

Zein–Poly(*N*-isopropylacrylamide) Conjugates

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Stable cross-linked gels were synthesized by polymerization of *N*-isopropylacrylamide (NIPA) blended with a seed protein (zein) and 4,4'-diazido-2,2'-stilbenedisulfonic acid (DAS) in dimethyl sulfoxide following irradiation. Grafting of DAS–zein chains on polyNIPA backbone was proposed. The gels were shown to undergo discontinuous volume transition at around 32 °C corresponding to lower critical solution temperature of NIPA. The gel swelling was pH-sensitive. Sharp maximum on viscosity vs temperature plots was observed in suspensions of the gel particles at 32 °C due to interparticle network formation caused by hydrophobic interactions between polyNIPA domains.

Introduction

Gels' ability to drastically change their volume in response to external stimuli^{1–6} has resulted in a novel class of intelligent materials for advanced applications, including biomedical systems.^{7–9} Among other polymers, poly(*N*-isopropylacrylamide) (polyNIPA) has championed the field of temperature-sensitive materials because of its well-defined lower critical solution temperature (LCST) in water.^{10–12} Cross-linking of polyNIPA results in gels capable of drastic swelling and collapse in aqueous solvents. Copolymers of NIPA with ionizable monomers can possess sensitivity to pH, metal ions, and temperature.^{6,13,14} If a protein is covalently conjugated onto a responsive polymer, a hybrid intelligent copolymer may be possible that is responsive to the changes in its environment and yet biologically active.¹⁵ In this study, we have taken interest in conjugating zein and polyNIPA with the goal of achieving responsive, potentially biodegradable gels that can be readily processed into fibers. Zein is a group of proline-rich proteins extractable from plant seeds;^{16–18} it represents an important exception from the conventional notion of insolubility of unmodified proteins in organic solvents.^{19–21} Zein is soluble in ethanol, 2-propanol, acetic acid, phenol, and dimethyl sulfoxide (DMSO).^{17,18} We recently found that polymerization of a solution of zein in *N,N*-dimethylacrylamide results in blends or semi-interpenetrating networks yielding thermoplastic hydrogels.²¹ There, water-insoluble zein molecules are finely dispersed within the network, providing stability without phase separation. However, increase of zein solubility upon increasing pH leads to its diffusion from the gels. Therefore, in order for the hybrid gel to be stable within a wide range of pH zein should be covalently attached to the polymer. In this study, we addressed this problem by introducing a photopolymerizable cross-linking agent, 4,4'-diazido-2,2'-stilbenedisulfonic acid,^{22–24} into the solution of NIPA and zein in an organic solvent (DMSO). Intimate mixing of NIPA, DAS, and zein in a common solvent provided better stability due to entanglements between zein and polyNIPA, when the latter was formed. Irradiation of the solution led to formation of stable gels, as outlined below.

Experimental Section

Materials. *N*-isopropylacrylamide (NIPA, Kodak) was repeatedly recrystallized (mp 64–66 °C) from a 65/35 mixture

of hexane and benzene.¹¹ Zein from maize seed (Aldrich) was obtained as a purified powder (formula weight 38 000, >93% protein by biuret). The yellow powder was dissolved in ethanol and washed repeatedly with petroleum ether and acetone until all color was removed. The resulting white material was dissolved in ethanol, precipitated at 4 °C by deionized water, washed by water, and freeze-dried. The amino acid content of the resulting protein has been reported previously.²¹ 4,4'-Diazido-2,2'-stilbenedisulfonic acid, disodium salt tetrahydrate (DAS, Aldrich) (97%), was used without further purification. All other chemicals, organic solvents, and gases were obtained from commercial sources and were of the highest purity available.

