Poly(vinyl alcohol)-Based Hydrogels Formed by "Click Chemistry"

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ABSTRACT: Novel poly(vinyl alcohols) (PVA) functionalized with pendant acetylene and azide groups were prepared by carbonyldiimidazole (CDI)-mediated couplings of the amines terminated with functional groups, 1-azido-2-aminoethane, propargylamine, or *N*-methylpropargylamine, to PVA. Low degrees (1−5%) of PVA modification were required in order to retain solubility in water. Azide-modified PVA and alkyne-modified PVA components were cross-linked by mixing of their solutions together with Cu(I) catalyst, a type of Huisgen's 1,3-dipolar azide−alkyne cycloaddition, recently defined as a powerful "click" chemistry. Reaction of the two different polymers results in a chemoselective coupling between alkynyl and azido functional groups with the multiple formation of triazole cross-links to give hydrogel formation. In another version the PVA-based hydrogels were obtained by cross-linking of alkyne-modified PVA with the telechelic bifunctional poly(ethylene glycol)−diazide cross-linker. The hydrogels prepared by these two methods were characterized by their equilibrium swelling in water, by their viscoelastic properties in the swollen state, and by their soluble fraction. It was demonstrated that network properties are affected by the type of cross-linker with polyfunctional PVA cross-linkers having higher gelation capacity than the bifunctional PEG cross-linker. The approach we describe here presents a promising alternative to a common chemical hydrogel preparation technique, which utilizes bifunctional low-molecular-weight cross-linkers.

Introduction

Bioorthogonal chemical reactions, in which the chemical partners react selectively without interference by biological functionality, have become important tools in construction of intelligent materials applicable in the biomedical field. For example, such materials are of interest as controllable drug delivery systems,[1] as cell-encapsulating materials,[2] and, in the new field of "tissue engineering", as matrices for repairing and regenerating a wide variety of tissues and organs.[3] The incorporation of the bioorthogonal chemical groups into biological molecules can create unique points of addressable reactivity in large and complex targets.[4] We are currently investigating different cross-linking chemistries, which could afford in-vivo hydrogel formation and cell growth. Cell applications impose, however, certain limitations to make the right choice of cross-linking chemistry: (i) Cross-linking has to be obtained in aqueous solution at physiological temperature and pH. (ii) Cross-linking reaction should be addition reaction or condensation without the release of harmful side product. (iii) The ligation reaction must be chemoselective and bioorthogonal with narrow distributions of reagents reactivity. Particularly, coupling functional groups should be stable toward hydrolysis and oxidation and recognize only each other while ignoring their cellular surroundings. (iv) Cross-linking kinetics has to be slower than the diffusion rate to allow proper mixing of polymer solutions but fast enough to give a hydrogel within a few minutes.

Some chemoselective cross-linking reactions initiated by simple mixing of polymer components solutions have been applied for hydrogel formation. For example, coupling between an aldehyde and a hydrazide group has been used for hyaluronic acid hydrogel preparation.[5−7] An interesting variation of this reaction, the linkage between an aldehyde and a cysteine 1,2-aminothiol group with the formation of thiazolidine ring, belongs to a family of peptide ligation reactions,[8] and it has recently been invented for hydrogel formation.[9] Michael addition of thiols to acrylates[10,11] or vinyl sulfones[12,13] were also applied for hydrogel formation. These reactions can be carried at physiological temperature and pH and are selective versus biological amines.[14] A major drawback, however, in using thiol-functionalized compounds is their sensitivity toward oxidation. Beyond the listed above examples, there are some biocompatible reactions which have never been applied for hydrogel preparation: Staudinger ligation between a phosphine and an azide, used in chemoselective modification of cell surface,[4] and Cu(I)-catalyzed version of Huisgen [3 + 2] cycloaddition of azides to alkynes.[15] The last reaction was elegantly utilized in recent years in preparation of polymeric triazoles by solution-phase polymerization,[16] dendrimers,[17,18] dendronized linear polymers,[19] and preparation of AB-type block[20] and AB_n-type graft[21] copolymers. Matyjaszewski and co-workers[22] have employed step-growth "click" coupling to the α-alkyne-ω-azido- or α,ω-diazido-terminated polymers to give triazole-linked supramolecular polymers. The utility of "click" chemistry inside living cells has also recently been demonstrated.[23,24] One may therefore envision incorporation of azide and alkyne functional groups into synthetic or natural biodegradable polymer for the purpose of in-situ hydrogel formation.

To create three-dimensional networks, the reactants must have an average functionality of more than 2. The most common chemical hydrogel preparation technique utilizes cross-linking of the pendant functional groups of a hydrophilic polymer by low molecular weight cross-linker normally having two, sometimes more, reactive groups.[25] The use of such bifunctional cross-linkers is accompanied by large degree of intramolecular cross-links formation, which makes high excess of cross-linking agent necessary in order to reach the gelation point. This obviously limits in-vivo application of the cross-linking method. The probability of intermolecular cross-links in the reactions between multifunctional oligomer and bifunctional cross-linker can be increased by increasing (i) concentration of polymer and cross-linker and (ii) distance between reactive groups of the

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bifunctional cross-linker, in other words, by using telechelic bifunctional polymer as a cross-linker. The advantage of the concept of two-component hybrid hydrogels was established for alginate cross-linking by PEG-diamines.[26] The authors found that hydrogels prepared with low-molecular-weight oligo(ethylene glycols) possessed elastic moduli much lower than that prepared with PEG of molecular weight 1000, which gives less fraction of intramolecular cross-links (loops). Hydrogel preparation by cross-linking with macromolecular bifunctional cross-linker is widely established in the literature.[5,9−13] At the same time, extensive functionalization of a linear macromolecule with many cross-linkable groups should obviously increase subsequent intermolecular cross-linking density of the resultant hydrogel. In this case both polymeric components equally act as cross-linkers toward many macromolecules of complementary reactivity. This approach was not explored yet in a systematic way by comparison with other cross-linking techniques.

Here we introduce azide and alkyne pendant groups onto poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) in order to investigate the possibility of hydrogel formation by click chemistry and to derive structure−property relationships for gels prepared by two cross-linking methods. In the first approach we used telechelic PEG-diazide as a cross-linker for the PVA multiply functionalized with complementary alkyne groups. In the second approach the hydrogels were obtained by the reaction between two PVA components multiply functionalized with azide and alkyne groups. Moreover, we establish a simple and efficient method for the low degree PVA hydroxyl groups functionalization with the reactive pendant groups. Low percent derivatization of biologically active polymers should cause minimal alteration of their structural homogeneity which is particularly relevant in biomaterial applications where the properties of hydrogel-forming polymers and their degradation products are of critical importance. The established here introduction of azide and alkyne groups into PVA could in principle be applicable to other hydroxyl-containing polymers, including naturally occurring polysaccharides.

Results and Discussion

Synthesis of Poly(vinyl alcohols) with Pendant Groups. PVA, like low-molecular-weight alcohols, can be relatively easily derivatized via the hydroxyl groups. Two of the most common PVA modifications reactions used so far are esterification and etherification of the hydroxyl groups. Esterification of PVA with acid chlorides,[27,28] anhydrides,[29−33] and carboxylic acid active esters[34] has widely been employed. The ester bond is, however, easily hydrolyzed, and chemical modification with ether linkages may be an alternative approach. PVA is reported to react with epoxides[35] and alkyl halides according to the Williamson ether synthesis.[36,37] The carbonyl group in aldehydes can be coupled to PVA via acetal rings.[38,39] However, harsh conditions normally associated with either ether or acetal linkage formation cannot be applied for appending of sensitive functionalities. Although hydroxyl groups in PVA show little reactivity at low temperatures, we have been interested in chemical modification of PVA hydroxyl groups under mild conditions, which would permit efficient incorporation of a broad range of chemical functionalities.

