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Mechanistic and Catalytic Studies of β-Nitroalcohol Crosslinking with Polyamine

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ABSTRACT: β-Nitroalcohols (βNAs) are promising corneoscleral crosslinking agents for the treatment of diseases such as keratoconus and myopia. Although it is believed that formaldehyde is released from the crosslinking reactions of βNAs, the mechanism by which βNAs react with amine-functionalized polymers has yet to be known. In this study, we present the reaction mechanism of the βNA crosslinking. Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) data provide strong evidence that formaldehyde is released during the reaction. Catalytic studies show that sodium bicarbonate ($NaHCO_3$) and salmon testes DNA accelerate the reaction while hydroxynitrile lyase from Arabidopsis thaliana decelerates the crosslinking reaction. These results suggest that βNAs are potential self-administered crosslinking agents for future clinical use. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 128: 3696–3701, 2013

KEYWORDS: biomedical applications; catalysts; crosslinking; gels; swelling

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INTRODUCTION

The cornea tissue of the eye suffers, due to a number of contributing factors over time, structural degeneration of the clear tissue covering the front of the eye.[1,2] It is believed that a defect in collagen causes a loss of strength and mechanical properties in the cornea that contributes to this degeneration.[1,2] *In vivo* corneal collagen crosslinking provides a potential treatment to recover this loss of strength and mechanical properties, which is available in the chemical (or photochemical) crosslinking of the proteins in the cornea.[3,4]

We recently reported the β-nitroalcohols (βNAs) to be promising chemical crosslinking agents for therapeutic corneoscleral crosslinking.[5–8] βNAs crosslink collagenous tissue under physiological pH and temperature. In addition, nitroalcohols are generally low toxicity and therefore amenable to clinical use.[9–12] It has been further hypothesized that the mechanism of crosslinking by βNAs involves the base-catalyzed release of formaldehyde (Scheme 1), which is the active crosslinking agent (Scheme 2). In this article, we provide evidence for the mechanism hypothesized in Schemes 1 and 2.[8,13–15] The βNAs investigated in this work are shown in Chart 1. These structures of the βNAs provide the basis for structure crosslinking ability. In addition, the polymer poly(allylamine) (PAA) was used as a model for the collagenous tissue.

Hydrogels are often used as scaffolds for tissue engineering.[16] Gel formation on reaction of βNAs with PAA was used as a mechanistic signature of the extent of crossing and establishment of increase in mechanical strength. In addition, transient and product species were examined by ^{1}H-nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR). The effect of selected bases on the rate and efficiency of gel formation was studied. Some conclusions concerning the mechanism of crosslinking were presented.

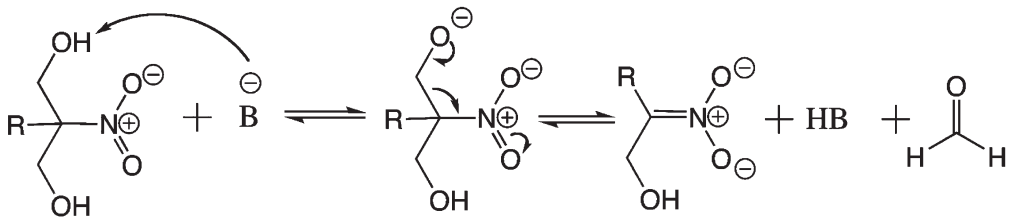
EXPERIMENTAL

Materials

All these chemicals: PAA (Aldrich, average Mw ~ 15,000), sodium phosphate monobasic (NaH_2PO_4, Aldrich, 99%), sodium phosphate dibasic (Na_2HPO_4, Aldrich, 99%), 2-nitroethanol (Aldrich, 97%), 2-methyl-2-nitro-1,3-propanediol (Alfa, 97%), tris (hydroxymethyl) nitromethane (Aldrich, 98%), 2-bromo-2-nitro-1,3-propanediol (Aldrich, 98%), deoxyribonucleic acid sodium salt, from salmon testes (Aldrich), hydroxynitrile lyase from *Arabidopsis thaliana* (AtHNL; Aldrich), and paraformaldehyde (PFA; Aldrich, 95%) were used as received. A buffer solution of pH 7.4 was prepared by mixing 81% of 0.2*M* Na_2HPO_4 and 19% of 0.2*M* NaH_2PO_4 aqueous solution.