Syntheses. A mixture of specified amounts of NIPA, zein, and DAS was dissolved in 20 mL DMSO by stirring overnight at 4 °C in the dark. The resulting transparent, yellowish solution was purged from air by nitrogen flow for 8 h at 20 °C and mixed under nitrogen atmosphere with 150 μ L of freshly prepared solution of ammonium persulfate (APS) in DMSO (300 mg/mL) and 50 μ L TEMED and then kept at 20 °C for 24 h to form a flowable viscous gel. The gel was then illuminated by a Light-Welder 3010 EC UV spot/wand lamp (spectral output of 300–500 nm and intensity of 6000 mW/cm², Dymax Co., Torrington, CT) for 2 h under constant flow of nitrogen and periodic shaking. The resulting dark red gel was kept at 70 °C for another 24 h to complete polymerization. In a separate series of experiments, the same procedure was applied to the mixtures of either NIPA and DAS or zein and DAS.

For fiber synthesis, the zein-containing gel was extruded under a pressure of 80 psi through a stainless steel cylinder with a single hole at the bottom (100 μ m internal diameter) onto a stainless steel winding cylinder (diameter 5 cm) rotating in a water bath at 20 °C. Upon contacting water, the gel hardened, forming thin fibers and changing its color from dark red to yellow. Fibers were extensively washed with deionized water, cut into small pieces, and finally dialyzed against constantly changing deionized water for 3 days to remove DMSO and other residues. FTIR (KBr): 3900, 3330, 1710, 1650, 1450, 1390, 1170, 750, and 680 cm⁻¹. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 4.0 (s, HC–N– of isopropyl group of NIPA), 7.2 (m, stilbene), and 10.2 ppm (m, –NH–CO of zein).

Suspensions were made by washing the zein-containing gel with deionized water and by shredding the hardened gel particles, which were dialyzed against deionized water and homogenized to result in a suspension stable at 20 °C for at least 2 days. Effective mean size of the suspended particles

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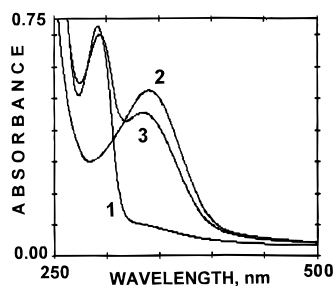


Figure 1. Absorption spectra of aqueous solutions resulting from 0.8 g/mL suspensions of purified zein (1), polyNIPA–DAS gel (2), and polyNIPA–DAS–zein gel (3) in 2 M NaOH. Suspensions were shaken at 20 °C for 10 days and filtered off.

was measured at 20 °C to be $160 \pm 60 \mu\text{m}$. Suspensions were dried under vacuum at 40 °C for measurement of the solids content.

Procedures. Volume transitions of cylindrical fiber gels were monitored in a transparent, temperature-controlled cell under a microscope using a microscaler to fit to the boundaries of the gel on a video monitor. Kinetics of volume transitions was monitored, and only equilibrium data are further discussed. Volume transitions were characterized by d/d_0 , where d and d_0 are the diameters of the gel at given and reference temperature and pH, respectively.

Properties of suspended gels were assessed using a PC-equipped Brookfield RVDV II+ Digital Viscometer with a 2 mL thermostated adapter (chamber dimensions, 12.70 mm diameter, 28.19 mm depth) and a SC4-14/6R spindle (8.74 mm diameter, 8.64 mm side length) adopting coaxial-cylinder geometry. The suspension was loaded into an adapter cup and thermostated at the desired temperature while the viscosity was measured at different shear rates. Shear rate (γ , s^{-1}) was found on the surface of the spindle.

Fluorescence spectra were measured using a Shimadzu RF-5301PC spectrofluorophotometer. Chromatography was run at 20 °C on a Shimadzu LC-10A Series HPLC system, which included an RF-551 fluorescence HPLC monitor, LC-10AD solvent delivery unit, and an SCL-10A system controller. Solutions of zein, polyNIPA, and DAS in DMSO were loaded onto a C_{18} column and then eluted with acetonitrile using a pump speed of 0.2 mL/min. Detection at 440 nm was used throughout. Intensity of the fluorescent fraction was measured and normalized to that of the sample irradiated for 2 h.