An alternative opportunity for PVA hydroxyls functionalization is via a carbamate linkage, easily generated from primary or secondary alcohols by first linking to an appropriate carbonic acid derived coupling reagent followed by treatment with an amine. PVAs containing carbamate linkages have been reported previously.[40,41] Poly(vinyl alcohol) having amino sugar as the pendant group was obtained by partial functionalization with 4-nitrophenyl carbonate groups and subsequent addition of amino sugar.[40] Some amines first activated with 1,1′-carbonyldiimidazole (CDI) were added to PVA in order to prepare tertiary amine-modified PVA.[41] However, the reported elevated temperatures and prolonged reactions times for such carbamate linking procedure preclude inclusion of sensitive chemical groups required during in situ preparation of the hydrogel. We have modified the CDI-mediated coupling procedure by changing the addition order of reagents. A general approach is outlined in Scheme 1. The starting PVA was treated with an excess of CDI (0.5 equiv/hydroxyl) in dry DMSO at room temperature to give *O*-(imidazol-1-ylcarbonyl)-activated PVA which was then in situ reacted with the appropriate amount of amine (0.02−0.1 equiv/hydroxyl). Finally, the reaction mixture was stirred with aqueous ammonia at room temperature to hydrolyze all *O*-(imidazol-1-ylcarbonyl)-activated hydroxyls which did not react with amine. Aliphatic carbamates were reported to be stable under hot (55 °C) ammoniacal treatment in contrast to aniline-derived carbamates,[42] which ensures the stability of our carbamate-modified polymers in aqueous ammonia at room temperature.

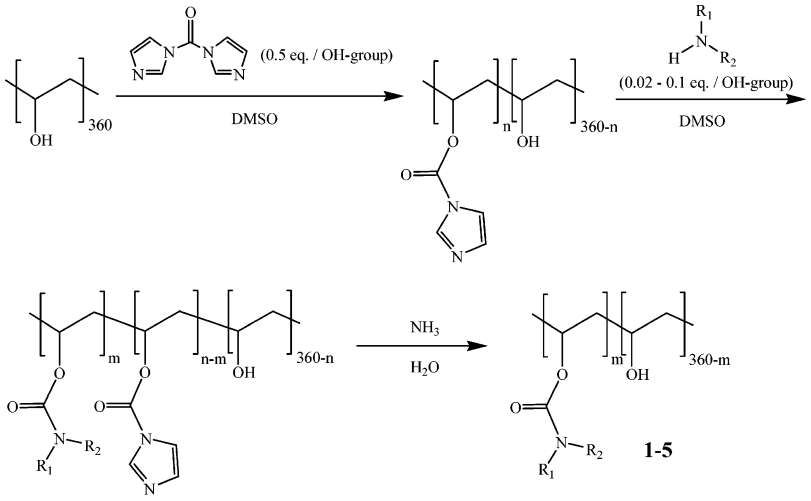
The functionalized polymers were recovered by precipitation of DMSO solutions in diethyl ether followed by dissolving the precipitates in water and repeated precipitation of water solutions in ethanol. Final precipitates were redissolved in water and dialyzed against water. The compositions of the PVAs after grafting and extent of incorporated functional groups were determined from their ^{1}H NMR spectra. To further confirm that the synthetic procedure presented here gives the expected polymers, we prepared model carbamates **6** and **7** (Scheme 2) by condensation of the corresponding *N*-methylpropargylamine and 2-azidoethylamine with methanol under the same conditions, which were used in polymer modification. For example, in comparison with parent PVA (Figure 1A), proton NMR spectra of **3** (Figure 1B) showed the appearance of two signals at δ 3.2−3.3, which corresponds to those of the methylene groups of $-NHCH_2CH_2N_3$ fragment in compound **7** (Figure 1C, a little shift was due to that compound **7** was analyzed in $CDCl_3$ while polymer **3** was recorded in D_2O). Consequently, integration of these signals against the signals at δ 3.6−4.0 and δ 1.3−2.0 (corresponding to C*H* and CH_2 of the polymer backbone) allowed to calculate the degree of modification. Other pendant groups listed in Scheme 1 also have clearly distinct ^{1}H NMR signals, allowing their unambiguous assignment and quantization (see Experimental Part). The molar ratios of introduced alkyne (azide) group to the initial PVA hydroxyl groups ([pendant group]/[−OH]$_0$) are plotted in Figure 2 as a function of the feed molar ratio of *N*-methylpropargylamine (2-azidoethylamine) to the hydroxyl group of PVA ([reagent]/[−OH]$_0$).

Thus, a mild approach, suitable for chemical linking of a wide range of sensitive functional groups to PVA, was developed through activation of PVAs hydroxyl groups with 1,1′-carbonyldiimidazole.

Synthesis of Poly(ethylene glycol)-Diazide. Diazide-terminated poly(ethylene glycol) was synthesized in a two-step reaction following a general procedure[43] utilizing PEG conversion to its bismesylate **8** derivative followed by substitution to PEG-diazide **9**, as outlined in Scheme 3.

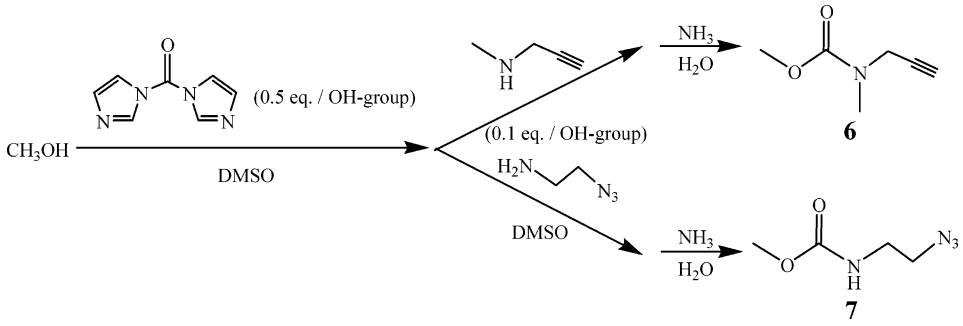
Model 1,3-Cycloaddition Reactions. The ability of the synthesized functional polymers to participate in 1,3-dipolar cycloaddition reactions was confirmed prior to their cross-linking to give hydrogels. Reactions of alkyne-modified PVA **2** with 2-azidoethylamine and azido-modified PVA **3** with

Scheme 1. Preparation of Partially Substituted via Carbamate Linkage Poly(vinyl alcohols)



Compound	R_1	R_2
1	H	$-CH_2-C\equiv CH$
2	Me	$-CH_2-C\equiv CH$
3	H	$-CH_2-CH_2-N_3$
4	H	$-CH_2$-furan
5	H	$-CH_2-NH-C(=O)-O-C(CH_3)_3$

Scheme 2. Structure of Model Carbamates 6 and 7 Synthesized Following the Same Procedure Proposed for PVA Modification



propargylamine in water in the presence of catalyst result in the formation of triazole-modified PVAs **13** and **12**, respectively (Scheme 4). Their structures were then established by ^{1}H NMR spectroscopy. Two model adducts **10** and **11** were also synthesized to facilitate the assignment of all NMR signals. For both polymers **12** and **13**, the key triazole resonances occurred essentially at the same chemical shift (7.9 ppm). Figure 3 shows that additionally to the methyl protons, which are characteristic for alkyne-modified PVA **2** (Figure 3A), the spectrum of **13** revealed signals at 3.25 and 4.50 ppm (Figure 3B). These additional peaks were assigned as α-CH_2 protons and β-CH_2 + γ-CH_2 protons, respectively (see Figure 3B for numbering), by comparison with NMR signals corresponding to the analogous groups of compound **10** (Figure 3C). As for the coupling of **2** with 2-azidoethylamine, an inspection of the ^{1}H NMR spectrum of the product **12** provided the positive evidence of the ability of alkyne- and azide-modified PVAs to enter into 1,3-cycloaddition reaction with corresponding counterparts.