Hydrogel Formation and Measurements of the Degree of Swelling

The chemical structures of the nitroalcohols and PAA studied in this work are shown in Chart 1. As discussed above, gel formation of a polymer can be used as a measure of the extent of



Scheme 1. A proposed mechanism of formaldehyde released from β-nitroalcohol (βNA). βNA undergoes deprotonation on hydroxyl group and subsequent decomposition to give formaldehyde by a base-catalyzed reverse Henry reaction.

crosslinking of the polymer. The rate of formation of the gel is qualitatively measured by time taken for a liquid sample undergoing gel formation and maintaining its shape against gravity, i.e., the sample in a vial containing the original reaction liquid initially can maintain its shape for hours after inversion of the vial as pictured in Figure 1 for gel before washed and dried.[17]

In a typical experiment, PAA (110 mg) and βNA (Table I) were dissolved in 0.3 mL of pH 7.4 phosphate buffer solution, and the mixture was heated to 37°C. Gel precipitates were formed after several hours depending on the conditions. The reaction was maintained until a firm gel formed. Unreacted nitroalcohol and PAA were removed by washing with H_2O/ethanol. Four kinds of ratios of 100, 50, 30, and 0% H_2O in ethanol were used for the washings. The washed gel was then dried under vacuum overnight at room temperature and then characterized by FTIR, physical measurements of the weight of water absorption, and the degree of swelling.

Swelling experiments were conducted in H_2O as shown in Figure 1. The dried gel (~40 mg) was immersed into 5 mL water at room temperature for 1 h. The weight of swollen samples was measured after the excess surface water was removed by filter paper. The degree of swelling was calculated using the swell ratio W/W_0, where W and W_0 are the weight of the swollen and dried gel, respectively.[8,18]

Instruments for Characterization of Gel Formation

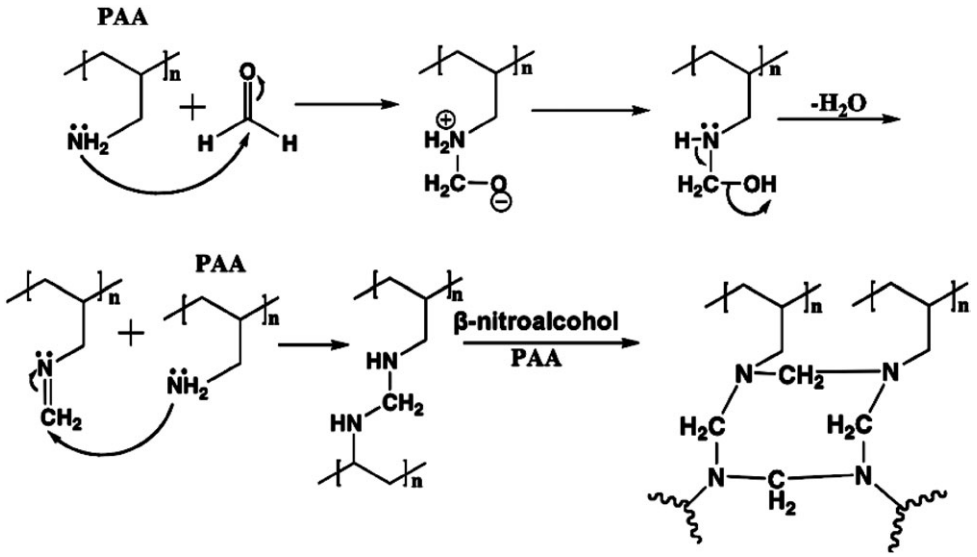
FTIR spectra were collected on a Nicolet Nexus 870 FTIR with a Thermo Multibounce HATR accessory with a ZnSe crystal. The pH of phosphate buffer solution was measured on a WTW Measurement System, Chekmite pH-20 sensor. ^{1}H-NMR spectra were obtained by Bruker 400-MHz NMR spectrometer.