NMR spectra were recorded on a Bruker AMX400 spectrometer with tetramethylsilane as an internal frequency lock. Infrared spectra of dry polymeric samples dispersed in KBr were recorded in a water-free atmosphere on a Perkin-Elmer Model 1600 spectrometer. Electronic absorbance spectra were measured on a Shimadzu UV-1601 spectrophotometer.

Results and Discussion

Gel Synthesis. Typical gels formed by polymerization of 5 g NIPA in the presence of 5 g zein and 0.2 g DAS in 20 mL DMSO following irradiation and the gel resuspension in water and dialysis (for the procedure, see Experimental Section) were tested for their stability. Thus, periodically weighed gels of well-defined cubic shape containing zein, polyNIPA, and DAS were kept at 20 °C in excess deaerated deionized water with the pH adjusted by 1 M NaOH or HCl to a desired value. The weights of the gels at pH range 1–11 remained constant for at least 60 days after 5–15 days necessary to reach equilibrium swelling. No deterioration of the gels' shape was observed. Under extreme conditions (10 days in 2 M NaOH), a yellow component leached from the gels (Figure 1). The dissolved

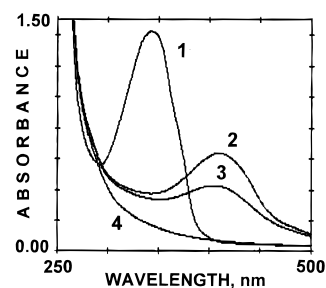


Figure 2. Effect of irradiation on electronic absorption spectra of a solution of zein, polyNIPA, and DAS (concentrations of 0.1, 0.1, and 0.02 mg/mL, respectively) in DMSO. The solution was purged from air by nitrogen and then kept in a sealed quartz cell throughout the experiment. $T = 20$ °C. Irradiation times are 0 (1), 1 min (2), 15 min (3), and 2 h (4).

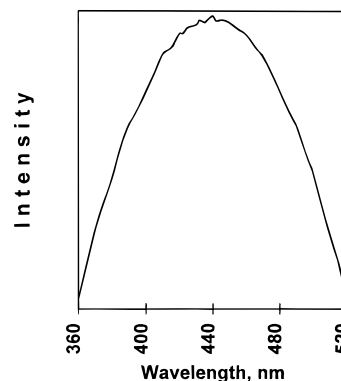


Figure 3. Fluorescence of a solution of zein, polyNIPA, and DAS (concentrations of 0.1, 0.1, and 0.02 mg/mL, respectively) in DMSO irradiated for 2 h in oxygen-free atmosphere.

component (comprising only 9 wt % of the dry gel) contained both protein and DAS (peaks at 292 and 334 nm, respectively). The gel recovered from NaOH solution was resuspended in a fresh portion of 2 M NaOH and was kept there for another 10 days. No detectable amount of leachate was observed. In contrast, a gel made of zein and polyNIPA without DAS was completely soluble in 2 M NaOH. Hence, the gels made of polyNIPA–DAS–zein appeared to be sufficiently stable for further study.

To elucidate the mechanism of the observed formation of stable gels, a model experiment with a dilute solution of polyNIPA, DAS, and zein in DMSO was conducted. PolyNIPA was preformed in order to eliminate interference of TEMED and APS. Irradiation was observed to eliminate the peak at 340 nm characteristic of the azido groups in DAS,²⁵ giving rise to a peak at 405 nm assignable to the upper energy excited state of nitrene^{26,27} (Figure 2). As nitrene radicals were consumed, the latter peak disappeared after about 15 min of irradiation. At this point fluorescence of the solution with the maximum at 440 nm was detected (Figure 3) and was observed to intensify further as the irradiation went on (Figure 4). The fluorescence spectra (Figure 3) were essentially identical with the ones of 4,4'-diamine-2,2'-stilbenedisulfonic acid,²⁵ thus indicating accumulation of arylamino products in the solution upon irradiation.