Hydrogel Formation by "Click Chemistry". On the basis of 1,3-cycloaddition reactivity of alkyne- and azide-modified PVAs, we reasoned that it, therefore, should be possible to prepare hydrogels by simply mixing of the prepared PVA components in the presence of Cu^+ catalyst. In our initial studies we focused on the use of the functionalyzed PVAs **2** and **3** as the cross-linking components. As expected, the gelation was observed in both DMSO and aqueous solutions within a minute

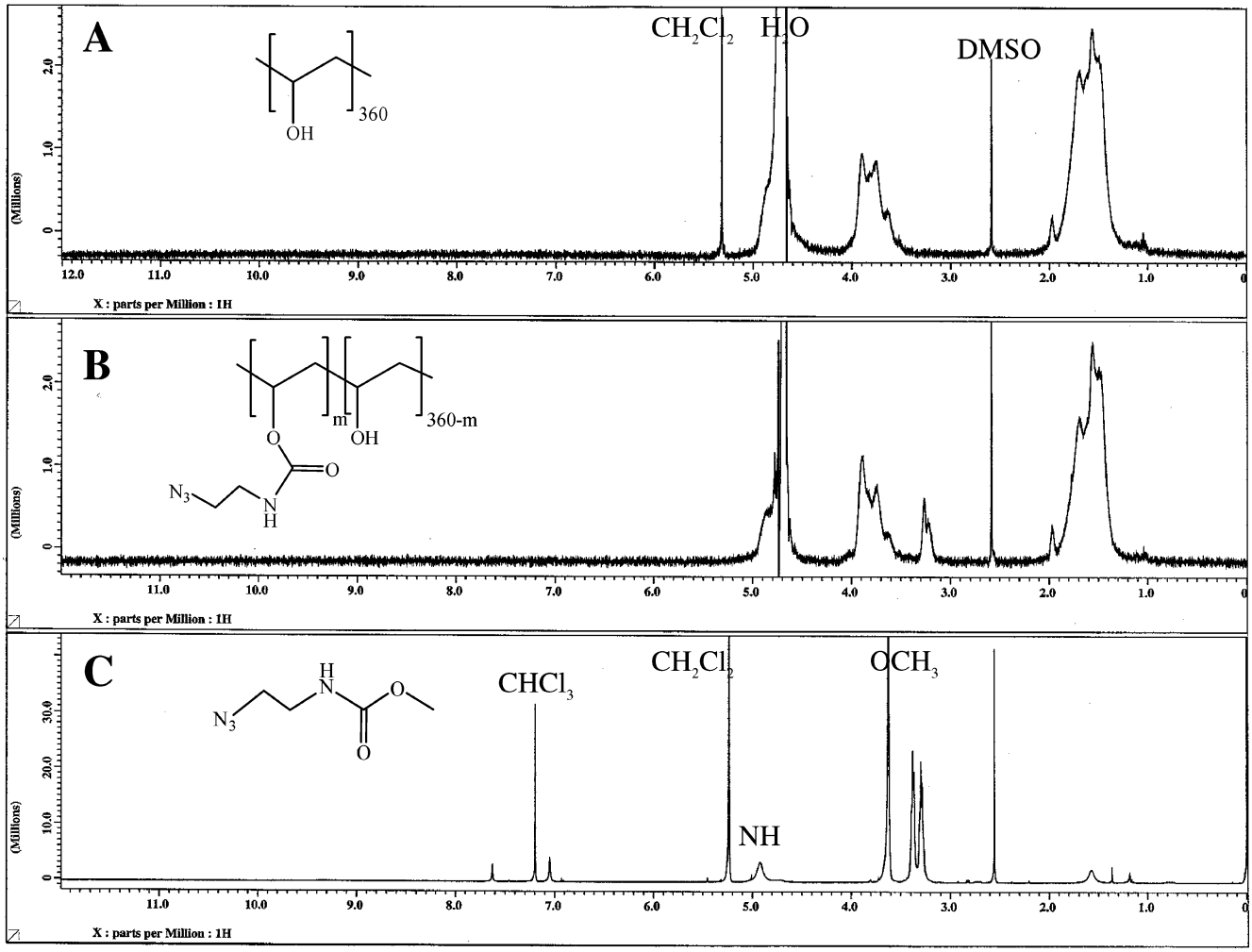


Figure 1. NMR spectra of (A) PVA in D_2O, (B) azide-modified PVA **3** in D_2O, and (C) methyl 2-azidoethylcarbamate **7** in $CDCl_3$. Degree of substitution (DS = $[N_3]/[-OH]_0$) for azide-modified PVA **3** is 0.045.

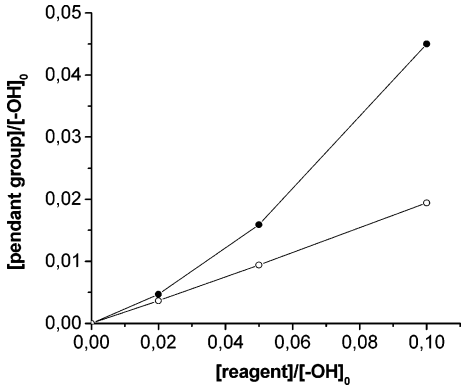


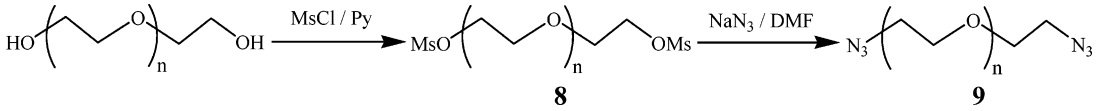
Figure 2. Changes of degree of substitution ([pendant group]/$[-OH]_0$) values of azide-substituted PVA **3** (●) and alkyne-substituted PVA **2** (○) as a function of feed molar ratio [reagent]/$[-OH]_0$ where reagent is *N*-methylpropargylamine or 2-azidoethylamine, respectively.

after addition of catalyst to the mixture of **2** and **3**. Hydrogels was thus formed by chemoselective 1,3-cycloaddition between alkynyl and azido functional groups of PVA components with the multiple formation of triazole cross-links (Figure 4A). Chemically cross-linked PVA hydrogels have been mostly prepared using bifunctional cross-linking agents, such as glutaraldehyde and hexamethylene diisocyanate, which react directly with PVAs hydroxyl groups.[38,44] As was mentioned above, cross-linking with low-molecular-weight toxic reagents is not practicable for in-situ hydrogel formation. Photo-cross-linking of PVA functionalized with the photo-cross-linkable groups such as acrylate[33,35,45,46] or furan-based photosensitive chromophores[39] was employed recently in order to preserve the biocompatibility of PVA-based chemical hydrogels.