RESULTS AND DISCUSSION

FTIR Spectra Analysis of Gel Formation

Figures 2 and 3 show FTIR spectra of the hydrogels from crosslinking reactions. The FTIR spectra of the hydrogels were taken immediately after reaction (dashed lines), and the hydrogels were washed with water/ethanol and dried, then the FTIR spectra were taken again (solid lines). As a control experiment, the PAA reacted with PFA, which releases formaldehyde in solution. The IR peak at 1632 cm^{-1} of the resulting hydrogel before washing in Figure 2 (dashed line) was assigned to the carbonyl group (—C=O) of formaldehyde, which was consistent with the IR spectrum of PFA in pH 7.4 phosphate buffer solution. The peak disappeared after washed with water/ethanol and dried (Figure 2: solid line).

The crosslinking experiments of PAA with nitrotriol [2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD), Chart 1] were carried out under the same conditions. The IR peaks at 1540 and 1070



Scheme 2. The proposed mechanism of crosslinking in PAA with β-nitroalcohol. An amino group of a PAA side chain with the lone pair attacks the electrophilic carbonyl carbon of formaldehyde from βNA to form a Schiff base, which can then go on and react with another PAA and formaldehyde to complete the crosslink.

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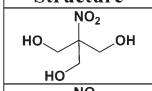
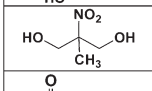
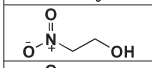
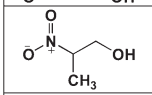
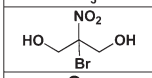
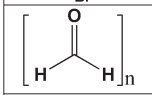
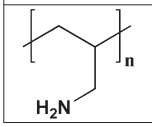
Structure	Chemical Name	Abbreviation
	2-hydroxymethyl-2-nitro-1,3-propanediol	HNPD
	2-methyl-2-nitro-1,3-propanediol	MNPD
	2-nitroethanol	2NE
	2-nitro-1-propanol	2NPROP
	2-Bromo-2-nitro-1,3-propanediol	BRONOPOL
	paraformaldehyde	PFA
	Poly(allylamine)	PAA

Chart 1. The chemical structures of the β-nitroalcohols and polyamine studied in this work.

cm^{-1} in Figure 3 (dashed line, before washing) were assigned to $-NO_2$ symmetric stretching and $-C-NO_2$ bending, respectively. There is also a peak at 1632 cm^{-1}, which was assigned to a $-C=O$ stretch. These peaks disappeared after washed and dried the hydrogel (solid line). The FTIR spectra in dash line (before washed and dried) has the same characteristic peak corresponding to the $-C=O$ group assigned to formaldehyde. This indicates that a carbonyl group was produced in both the crosslinking reactions. These results are consistent with the release of formaldehyde during the PAA crosslinking reaction using both PFA and nitrotriol as crosslinking reagents. The similar IR spectra of dried hydrogel (solid line) for both reactions indicate that a similar crosslinking product was formed. Furthermore the absence of IR peaks for the nitro group after washed and dried demonstrates that the crosslinking occurred between PAA and formaldehyde released from PFA and HNPD, and that the nitroalkyl side products were not a portion of the polymer chain and the gel.

^{1}H-NMR Analysis of Formaldehyde Released from β-Nitroalcohols

As shown in Schemes 1 and 2, formaldehyde has been hypothesized as a reactive intermediate in the crossing of PAA. This is partially evidenced by IR spectra of the hydrogels taken immediately after the crosslinking reactions ($-C=O$ group at 1632 cm^{-1}). To further confirm the formation of formaldehyde during the PAA crosslinking, ^{1}H-NMR spectra were taken to provide a direct measurement of formaldehyde, which exhibits a characteristic chemical shift around 8.48 ppm. Figure 4 shows the ^{1}H-NMR spectrum of nitrotriol after 20 h at 37°C and pH 12.7 (top). The chemical shift at 8.48 ppm is clearly seen in Figure 4. Furthermore, when adding PFA to the sample and heating to 37°C for 10 min, the NMR spectrum showed that the relative intensity of the peak increased at around 8.48 ppm and only one peak appeared in the region. These results indicate the generation of formaldehyde from nitrotriol. Figure 5 provides a comparison of ^{1}H-NMR spectra of formaldehyde produced by the base-catalyzed decomposition of nitroethanol. We note that the concentration of formaldehyde released from nitrotriol (higher order βNA) is much higher than that from nitroethanol mono-beta-nitroalcohol (mono-βNA). This can be explained by the fact that the nitrotriol possesses three potential equivalents of formaldehyde when compared with only one equivalent for nitroethanol (2NE). We also found that the amount of formaldehyde did not change with increasing time by comparing the integration of chemical shift of formaldehyde and βNA. This observation suggests that formaldehyde release occurs through a base-catalyzed and thermally driven reverse Henry Reaction until the reaction reaches an equilibrium.[13] As outlined in Scheme 1, the reaction of formaldehyde forming from βNAs starting material is reversible.[6,8,12]