Massive formation of singlet nitrenes in the absence of oxygen manifested by the absorbance peak at 405 nm (Figure 2) should cause insertion of DAS residues into C–H bonds of both polyNIPA and zein, thereby producing useful cross-linking. Electrophilic attack of singlet nitrenes on (mostly tertiary) C–H groups is known to be a prevalent and fairly selective reaction.²⁷ Minor reactions of singlet nitrenes may comprise nitrene recombination (which is rarely observed in the continuous

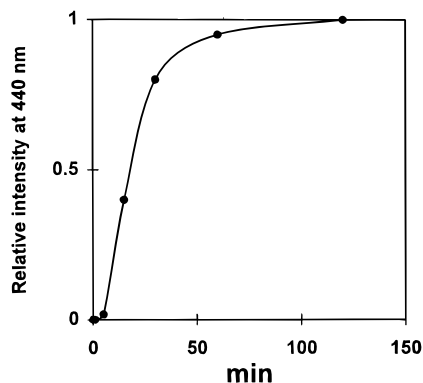
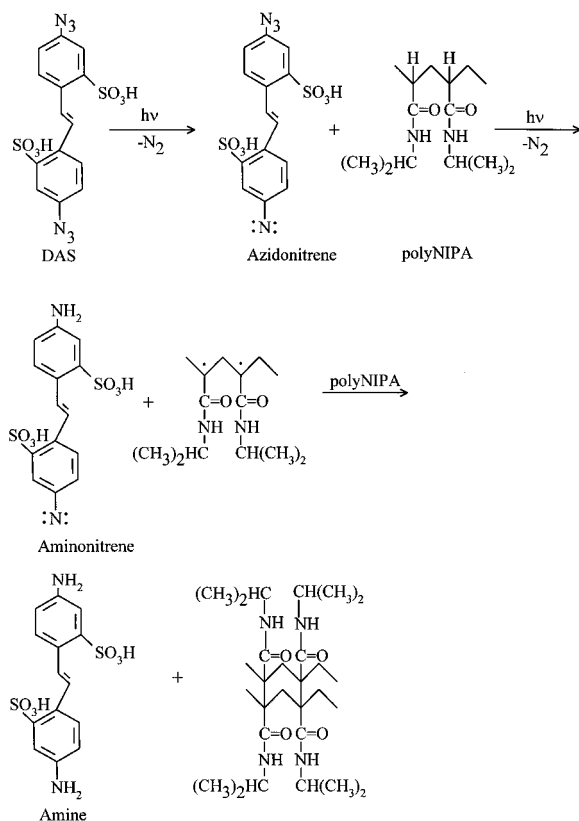


Figure 4. Relative intensity of fluorescence at 440 nm vs time of irradiation for a solution of zein, polyNIPA, and DAS in DMSO. For concentrations, see legend to Figure 2. Intensity at 440 nm was normalized to that after 2 h of irradiation.

SCHEME 1



photolysis), insertion into N–H bonds of amino groups of zein, and insertion into O–H groups (more common for carbonyl nitrenes). Also, reaction of singlet nitrenes with residual oxygen may produce triplet nitrenes that can abstract hydrogen from C–H groups of primary amines.²⁷ These, however, should be removable from the resulting gels by water (see below). In Schemes 1 and 2, consistent with the observed spectra (Figures 2 and 3), we propose prevalent mechanisms of cross-linking along with formation of arylamine side-products (Scheme 1). For brevity, the polymer that undergoes cross-linking was drawn in Scheme 1 to be polyNIPA, but it could be also zein and zein–polyNIPA.