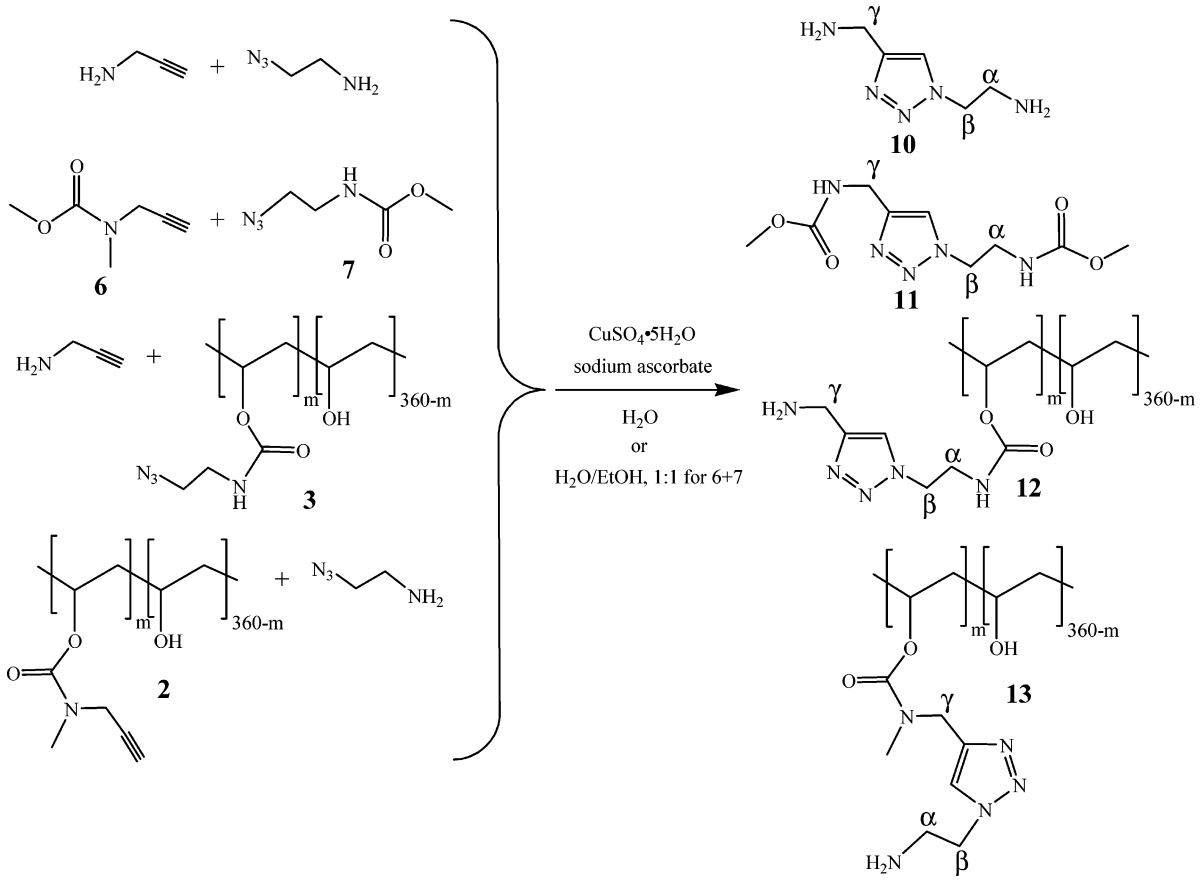
The novel fast-gelling PVA hydrogel developed herein is formed via bioorthogonal "click" reaction between chemoselective pendant groups which are separately incorporated into two PVA components. This is a new approach which should provide several advantages with respect to the gel formation including high cross-linking rates and should affect the macroscopic properties of the obtained hydrogels. To compare the presented here approach with the most common chemical hydrogel preparation technique nowadays, we also prepared hydrogels from multifunctional PVA component and bifunctional PEG cross-linker, as in the mixture **2** + **9** (Figure 4B).

Gel Characteristics as a Function of Polymer Components Structure and Preparation Conditions. Hydrogel properties are expected to be highly dependent on polymer components structure, their % w/w concentration, stoichiometry, and catalyst concentration. Table 1 compares the solid contents, swelling ratio, and storage and loss moduli (G' and G'') of the gels obtained using different preparation conditions and polymer components structure. In the first set of experiments (no. 1−3 in Table 1) the azide-PVA **3** and alkyne-PVA **2** components with the decreasing degree of substitution (DS) were cross-linked to determine how the functional groups concentration would affect cross-linking efficiency of starting polymer

Scheme 3. Preparation of Functional Poly(ethylene glycol)



Scheme 4. 1,3-Cycloaddition Model Reactions with Functional PVAs 2 and 3 and between Low-Molecular-Weight Substrates



components and the mechanical properties of the resulting gels. Generally, increased functional group concentration leads, as expected, to a higher gel fraction content for a given PVA of molecular weight 16 000 g/mol (increasing as follows: experiment 3 → 2 → 1). This effect is obviously more pronounced for polymer components with low DS (compare gel fractions for experiments 3 and 2), whereas the gelation of higher substituted PVAs becomes independent of the polymer functionality (compare gel fractions for experiments 2 and 1). It is evident that some minimal number of functional groups per polymer chain is required to make any given polymer molecule acting as an intermolecular cross-linker for at least two other macromolecules with complementary reactivity. Further increase in polymer functionality will not affect its gel-forming ability, but instead it will only lead to the increased cross-linking density as seen from lower swelling ratio of the gel formed in experiment 1 compared to that in experiment 2 (Table 1). G' was found to be strongly dependent on the number of cross-links formed, which is therefore finely controlled by functionality of the cross-linking polymer components.

Next, the PEG-diazide **9** with the least functionality (= 2) was used as one of the polymer components (experiment 4 in Table 1). It is noteworthy that for both gels no. 4 and 1 the concentration of functional groups and preparation conditions are almost the same. This was achieved due to the fact that both PEG-diazide **9** and azide-modified PVA **3** with the DS = 4.5% have almost the same specific molecular weight $M/f \approx 1050$, where M is the polymer molecular weight and f is the mean number of functional groups per polymer chain. In other words, one azide-modified PVA molecule with the DS = 4.5% ($f = 16.2$) can be mentally "constructed" from approximately eight PEG-diazide molecules ($f = 2$) by "linking" them together into one linear chain. Consequently, "disconnection" of such azide-modified PVA molecule onto eight parts will be "equivalent" of getting eight PEG-diazide molecules. Nevertheless, eight PEG-diazide molecules are not really equivalent to one of the 4.5% azide-modified PVA in hydrogel formation. First of all, the gelation by PEG-diazide **9** was much slower and occurred within 1 h while hydrogels from both PVA components were formed immediately after addition of copper catalyst. Second, the gel fraction of the PEG-diazide cross-linked gel is approximately half of that of the gel formed from the 4.5% azide-modified PVA (compare experiments 4 and 1). This observation indicates that the bifunctional cross-linkers are much more prone to form single-end linkages or intramolecular cross-links which reduce intermolecular cross-linking efficiency. While increase in the macromer functionality f led to the increase of number of intermolecular cross-links, structural differences in the network may also result. As a consequence of the decreased cross-linking density of the gel formed from PEG-diazide, the swelling ratio increases substantially from ~3.6 for gel 1 to ~13.7 for gel 4. Conversely, the elastic modulus drops down by ~80%. This finding confirms that cross-linking density of hydrogels, i.e., their stiffness, can be regulated by changing the