Effect of Different Nitroalcohol Structures on the Gel Formation in the Crosslinking Reaction of βNAs with PAA

The degree of gel formation in the reaction of βNAs with PAA was examined for different structures of nitroalcohols as summarized in Table I. The reaction conditions were optimized by using the mass ratio of crosslinker (nitrodiol) to polymer (PAA) of 1.[8] For example, according to the equivalent of hydroxyl groups of βNAs compared with nitrodiol, 60 mg of nitrotriol

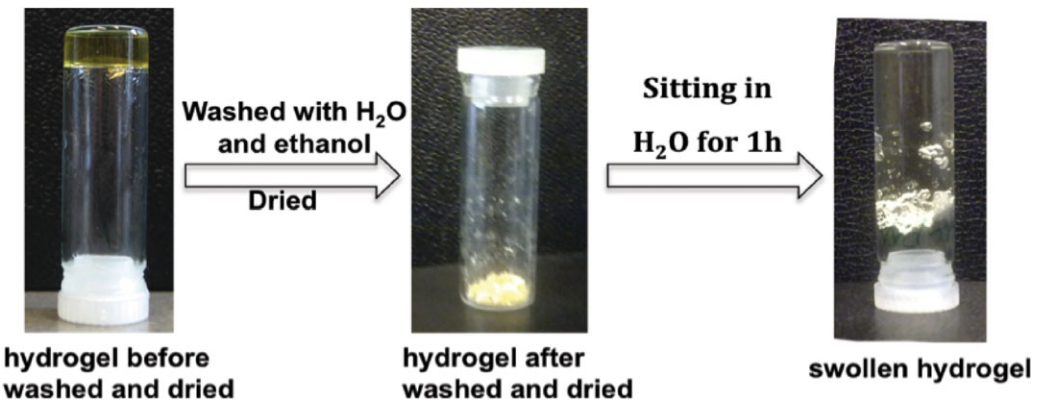


Figure 1. Three kinds of gels during the swelling process are pictured including hydrogel before (left) and after (middle) washed and dried, and swollen gel (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. The Gelation Time and the Degree of Swelling of Hydrogels Prepared with Nitroalcohols and Poly(allylamine)

Reactants (with PAA)	Concentration (M)	Gelation time (h)	Degree of swelling
paraformaldehyde	2.0	24	25 ± 4
nitro-triol	1.3	29	28 ± 5
nitro-diol	2.7	66	22 ± 4
nitro-ethanol	7.0	48	21 ± 3
nitro-propanol	7.0	2 weeks (partial gel)	20 ± 3
Bronopol	2.0	42	15 ± 2

The amount of PAA was kept to 24 m*M* for all experiments. The experimental conditions were 37°C and pH 7.4. The error of the measured time was 20 min.

and 220 mg of mono-nitroalcohol were used with 110 mg PAA separately.

From Table I, it is seen that the rate of gelation decreased with the number of hydroxyl group in the nitroalcohols following the order of triol, diol, and monol. In addition, the substituent of nitroalcohols also influenced the rate of gel formation. It was found that the substituent with electron-donating group decreased the rate. For example, it took 2 weeks for the methyl-substituted nitropropanol to form gels, when compared with the gelation time of 48 h for the unsubstituted nitroethanol. The nitrodiol is expected to have a faster time of gel formation than monoalcohol nitroethanol does. However, the methyl-substituted nitrodiol significantly slowed down the gel formation with a time of 66 h in comparison with the gel formation time of 48 h for nitroethanol. It is likely that methyl group has an electron-donating feature, which made the intermediate unstable (Scheme 1) and decreased the reaction rate. On the other hand, the substituent with electron-withdrawing feature increased the rate, and the equilibria were more favorably achieved toward