Gel Performance. Having obtained stable, cross-linked gels, we embarked on a study of their performance in aqueous solutions. Figure 5 depicts temperature-induced volume transitions of the polymer–protein gels. It can be seen that discontinuous transitions were observed at around 32 °C and are independent of the pH of the solution. Remarkable pH

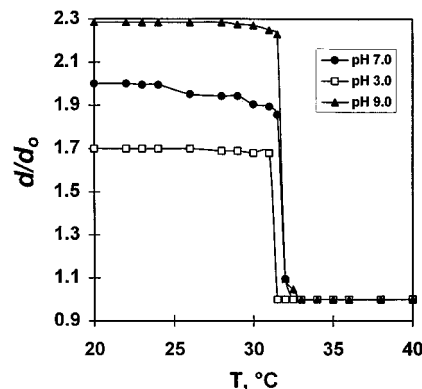
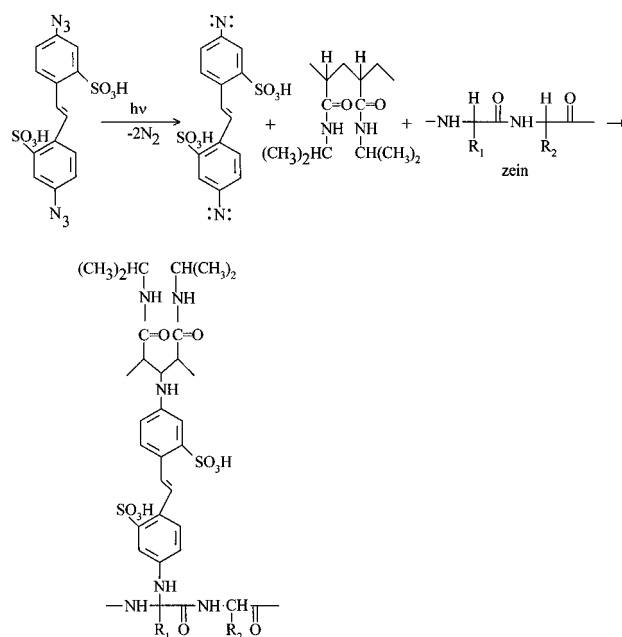


Figure 5. Effect of temperature on d/d_0 characterizing volume phase transitions of polyNIPA–DAS–zein gel fibers in water at different pH. Initial concentrations of NIPA, zein, and DAS in DMSO were 0.25, 0.25, and 0.025 g/mL, respectively. Polymerization was carried out as described in Experimental Section.

SCHEME 2



insensitivity of the temperature of transition may be correlated with the gel structure. As mentioned in the Introduction, the rationale for the use of poly(*N*-isopropylacrylamide) as a gel constituent was its uniquely sharp, discontinuous volume transition at around 32 °C. Random copolymerization of NIPA with ionizable monomer usually leads to a drastic shift of the volume transition of the resulting copolymer to higher temperatures, when the pH is higher than pK_a of the ionizable component.⁸ This is because of the solubility that the ionized component conveys to the incipient formation of insoluble, temperature-sensitive aggregates of NIPA.⁶ It has been shown that in order to retain a reversible temperature-induced volume phase transition of an ionizable copolymer over a broad range of pH, the latter should contain long enough segments of a hydrophobic component.^{6,28} The hydrophobic segments will provide entropy-driven aggregation of the entire copolymer at temperatures not significantly different from those of the hydrophobic component itself, whereas ionizable segments will render the copolymer ion-sensitive. The block or graft copolymers where pH- and temperature-sensitive segments either alternate along the chain or are grafted onto each other are therefore the only type of copolymers where volume phase transitions can be due to pH, temperature, or both.⁶ The

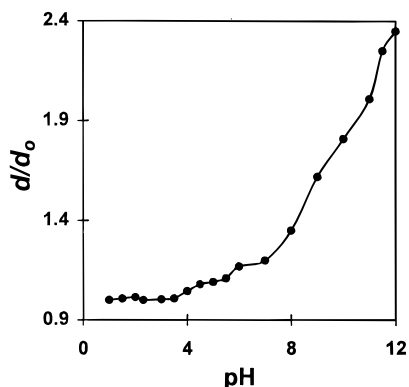


Figure 6. Effect of pH on d/d_0 characterizing volume phase transitions of polyNIPA-DAS-zein gel fibers in water at 20 °C. For initial concentrations of the gel components, see legend to Figure 5.

