity with time becomes asymptotic, indicating that the establishment of a percolation process is reached for polymer concentrations higher than 5% (w/v). Below this concentration, the coupling between thiol end-capped PVA and methacrylated PVA provides an increase of relative viscosity with a sigmoidal trend, leveling off at a constant value that depends on the total polymer concentration. The cross-linking reaction triggered by irradiation with a 40 W source at 360 nm on the methacryloyl-substituted PVA in the presence of photoinitiator was studied in a previous paper by us.[2] The gelation times displayed by the Michael-type addition reaction and the free radical cross-linking reaction are comparable as well as the gels transparencies.

The gel phase behavior was characterized by elastic modulus and swelling measurements.

According to the affine network model,[21,22] in the small strain region the elastic modulus (E) is related to the density of polymer chains (ν_e/V_0) of effectively contributing chains to the elastic properties by

$$\frac{\nu_e}{V_0} = \frac{E}{3RT} \phi_0^{-2/3} \phi_2^{-1/3} \qquad (2)$$

where ϕ_0 and ϕ_2 are the polymer volume fraction before and after the networking process, respectively. In this model the polymer chain density in eq 2 does not fluctuate upon strain,

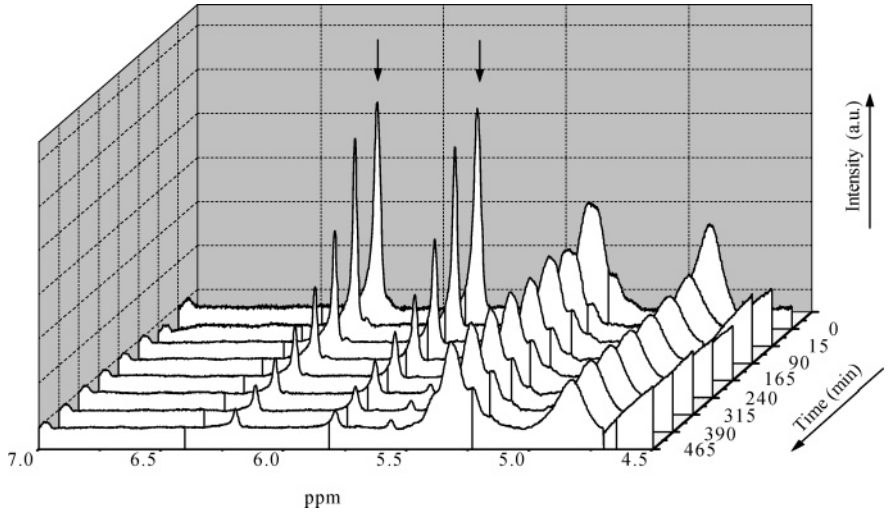


Figure 2. ^{1}H NMR of 6% (w/v) thiol end-capped PVA and PVA-MA solution at 40 °C and at different reaction times. Arrows indicate the vinyl protons' consumption.

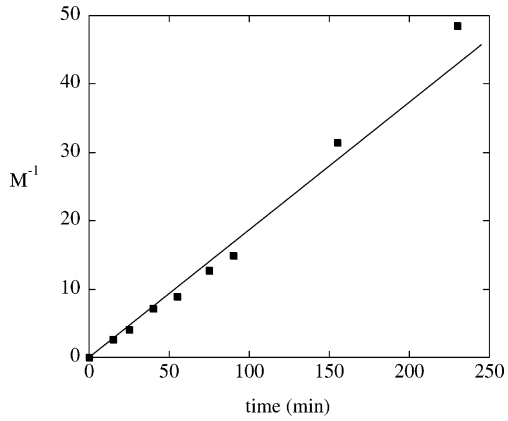


Figure 3. Second-order kinetics of a Michael-type addition reaction of PVA-MA with thiol end-capped.

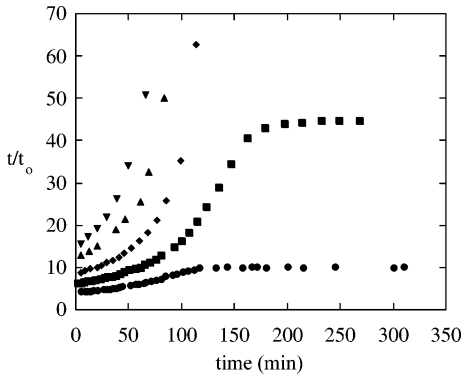


Figure 4. Time increase of viscosity for different total polymer concentrations: ▼ 8% (w/v), ▲ 7% (w/v), ♦ 6% (w/v), ■ 5% (w/v), ● 4% (w/v).

and it can be related to the molecular weight between cross-links (M_c) according to

$$M_c = \frac{\rho_2}{\frac{\nu_e}{V_0} + 2\frac{\rho_2}{M_n}} \quad (3)$$

with the density of atactic dry PVA (ρ_2) and the number average molecular weight of the PVA-MA chains participating in the network formation (M_n).