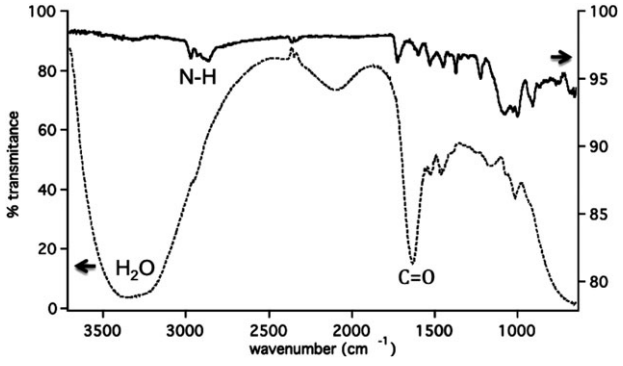


Figure 2. Attenuated total reflectance (ATR–FTIR) of poly(allylamine) crosslinked with paraformaldehyde at pH 7.4 and 37°C before (----) and after (—) washed with ethanol and water, and dried.

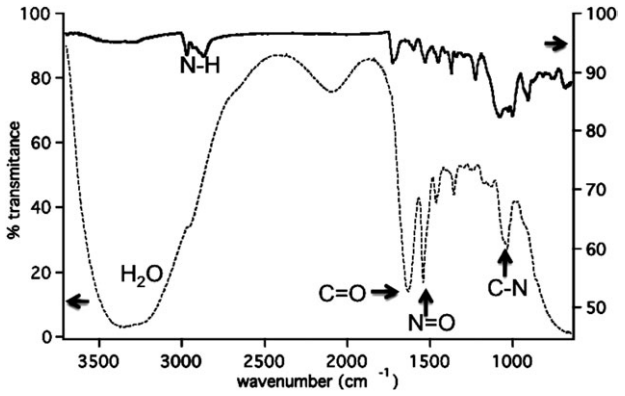


Figure 3. ATR–FTIR of poly(allylamine) crosslinked with nitrotriol at pH 7.4 and 37°C before (----) and after (—) washed with ethanol and water, and dried.

the product of gel due to the stabilized intermediate as shown in Scheme 1. It is seen in Table I that bronopol with —Br group formed gels with a time of 24 h, which is faster than that for the methyl-substituted nitrodiol of 66 h. PFA produces formaldehyde on heating. To compare crosslinking of the nitroalcohols, the gel formation for formaldehyde using PFA with PAA was studied. The gel formation time for PFA was only 24 h, which is faster than that for the nitrotriol. This suggests that formaldehyde is the direct crosslinking agent that is released from *β*NAs.

The degree of swelling is used as a measure of the degree of crosslinking.[17,19,20] The degree of swelling at equilibrium depends on several factors such as temperature, length of the network chains, number of the crosslinks, size of the solvent molecules, and the strength of thermodynamic interaction between the polymer chains and solvent molecules, etc.[19] All synthetic and biological crosslinking (or networks) can swell when exposed to low-molecular-weight solvents. The hydrogel swelling experiments were carried out in deionized H_2O at room temperature. As shown in Figure 1 and Table I, there is no big difference in the degree of swelling for the hydrogels produced by *β*NAs and PAA. This further supports that these crosslinking reactions were generated from the reaction of formaldehyde and PAA as evidenced by the aforementioned IR results. As shown in Scheme 2, the crosslinking of *β*NAs with PAA can be essentially understood as the reaction of formaldehyde with PAA. The electron-withdrawing substituent Br of bronopol may cause the smaller difference in the degree of swelling for bronopol crosslinking. Under the same experimental conditions, the structure of bronopol with withdrawing electrons may favorably make more crosslinks when compared with other *β*NAs. For polymers with the same structure and solvent, a larger number of crosslinks cause a smaller variation of chain length between the network junctions. Therefore, a smaller extent of these chains yields a smaller degree of swelling as volume increases.