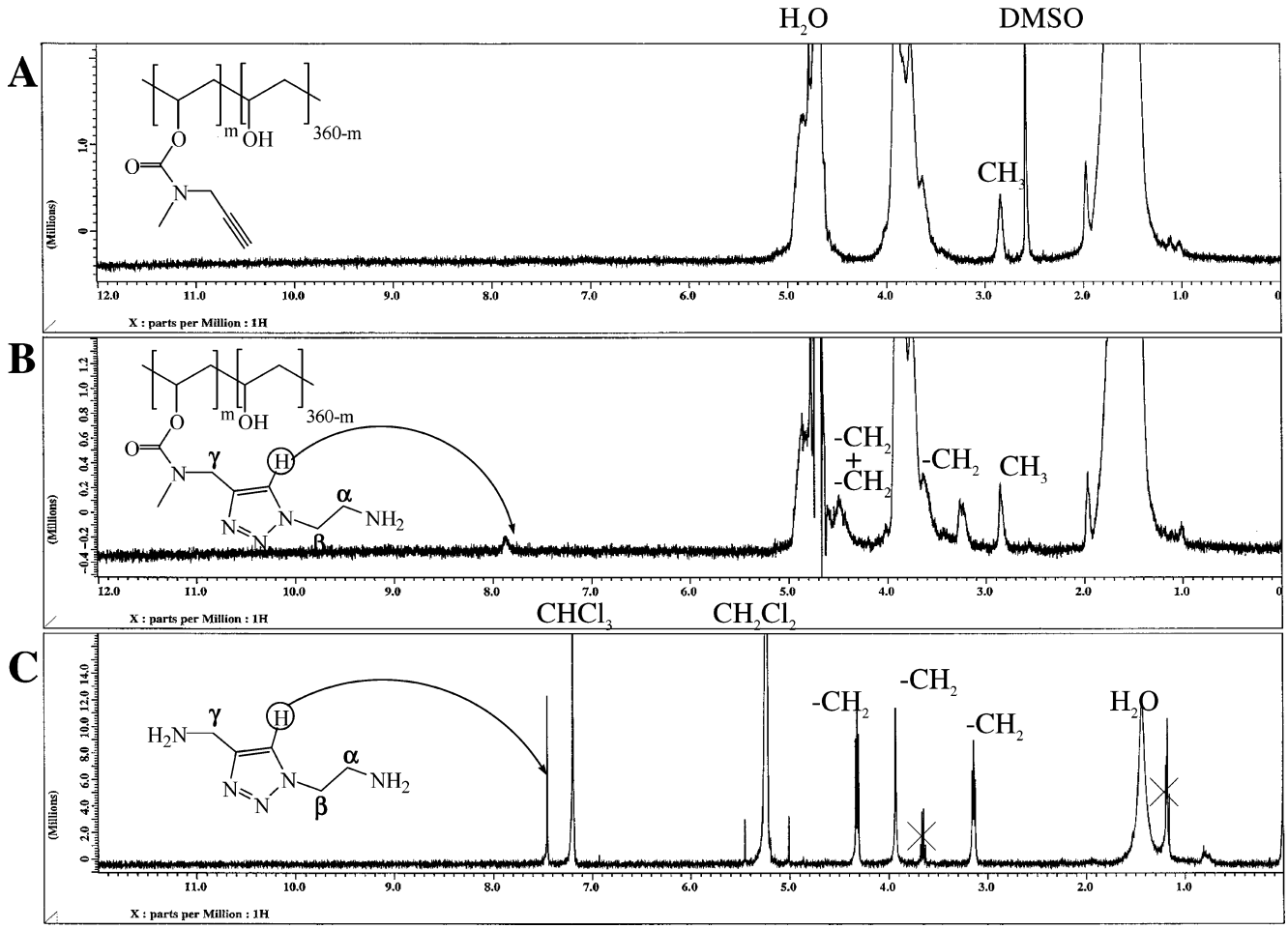


Figure 3. NMR spectra of (A) alkyne-modified PVA **2** in D_2O, (B) triazole-modified PVA **13** in D_2O, and (C) 1-(2-aminoethyl)-4-aminomethyl-1*H*-[1,2,3]-triazole **10** in $CDCl_3$. Degree of substitution (DS = [pendant group]/[−OH]$_0$) for alkyne-modified PVA **2** and triazole-modified PVA **13** are 0.0194 and 0.017, respectively.

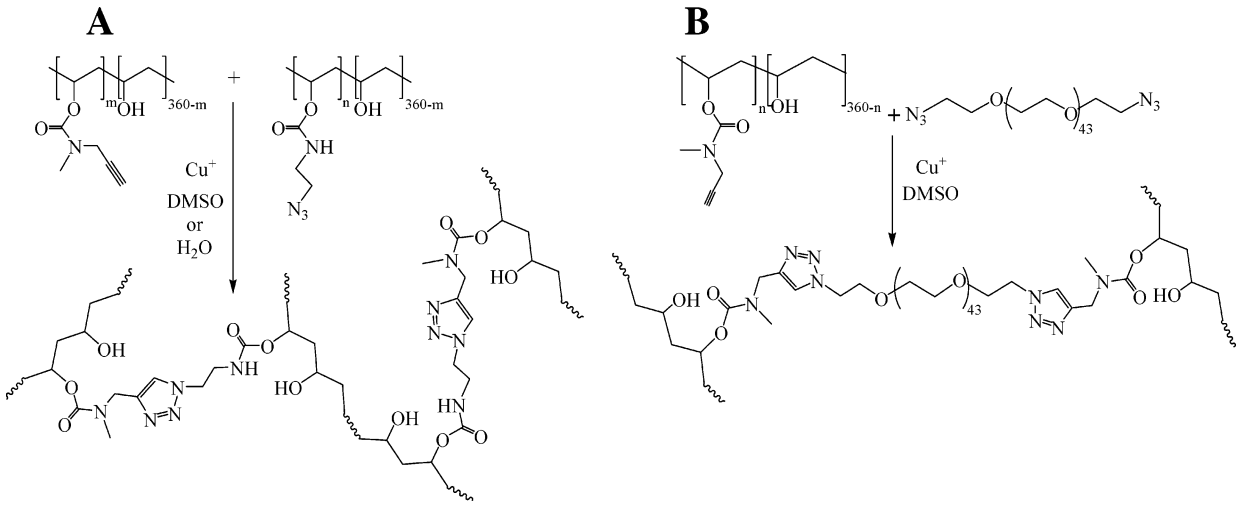


Figure 4. Cross-linking reaction between (A) alkyne-modified PVA **2** and azide-modified PVA **3** and (B) alkyne-modified PVA **2** and PEG-diazide **9** leading to gel formation.

functionality of the cross-linking macromer. Hence, as expected, the swelling ratio and accompanying elastic modulus of the hydrogel can be regulated by changing the number of cross-linkable groups in the second polymer component.

We next evaluated the effect of stoichiometry of the two polymer components on the hydrogel formation and their final properties. One can see that variation of the stoichiometric ratio from 0.7 to 1.4 (experiments 5, 2, and 6) only slightly affected the network formation from 1.6% azide-modified PVA and 1.7% alkyne-modified PVA components. Thus, deviation of the stoichiometry from 1:1 led to some decrease of gel fraction, giving the less densely cross-linked gels with slightly higher swelling ratio (compare experiment 2 with experiments 5 and 6). This result suggests that the hydrogels prepared from both multifunctional polymer components at high DS are not so sensitive to the preparation conditions, including stoichiometry and precursor concentration. The influence of the last parameter was examined in the set of experiments 3, 7, and 8 for a couple

Table 1. Comparison of the Solid Content and Swelling Ratio of Hydrogels Obtained Using Different Preparation Conditions and Polymer Components Structure[a]

no.	azide comp DS (%)	alkyne comp DS (%)	polymer concn[b] (%)	molar ratio of functional groups	functional group concn[c] (mM)	Cu^{2+} concn[d] (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	4.5	1.7	10	1:1	29.2	6	55.6	45.3 (82)	162.5	3.59	16900	715
2	1.6	1.7	10	1:1	19.9	6	55.6	44.7 (80)	190.9	4.27	9458	515
3	1.6	0.94	10	1:1	14.4	6	55.6	39.1 (70)	163.8	4.19	3407	192
4	PEG-di(N_3)	1.7	10	1:1	29.8	6	55.6	20.5 (37)	280.0	13.66	2070	100
5	1.6	1.7	10	0.7:1	16.3:23.4	6	55.6	38.2 (69)	172.5	4.52	4617	334
6	1.6	1.7	10	1:0.7	23.4:16.3	6	55.6	40.0 (72)	183.3	4.58	4167	111
7	1.6	0.94	20	1:1	28.8	6	111.2	98.8 (89)	332.2	3.36	12235	248
8	1.6	0.94	30	1:1	43.2	6	166.8	143.4 (86)	495.1	3.45	12770	352
9	PEG- di(N_3)	1.7	20	1:1	89.4	6	111.2	58.7 (53)	702.0	11.96	6561	168
10	PEG-di(N_3)	1.7	30	1:1	29.2	6	166.8	107.2 (64)	1085.2	10.12	7654	605
11	4.5	1.7	10	1:1	29.2	1.5	55.6	3.6 (7)	13.0	3.61		
12	4.5	1.7	10	1:1	29.2	24	55.6	49.5 (89)	180.1	3.64	18790	1013
13	1.6	0.94	10	1:1	14.4	6	55.6	31.8 (57)	197.0	6.20	2796	479

[a] Solvent used for mixing azide and alkyne components prior addition of the aqueous solutions of sodium ascorbate and copper(II) sulfate was DMSO for experiments 1−12 and H_2O for experiment 13. [b] Overall concentration of polymer components in the final cross-linking mixture. [c] Concentration of azide groups (= alkyne groups, except experiments 7 and 8) in the final cross-linking mixture. [d] Concentration of cupper(II) sulfate in the final cross-linking mixture. Concentration of sodium ascorbate was 5 times higher than $[Cu^{2+}]$ for all experiments.

of 1.6% azide-modified and 0.94% alkyne-modified PVA components and in experiments 4, 9, and 10 for 1.7% alkyne-modified PVA and PEG-diazide cross-linker. It is seen that, increasing the concentration of the precursor solution, one can improve the gelation efficiency of the polymer precursors and obtain more stiff hydrogels with decreasing swelling properties. This effect is very pronounced for gels composed of bifunctional PEG-diazide cross-linker. While no gel was formed from 5% **2** + **9** solution (data not shown), the same gel formed from more concentrated solutions (experiments 4 → 9 → 10) exhibited higher gel fractions and compression of the gel volumes. Contrary, hydrogels prepared form both PVA components (experiments 3 → 7 → 8) exhibited initial increase in the cross-linking density followed by leveling off after reaching the precursor concentration of 20%. It has been demonstrated[26] that elastic modulus increases with the increase of polymer components concentration as long as the theoretical molecular weight between cross-links (M_c) is higher than the molecular weight of the cross-linking molecule, but not above this point. Obviously, cross-linking by a second polyfunctional macromolecule should follow different theoretical model which has not been developed yet.