Table 1. Mechanical Properties of Two Hydrogels with Different Molar Ratios of Coupling Groups

gel sample	vinyl/thiol	ϕ_0[a]	ϕ_2[a]	ν_e/V_0 (mol/m^3)	E[a] (MPa)	M_c (g/mol)
1	1/1	0.10	0.07	11	5	2400
2	1/2	0.11	0.09	20	15	1700

[a] Errors within 10%.

The determination of M_c was carried out on gels obtained with two mixing ratios, vinyl/thiol = 1:1 and 1:2, respectively. The values of M_c shown in Table 1 were obtained by DMA analysis according to eq 3. There is a clear influence of M_c on the relative amount of the vinyl and thiol groups and on the presence of physical junctions spontaneously formed during the networking process. The increase of the cross-link density due to physical linkages promoted by the crystallization of a small region of PVA influences the overall mechanical properties of the network, and it can be only partially controlled.

The swelling response of the networks obtained with an equimolar mixture of PVA-MA/PVA-SH at a total polymer concentration of 6% (w/v) was studied in water and at an increasing concentration of NaCl, i.e., 0.1 and 0.5 M, a salt concentration range which encompasses the ionic strength values found in the anterior, central, and posterior parts of bovine vitreous.[23] The pH values in healthy human vitreous samples is approximately 7.1,[4] about a unit higher than that of the aqueous medium of the present study. In these conditions the

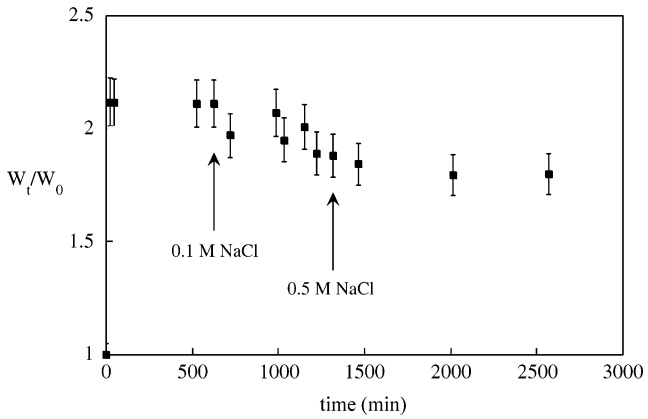


Figure 5. Swelling equilibrium of PVA hydrogels based on a Michael-type addition reaction. Arrows indicate the medium change.

pH difference should be of minor influence on the swelling behavior of the hydrogels due to the electrolyte screening action of the medium.

In Figure 5 the swelling ratio, i.e., the weight of swollen gel with respect to the initial gel mass, is measured by equilibrating the hydrogel against water, 0.1 and 0.5 M NaCl, respectively, as a function of time. The equilibrium is achieved generally in approximately 12 h independently from the ionic strength. Passing from water to 0.5 M NaCl, a shrinking of approximately 20% was observed indicating the screening effect of aqueous NaCl on the network net charge, assuming that the isoelectric point of the cysteinyl moiety of the end-capped PVA chains is similar to that of cysteine, i.e., 5.06.

Concluding Remarks

In a previous work we explored in situ formation of a PVA-based network by irradiating at 350 nm an aqueous solution of methacryloyl-grafted PVA in the presence of a photoinitiator.[2] However, the presence of transient radicals can represent an obstacle to the use of this method for the formation of a hydrogel directly in the ocular cavity. As an alternative route, in this work we studied the possibility of obtaining a network by the nucleophilic thiol−ene addition between the thiol-capped PVA chains and the vinyl moiety contained in a grafted methacrylic PVA as a potential candidate for surgical applications entailing in situ vitreous replacement. The gel point is reached in a suitable time, and the hydrogel is osmotically stable, reaching swelling equilibrium after approximately 12 h. Both polymeric components are readily soluble in aqueous medium and display a remarkable ease in their handling as well as in their injectability. Contrary to the photo-cross-linked PVA hydrogels, networks obtained by vinyl−thiol conjugation display higher elastic moduli at comparable total polymer concentrations. These two systems represent a new set of hydrogels among the yet small number of synthetic materials suitable for tissue replacement. The advantages offered by the hydrogel described in this work include the possibility to inject easy-to-handle solutions containing the polymers to couple, a suitable gelation time for standard surgery, and good chemical and physical stability.

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