Effect of Catalysts on Gel Formation

Henry reaction, consisting of condensation of aliphatic nitro compounds with aldehydes or ketones, is a widely used method for the synthesis of aliphatic nitroalcohols.[13] The reaction is

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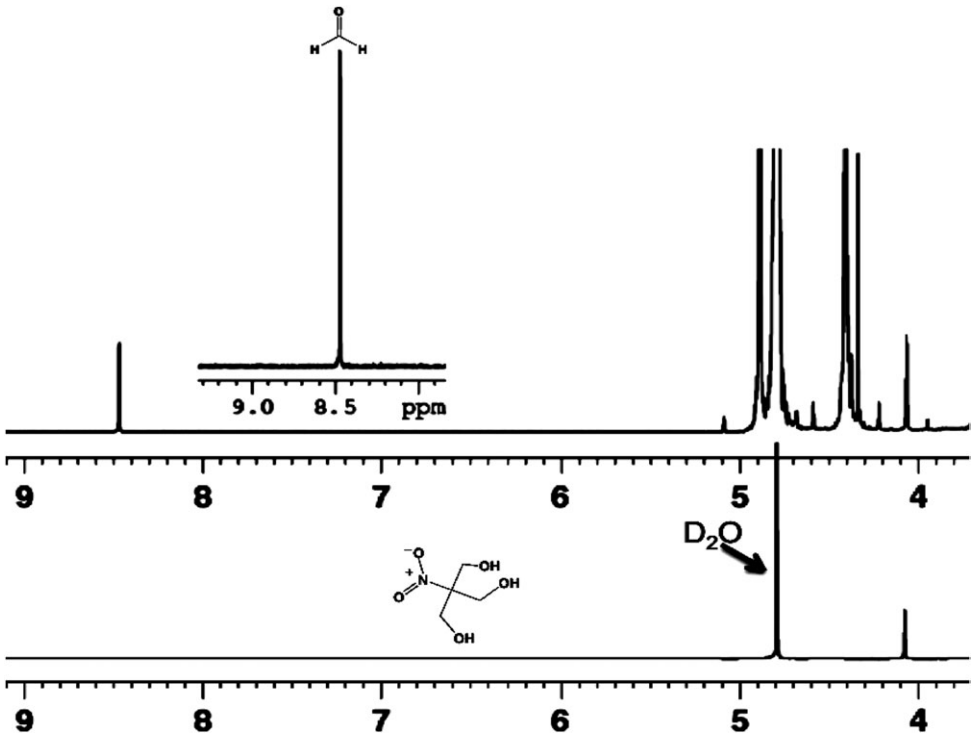

Figure 4. ^{1}H-NMR spectrum of nitrotriol taken after 20 h at 37°C and pH 12.7 is shown at the top. The original ^{1}H-NMR spectrum of nitrotriol is shown at the bottom. D_2O was the solvent for NMR measurement.

base catalyzed and reversible. On the other hand, the reverse Henry reaction of *β*NAs releases formaldehyde that can act as a crosslinking agent for PAA gel formation.[5,8] Although strong bases such as NaOH or KOH are good catalysts for the reaction,[13] mild catalysts are needed for clinical use. Here, we chose $NaHCO_3$, DNA, and hydroxynitrile lyase as catalysts for the crosslinking reaction to investigate effect of the catalysts on the gel formation of PAA with *β*NAs.

The PAA crosslinking was performed in phosphate buffer solution (pH 7.4) at physiological temperature of 37°C. As shown in Table II, with increasing the amount of $NaHCO_3$ aqueous solution (0.5*M*, pH 9.0), the gel formation times for PAA crosslinking with *β*NAs significantly decreased when compared with the reaction time without any catalysts. It is noted that the pH values of the phosphate buffer solution also increased with the addition of sodium bicarbonate ($NaHCO_3$) aqueous solution. Thus, it is suggested that the increases in pH value made the gel formation faster. This result is consistent with that we reported previously.[8]

DNA from natural sources has been used as catalysts to facilitate the Henry reaction under mild reaction conditions.[21] We chose salmon testes DNA as the catalyst for *β*NAs crosslinking, and the corresponding gelation times are shown in Table II. The rates of gel formation for three nitroalcohols increased when compared with that without any catalysts. Specifically, the gelation time for bronopol decreased significantly. These results indicate that salmon testes DNA is capable of catalyzing the crosslinking reaction of *β*NAs and PAA. However, the catalytic ability of salmon testes DNA is less effective than that of $NaHCO_3$.