In the last set of experiments (11, 1, and 12) 4.5% azide-modified PVA and 1.7% alkyne-modified PVA were used for hydrogel formation, and the concentration of the cupper(II) sulfate was varied from 5 to 80 mol % relative to the functional group concentration. Decreasing the catalyst concentration from 80 to 20 mol % resulted in no change in the cross-linking efficiency and cross-linking density (compare gel fraction and swelling ratio for experiments 1 and 12). However, further decreasing of the catalyst concentration to 5 mol % resulted in much smaller hydrogel yield (7% for gel 11). The substantial drop in cross-linking efficiency points at some Cu^{2+} involving side reaction which becomes rate-comparable to the main cycloaddition reaction when the Cu^{2+} concentration reaches some minimum limit. It could be possible that the forming triazole cross-links bind Cu^{2+} or/and actual catalytic Cu^{+} (created by reduction of Cu^{2+} with sodium ascorbate) ions, thus eliminating them from further catalytic cycles.

At last we performed the network formation exclusively in water in order to evaluate the solvent impact on the cross-linking process. Unfortunately, modified PVA with the DS > 2% are only partially soluble in water, which makes it difficult to use them for hydrogel formation. Our trials to perform cross-linking with turbid aqueous PVA solutions of high DS led to precipitation instead of continuous 3D-network formation. This effect indicates possible aggregation of the hydrophobically modified PVA monomer units which could cause cross-linking within local aggregates followed by their precipitation. By decreasing the degree of modification, we could obtain clean 10% aqueous solution of 1.6% azide-modified PVA and 0.94% alkyne-modified PVA precursors (experiment 13) which allowed us to get the gel after addition of the catalyst. This gel was obtained with slightly lower efficiency and was much softer compared to its preparation from DMSO solution. It was not transparent as all gels prepared from DMSO solution, indicating heterogeneities in the formed gel network.

It is seen that gel fractions for the gels prepared from both PVA components are higher (70−89%) than those corresponding to the gelation with bifunctional PEG-diazide (37−64%). However, one could expect 100% gelation efficiency of the multifunctional modified PVA polymers carrying about 3.5 to 16 functional groups per molecule. "Click chemistry" was shown to be highly regioselective, resulting in 1,4-disubstituted triazoles, and almost quantitative, thus allowing to conduct the reaction with stoichiometric amounts of azide and alkyne groups. Hence, it is very unlikely that not 100% gelation was due to incompletion of the 1,3-cycloaddition cross-linking reaction or because of some not cross-linking side reactions. Contrary, the reaction appears to be very quick, and the gels were formed essentially after addition of the copper catalyst to the polymers solution. We reason that it, therefore, could only be due to the difficulty of proper mixing of reagents, which start to react immediately as soon as two solutions are brought into contact. They form the gel "interphase", which becomes a barrier for the rest of the unreacted components to diffuse.

Determination of the Molecular Weight between Cross-Links. The shear modulus G' can be used to determine the molecular weight between cross-links M_c. If no additional cross-linking by secondary forces is present, the gel will behave like a simple rubberlike solid, and the extent of bonding can be calculated from the number of elastic chains ν between cross-links in the polymer network. For the affine network model G' is given by[47] $G' = \nu RT = cRT/M_c \times (1 - 2 \times M_c/M_n)$. Here, c is the concentration of polymer (g m^{-3}) in the cross-linking solution, M_c is the molecular weight between cross-links, and M_n is the primary chain molecular weight (g/mol). The second term in parentheses is a correction for dangling chains which

Table 2. Elastic Modulus and Molecular Weight between Cross-Links in Hydrogels 1−13

no.	molar ratio of functional groups	G', Pa	alkyne−polymer mass,[a] mg	M_c(alkyne),[b] g/mol	theor M_c(alkyne),[c] g/mol	azide−polymer mass,[a] mg	theor M_c(azide),[b] g/mol	M_c(azide),[c] g/mol	M_c,[d] g/mol	theor M_c,[e] g/mol
1	1:1	16900	39.6	4837	2709	16.0	3073	1100	3955	1905
2	1:1	9458	26.9	5220	2709	28.7	5358	2890	5289	2800
3	1:1	3407	34.8	7028	4823	20.8	6527	2890	6778	3857
4	1:1	2070	40.3	7634	2709	15.3	997	1025	4316	1867
5	0.7:1	4617	31.9	6674	2709	23.7	6271	2890	6437	2815
6	1:0.7	4167	22.0	6295	2709	33.6	6888	2890	6539	2784
7	1:1	12235	69.6	6328	4823	41.6	5571	2890	5950	3857
8	1:1	12770	104.4	6792	4823	62.4	6194	2890	6493	3857
9	1:1	6561	80.6	6946	2709	30.6	982	1025	3964	1867
10	1:1	7654	120.9	7126	2709	45.9	991	1025	4076	1867
11	1:1		39.6		2709	16.0		1100		1905
12	1:1	18790	39.6	4622	2709	16.0	2863	1100	3743	1905
13	1:1	2796	34.8	7206	4823	20.8	6790	2890	6998	3857

[a] Mass of polymer component in the final cross-linking mixture. [b] Molecular weight between cross-links in polymer component (alkyne or azide). M_c(component) was calculated from the equation $G' = cRT/M_c \times (1 - 2 \times M_c/M_n)$ where c is the concentration of alkyne or azide component (g m^{-3}) in the cross-linking solution. [c] Theoretical molecular weight between cross-links in the polymer component is assumed to be equal to M/f, where M is the polymer molecular weight f is the mean number of groups per polymer chain. [d] Average molecular weight between cross-links in hydrogel. $M_c = [n \times M_c(\text{alkyne}) + m \times M_c(\text{azide})]/(n + m)$, where n and m are the stoichiometric coefficients of the alkyne and azide components in the molar ratio of their functional groups (for equimolar ratios, as in experiments 1−4 and 7−13, $n = m$). [e] Average maximal molecular weight between cross-links in hydrogel. $M_c = [n \times M_c(\text{alkyne})_{\text{theor}} + m \times M_c(\text{azide})_{\text{theor}}]/(n + m)$.

originate from cross-links but not play a role in network elasticity. We applied this equation to calculate the molecular weight between cross-links along the linear polymer chain of each component (Table 2) using its corresponding concentration in the cross-linking solution. These data were compared with the theoretical M_c values under the condition when all cross-linkable chemical groups (azide or alkyne respectively) participate in the formation of the elastically active cross-links. In this case the theoretical molecular weight between cross-links for the particular polymer component is equal to its molecular weight divided by the mean number of functional groups per polymer component primary chain. The experimentally determined M_c's appeared, as expected, higher than the theoretical ones, indicating that the maximal cross-linking density was never achieved due to network defects, such as nonreacted groups and loops. It can be seen that such imperfections are less notable for hydrogels that were formed from two PVA components compared to hydrogels obtained by cross-linking with PEG-diazide second component. Second, the unexpected change of M_c was observed in the experiments, in which polymer concentration in the initial cross-linking solution was varied from 5 to 30% (see sets of experiments 3→7→8 and 4→9→10). Calculated M_c decreases for hydrogels with increasing polymer content up to 20%. Correspondingly, elastic modulus was shown to increase with the increasing polymer components concentration in this range. However, at higher concentration (30%), M_c starts to increase. This behavior is consistent with earlier observation for the systems where the cross-linking molecule occupies a large weight fraction in the sample,[26] and it is contradictory to the theory of rubber elasticity,[47] which was developed for zero length or short cross-linking molecules.