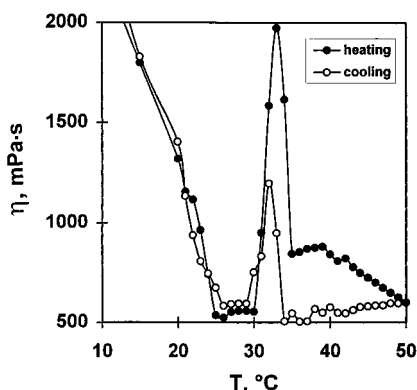


Figure 7. Thermothinning behavior ($\eta = f(T)$) of polyNIPA-DAS-zein suspension in water. Effective concentration of particles in water was 17 wt % at pH 8.0. Rate of heating/cooling was 2 °C/min, and the time of equilibration at each temperature was 5 min. $\gamma = 40 \text{ s}^{-1}$. For initial concentrations of components in gel particles, see legend to Figure 5.

temperature-induced volume phase transitions observed within the wide range of pH (Figure 5) correlate well with the proposed structure of protein-polymer networks (Scheme 2) where pH-sensitive segments (DAS-zein) are grafted onto a temperature-sensitive backbone (polyNIPA).

Transitions of the gel versus pH are shown in Figure 6. It can be seen that the gel started to swell at $\text{pH} > 4$ upon ionization of zein molecules that repulsed each other. DAS molecules containing strongly acidic sulfonyl groups were ionized throughout the entire pH range and therefore did not contribute to the observed volume transition. The differences in absolute values of the parameter d/d_0 , measured while varying temperature (Figure 5) and pH (Figure 6), are due to the (arbitrary) choice of d_0 as the effective diameter of the gel in the collapsed state. Thus, at pH 3.0 and 20 °C the gel is expanded when compared to its collapsed state at pH 3.0 and 40 °C (Figure 5), but it is still collapsed when compared to its state at pH 1.0 and 20 °C (Figure 6).

It is interesting to note that zein itself is temperature-sensitive because of the high content of hydrophobic amino acid residues.²¹ Thus, some zein properties may be observed for the gels outside the temperature range characteristic of the transitions of polyNIPA. Indeed, thermothinning of the gel suspension at 10–25 °C (Figure 7) can be attributed to a reversible association of the zein chains in water.²⁹ At elevated

temperatures zein molecules that have been bridging the particles fold, forming compact domains.²¹ Absent bridging chains, the particles will readily slide along each other. It can be seen that the viscosity of the suspension was minimal at 25–30 °C. However, at 31–33 °C a sharp increase of viscosity was observed that corresponded to the volume phase transition of polyNIPA. This remarkable phenomenon can be explained by a network formed due to hydrophobic interactions between polyNIPA domains belonging to different particles. This network was destroyed by shear flow. Upon cooling, a maximum in the viscosity vs temperature curve was observed at 32 °C.

Conclusions

Formation of cross-linked gels has been observed upon polymerization of *N*-isopropylacrylamide (NIPA) blended with a seed protein (zein) and 4,4'-diazido-2,2'-stilbenedisulfonic acid (DAS) in dimethyl sulfoxide following irradiation. The resulting gels were shown to be stable in aqueous solutions (pH 1–11). Grafting of DAS-zein chains on a polyNIPA backbone was proposed. Fiber gels were shown to undergo a discontinuous volume transition at around 32 °C corresponding to lower critical solution temperature of NIPA. The gel swelling appeared to be pH-sensitive. Thermothinning of suspensions of the gel particles was observed with a sharp maximum of viscosity around 32 °C due to interparticle network formation caused by hydrophobic interactions between polyNIPA domains belonging to different particles.

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