Enzymes as practical catalysts have been increasingly exploited for organic synthesis and biosynthesis due to their mild reaction conditions.[22] Hydroxynitrile lyase containing an *α*/*β*-hydrolase-fold from the noncyanogenic plant *A. thaliana* (AtHNL) can catalyze the reaction between nitromethane and aromatic aldehydes to yield active *β*-nitroalcohols (Henry reaction, nitroaldol reaction).[22–24] It is expected that the reversibility of Henry reaction (retro Henry) can work in nitroalcohol crosslinking. As shown in Table II, 0.1 mL of hydroxynitrile lyase (AtHNL) was added into the 0.3 mL pH 7.4 buffer reaction mixtures of the nitroalcohol and PAA, respectively. It is interesting to notice

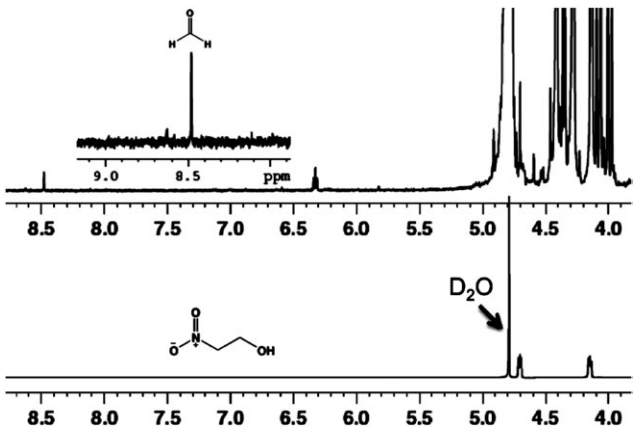

Figure 5. ^{1}H-NMR spectrum of nitroethanol taken after 20 h at 37°C and pH 12.7 is shown at the top. The original ^{1}H-NMR spectrum of nitroethanol is shown at the bottom.

Table II. Effect of $NaHCO_3$, Salmon Testes DNA, and Hydroxynitrile Lyase on Gel Formation of Nitroalcohols Crosslinking Reaction

Nitroalcohols	Reaction time without catalyst (h)	Reaction time with 0.5*M* $NaHCO_3$		Reaction time with salmon testes DNA		Reaction time with hydroxynitrile lyase	
 nitro-triol	29	25 μmol	14 h	3.4 mg	26 h	0.1 mL	40 h
		12.5 μmol	17.5 h				
 nitro-diol	66	25 μmol	29 h	3.1 mg	46 h	0.1 mL	96 h
		12.5 μmol	36 h				
 bronopol	42	25 μmol	6 h	3.6 mg	18 h	0.1 mL	72 h
		12.5 μmol	7 h				

For experiments without catalyst, 0.3 mL of pH 7.4 phosphate buffer solution was used.
The pH of the reaction mixture was 8.2 when 25 μmol of 0.5*M* $NaHCO_3$ was added, and the pH was 8.0 when 12.5 μmol of 0.5*M* $NaHCO_3$ was added.
The error of the measured time was 20 min.

that the reaction rates decreased in the presence of hydroxynitrile lyase. This indicates that hydroxynitrile lyase brings the reaction toward to the formation of nitroalcohols instead of catalyzing formaldehyde formation. It is likely that α/β-hydrolase plays a dominant role in the catalytic effect.

CONCLUSIONS

We have presented the mechanism of βNAs crosslinking with PAA, and catalytic effects of $NaHCO_3$, salmon testes DNA, and hydroxynitrile lyase on the crosslinking reaction. Formaldehyde was directly produced from βNAs by retro Henry reactions. βNAs as a formaldehyde donor crosslinked with the amine-functionalized polymer PAA to form hydrogel under physiological conditions. These findings indicate that the βNAs are very promising for *in vivo* induction of crosslinking in the clinical cornea. The working mechanisms for the crosslinking process and gel formation of βNAs have been proposed. We have also studied the alkali catalytic processes of βNAs crosslinking. It was found that $NaHCO_3$ is a mild and effective catalyst for topical pharmacologic corneoscleral or other collagenous tissue crosslinking.

ACKNOWLEDGMENTS

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