Peppas and Merill introduced the equation according to which the network structure can be derived from its swelling behavior and the interaction parameter between cross-linking polymer and the solvent used as cross-linking media.[48] We could not, however, apply this equation to our gels which were mostly formed in DMSO. The gels just after formation, in the relaxed state, were not strictly speaking real hydrogels. After gel formation the solvent was thoroughly exchanged to water by swelling in water with the following freeze-drying and the repeated reswelling in water. Thus, only after solvent exchange we got hydrogels which were used in the swelling and rheological mesurements.

Conclusion

We have elaborated partial functionalization of the PVA hydroxyl groups via carbamate linkages which allowed us to introduce different functional groups, including those which can be used for the cross-linking of the macromer molecules. The synthetic route was shown to have a large degree of flexibilitywhich likely can be expanded to other hydroxyl-containing polymers, including naturally occurring polysaccharides. Multiple introduction of azide groups to one PVA component as well as alkyne groups to the other one was further shown to yield transparent hydrogels upon mixing these components in the presence of the Cu^+ catalyst (click chemistry). Click chemistry was thus for the first time applied for the purpose of hydrogel formation. Taking into account the bioorthogonality and biocompatibility of the clicking chemistry, the hydrogels prepared by such a way could be implied for many biological targets, biomolecules, or cells. On the other hand, the hydrogels were prepared in a distinct way to the commonly used cross-linking technique, namely by mixing of two polymeric components multiply functionalized with the complementary reactive chemoselective groups. This approach was shown to be more effective in gel formation, giving higher values of the gel fractions compared to use of the bifunctional cross-linkers. At the same time, mechanical properties of the obtained hydrogels were also affected by the type of the cross-linking macromolecules. Thus, hydrogels obtained from two polyfunctionalized PVA components were characterized by higher elastic moduli compared to hydrogels prepared with the bifunctional cross-linker. Using bifunctional cross-linkers, especially with low molecular weight and short distance between functional groups, results in large amount of intermolecular cross-links and loop formation. In other words, one can control the mechanical properties of hydrogels not only by varying the weight fraction (concentration) of the cross-linking molecule but also by changing the functionality (number of functional group) of the cross-linking molecule.

Experimental Part

General. 2-Azidoethylamine[49] and *tert*-butyl 2-aminoethylcarbamate[50] were prepared according to literature procedures. Other reagents, 1,1′-carbonyldiimidazole, propargylamine, *N*-methylpropargylamine, and 2-furfurylamine, were purchased from Aldrich Chemical Co. and used as received. PVA with average molecular

weight 16 000 g/mol (degree of deacetylation: 98.0−98.8%) and methanesulfonyl chloride were from Fluka. PEG of molecular weight 2000 g/mol was obtained from Merck (Schuchardt). All solvents were of analytical quality (p.a.) and were dried over 4 Å molecular sieves. Dialysis membranes Spectra/Por 6 (cutoff 1000 g/mol) were purchased from VWR International. The NMR experiments (δ scale; J values are in Hz) were carried out on a JEOL JNM-ECP Series FT NMR system at a magnetic field strength of 9.4 T, operating at 400 MHz for ^{1}H. Fourier transform infrared (FTIR) spectra were recorded using a Spectrum One FT-IR spectrophotometer from Perkin-Elmer Instruments.

General Procedure for the Preparation of Modified PVAs. PVA (500 mg, 11.25 mmol of hydroxyl groups) was dissolved in dry DMSO (10 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 (913 mg, 5.63 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. After that the solution of corresponding amine (0.225−1.125 mmol, 0.02−0.1 equiv) in DMSO (1 mL) was added. Stirring was continued at room temperature for ∼20 h under an argon atmosphere. Afterward 5 mL of concentrated aqueous NH_3 was added, and the mixture was stirred for 1 h at room temperature. Finally it was diluted with 60 mL of water, filtered until clear, and reduced to ∼10 mL volume by rotary evaporation. The substituted polymer was precipitated from the residual DMSO solution by adding 10-fold excess of an 80/20 mixture of diethyl ether and ethanol. All samples were with degree of substitution (DS) up to 5% and were soluble in water. That is why the precipitated samples were redissolved in a small amount of water and dialyzed against water for 24 h. In some cases, to aqueous solution was added ethanol to precipitate substituted polymer again, which was then dissolved in water and finally dialyzed against water for 24 h. The dialyzed solution was subsequently freeze-dried to give a fine white powder.

N-Methylpropargylamine-Substituted PVAs **(2)**. *N*-Methylpropargylamine-substituted PVA was synthesized with three different DS: 0.00364 (yield 91%), 0.0094 (83%), 0.0194 (89%). ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m, partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.00−3.55 (1H, m, polymer backbone CH of unmodified unit), 2.85 ((DS × 3)H, s, NCH_3), 2.00 − 1.35 (2H, m, polymer backbone CH_2).

2-Azidoethylamine-Substituted PVAs **(3)**. 2-Azidoethylamine-substituted PVA was synthesized with three different DS: 0.0047 (yield 95%), 0.0159 (59%), 0.045 (73%). ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.00−3.55 (1H, m, polymer backbone CH of unmodified unit), 3.18−3.30 ((DS × 4)H, 2 × m, $-NHCH_2CH_2N_3$), 2.00−1.35 (2H, m, polymer backbone CH_2).

2-Furfurylamine-Substituted PVA **(4)**. DS 0.0393 (84%). ^{1}H NMR (D_2O): 7.35 ((DS × 1)H, s, furan), 6.30 ((DS × 1)H, s, furan), 6.19 ((DS × 1)H, s, furan), 4.90 ((DS × 1)H, m, partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.19−4.15 ((DS × 2)H, m, $-CH_2$-furan), 4.00−3.55 (1H, m, polymer backbone CH of unmodified unit), 2.00−1.35 (2H, m, polymer backbone CH_2).

tert-Butyl 2-Aminoethylcarbamate-Substituted PVA **(5)**. DS 0.047 (69%). ^{1}H NMR (D_2O): 4.90 ((DS × 1)H, m, partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.00−3.55 (1H, m, polymer backbone CH of unmodified unit), 3.08 ((DS × 4)H, m, $-NHCH_2CH_2NHBoc$), 2.00−1.35 (2H, m, polymer backbone CH_2), 1.32 ((DS × 9)H, s, *tert*-butyl).

Methyl N-Methyl-N-(2-propynyl)carbamate **(6)**. Model compound **6** was prepared according to the same procedure as was proposed for PVA modification. Methanol (144 mg, 4.50 mmol), DMSO (4 mL), CDI (365 mg, 2.25 mmol), and *N*-methylpropargylamine (31 mg, 0.45 mmol) were used in the synthesis. After treatment with 2 mL of concentrated aqueous NH_3 and rotary evaporation of the mixture to ∼4 mL volume, it was diluted with water and extracted with diethyl ether. The ether phase was separated, dried over Na_2SO_4, and evaporated to dryness to give an oily product. Yield: 100%. ^{1}H NMR ($CDCl_3$): 4.05, 3.99 (2H, 2 × broadened overlapping s, CH_2), 3.65 (3H, s, OCH_3), 2.90 (3H, s, NCH_3), 2.17 (1H, t, $HC\equiv$, J = 2.4 Hz).

Methyl 2-Azidoethylcarbamate **(7)**. Model compound **6** was prepared according to the same procedure as was proposed for PVA modification. Methanol (144 mg, 4.50 mmol), DMSO (4 mL), CDI (365 mg, 2.25 mmol), and 2-azidoethylamine (39 mg, 0.45 mmol) were used in the synthesis. Workup procedure and isolation were the same as for preparation of **6**. Yield: 100%. ^{1}H NMR ($CDCl_3$): 4.92 (1H, broadened s, NH), 3.62 (3H, s, OCH_3), 3.38 (2H, t, $NHCH_2CH_2N_3$, J = 5.1 Hz), 3.29 (2H, quartet, $NHCH_2CH_2N_3$, J = 5.4 Hz).

Poly(ethylene glycol)-Diazide **(9).** PEG of molecular weight 2000 g/mol (2 g, 1 mmol) was dried by coevaporation with dry pyridine twice and dissolved in the same solvent (8.5 mL). The pyridine solution was cooled to 0 °C, and methanesulfonyl chloride (0.39 mL, 5 mmol) dissolved in dry dichloromethane (4 mL) was added dropwise over 5 min at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. Solvent was removed by rotary evaporation. The residue was worked up with aqueous saturated $NaHCO_3$ and extracted with CH_2Cl_2, and the organic phase was dried over Na_2SO_4. The product was finally precipitated by addition of diethyl ether. The pale solid material was obtained by filtration and dried under vacuo to give 1.976 g (91%) of PEG-dimesylate **8** as judged by ^{1}H NMR ($CDCl_3$): 4.35 (4H, m, 2 × $MsOCH_2$), 3.72 (4H, m, 2 × $MsOCH_2CH_2$), 3.66−3.45 (172H, m, $[CH_2CH_2O]_{43}$), 3.02 (6H, s, 2 × CH_3SO_2O-). A mixture of PEG-dimesylate **8** (1.976 g, 0.91 mmol) and sodium azide (296 mg, 4.55 mmol) in dry DMF (10 mL) was stirred under argon at 105 °C for 4 h and then at room temperature for another 18 h. The solid salts were removed by filtration through Celite, and the filtrate was concentrated by rotary evaporation. The polymer was recovered from DMF concentrated solution by precipitation with diethyl ether and filtration. The precipitate was partitioned between CH_2Cl_2 and water. After stirring for 10 min the organic phase was separated from water, dried over Na_2SO_4, and evaporated to dryness. The residue was redissolved in water and dialyzed against water for 24 h. The dialyzed solution was subsequently freeze-dried to give 1.438 g (77%) of **9**. ^{1}H NMR ($CDCl_3$): 3.50−3.69 (176H, m, $CH_2[CH_2CH_2O]_{43}CH_2$), 3.33 (4H, t, 2 × CH_2N_3, J = 5.2 Hz).

Model 1,3-Cycloaddition Reactions with Low-Molecular-Weight Substrates. Alkyne (0.5 mmol) and azide (0.5 mmol) components were dissolved in water (2 mL) or in a 1:1 mixture of water and ethanol (2 mL). Sodium ascorbate (9.9 mg, 0.05 mmol) was added followed by copper(II) sulfate pentahydrate (2.5 mg, 0.01 mmol). The heterogeneous mixture was stirred vigorously overnight (∼17 h), at which point it cleared. The reaction mixture was diluted with water and extracted with CH_2Cl_2 (3 times). Organic phase was separated, dried over Na_2SO_4, and evaporated to dryness, affording an oily residue which was analyzed by NMR (see Scheme 4 for protons numbering).

Triazole Derivative **(10)**. Propargylamine and 2-azidoethylamine were used in the synthesis to give 70 mg (100%) of compound **10**. ^{1}H NMR ($CDCl_3$): 7.46 (1H, s, triazole), 4.32 (2H, t, β-CH_2, J = 5.9 Hz), 3.93 (2H, s, γ-CH_2), 3.14 (2H, t, α-CH_2, J = 5.9 Hz).

Triazole Derivative **(11)**. Methyl *N*-methyl-*N*-(2-propynyl)-carbamate **6** and methyl 2-azidoethylcarbamate **7** were used in the synthesis to give 47 mg (34%) of compound **11**. ^{1}H NMR ($CDCl_3$): 7.52, 7.38 (1H, 2 × s, triazole), 5.12 (1H, broadened m, NH), 4.46 (2H, s, γ-CH_2), 4.39 (2H, m, β-CH_2), 3.62 (6H, s, 2 × OCH_3), 3.28 (2H, quartet, α-CH_2, J = 5.4 Hz), 2.90 (3H, s, NCH_3).

Model 1,3-Cycloaddition Reactions with Polymers. PVA **2** (DS 0.0194) or **3** (DS 0.045) (0.0176 mmol of pendant groups) was dissolved in water (6 mL), and the complementary reagent (2-azidoethylamine for **2** or propargylamine for **3**, 0.176 mmol) was added to the aqueous solution. Sodium ascorbate (5.2 mg, 1.5 equiv/pendant group) followed by copper(II) sulfate pentahydrate (1.3 mg, 0.3 equiv/pendant group) were added then. The heterogeneous mixture was stirred vigorously overnight (∼17 h), at which

point it cleared. The reaction mixture was diluted with water and dialyzed against water for 24 h. The dialyzed solution was subsequently freeze-dried, and the residue was analyzed by NMR (see Scheme 4 for protons numbering).

Triazole-Substituted PVA **(12)**. DS 0.042 (82%). ^{1}H NMR (D_2O): 8.00−7.80 ((DS × 1)H, broadened signal, triazole), 4.90 ((DS × 1)H, m, partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.56−4.29 ((DS × 2)H, m, β-CH_2), 4.15−3.35 (1H, m, polymer backbone CH of unmodified unit + γ-CH_2 + α-CH_2), 2.00−1.35 (2H, m, polymer backbone CH_2).

Triazole-Substituted PVA **(13)**. DS 0.017 (41%). ^{1}H NMR (D_2O): 7.88 ((DS × 1)H, s, triazole), 4.90 ((DS × 1)H, m, partially overlapped with H_2O signal, polymer backbone CH of modified unit), 4.58−4.37 ((DS × 4)H, m, β-CH_2 + γ-CH_2), 4.00−3.55 ([1 + (DS × 4)]H, m, polymer backbone CH of unmodified unit), 3.30−3.19 ((DS × 2)H, m, α-CH_2), 2.85 ((DS × 3)H, s, NCH_3), 2.00−1.35 (2H, m, polymer backbone CH_2).

Hydrogel Formation. Hydrogels were formed either between multifunctional alkyne-PVA (**2**) and azide-PVA (**3**) or by cross-linking of **2** with bifunctional PEG derivatives **9**. For each experiment desired amounts of both alkyne and azide components were dissolved in 350 μL of DMSO (or water). Freshly prepared aqueous solution of sodium ascorbate (50 μL, 0.3 M) was added, followed by aqueous solution of copper(II) sulfate pentahydrate (100 μL, 30 mM) so that the final concentrations of sodium ascorbate and copper(II) sulfate in the mixture were 30 and 6 mM, respectively. In the case of catalyst concentration dependence studies, the concentrations of sodium ascorbate and copper(II) sulfate pentahydrate were varied as specified in the subsequent section of the Results and Discussion. The ratio of azide and alkyne groups was 1:1 for all experiments with the exception of those where the influence of stoichiometric ratio was studied (see Results and Discussion for details). The hydrogels began to form immediately or within 1 h after addition of copper(II) sulfate, depending on the preparation conditions used (total concentration of polymer components, catalyst concentration, structure of polymer components, and solvent). The mixtures were agitated for further 24 h to obtain a solid, uniform hydrogels.

Determination of the Gel Sol Fraction and the Equilibrium Swelling in Water. The prepared gel after cross-linking reaction was swollen for 48 h in deionized water during which the nonincorporated network fraction (= sol fraction) and the catalyst were 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 cross-linking 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.

Rheological Characterization of Hydrogels. The mechanical properties of the swollen gels were measured on the AR2000 rheometer (TA Instruments Inc., 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 ca. 150 mN and 25 °C. Data are reported at a frequency of 0.5 Hz.

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