JOURNAL OF MOLECULAR RECOGNITION, VOL. 9, 549–557 (1996)

Synthesis and Characterization of Sol–Gel Phase-reversible Hydrogels Sensitive to Glucose

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A new type of hydrogel capable of sol–gel phase-reversible transition by changes in the environmental glucose concentration has been synthesized. The hydrogel consists of vinylpyrrolidinone–allylglucose (VP/AG) copolymer and concanavalin A (Con A). The formation of the hydrogel is based on the specific interaction between glucose on the copolymer and glucose receptor sites on Con A. Hydrogels were formed immediately after mixing the copolymer and Con A. The critical factors in the formation of hydrogel were the concentrations of the copolymer, of glucose on the copolymer and of Con A. In general, the formation of a hydrogel became more efficient as the concentration of glucose on the copolymer decreased and/or as the Con A concentration increased. The hydrogel formed became a sol in the presence of glucose in the environment. The environmental glucose concentration necessary to dissolve the gel to sol was approximately four times higher than the concentration of glucose on the copolymer. Upon removal of the environmental glucose by dialysis, the sol became a gel again and the sol–gel phase transition could be repeated.

Introduction

A hydrogel is a three-dimensional polymer network which swells in water. Because of their high water-absorbing ability, hydrogels have been used in various applications. One of the important applications has been in the area of controlled drug delivery. Although many drugs are effective by continuous delivery at a certain rate, there are many drugs which need to be delivered in a non-continuous fashion. For example, the delivery of insulin has to be temporal, rather than continuous, to be clinically effective. Such a specific demand for drug delivery requires hydrogels which can respond to external signals. Those hydrogels which can respond to various external stimuli are known as smart hydrogels. The environmental stimuli which have been frequently used in smart hydrogels include pH, temperature, light, electric field, solvent, external stress, specific ligand, enzymes and combinations of these (Park *et al.*, 1993).

The currently available smart hydrogens undergo volume changes, i.e. either swelling or shrinking, in the presence of external stimuli. The volume change of a hydrogen generally, occurs abruptly with only a small change in the environmental factors. Such a discrete volume change has been called a volume phase transition since the volume change profile is similar to that of gas–liquid phase transition (Tanaka, 1992). It is noted, however, such a volume phase transition is not really a phase transition since the volume change occurs in the same gel phase. We were interested in the development of hydrogels which undergo true phase transition between sol and gel states. Furthermore, we were interested in hydrogels which undergo phase transition by responding to a specific environmental signal. We chose glucose as a model environmental stimulus in a hope that the phase-reversible hydrogels can be used for temporal delivery of insulin or for sensing of glucose molecules in the environment in the future. This paper describes the synthesis and characterization of sol–gel phase-reversible hydrogels sensitive to glucose.

Sol–Gel Phase-Reversible Hydrogel Sensitive to Glucose

The preparation of glucose-sensitive phase-reversible hydrogels demands two fundamental requirements: reversible cross-linking (i.e. physical cross-linking) and glucose specificity. A highly specific interaction between glucose and concanavalin A (Con A) was used to form physical crosslinks between glucose-containing polymer chains. The glucose molecules which are attached to the polymer backbone react with Con A. Since Con A exists as a tetramer at physiological pH and each subunit has a glucose binding site, Con A can function as a cross-linking agent for glucose-containing polymer chains. Because of the non-covalent interaction between glucose and Con A, the cross-links formed are reversible. Individual free glucose molecules can compete with the polymer-attached glucose molecules. Hence, the maintenance of the cross-links depends on the relative concentration of free glucose in the environment. This concept is illustrated in Fig. 1. The gel is formed by mixing glucose-containing polymers with Con A. Upon addition of free glucose molecules, the hydrogel dissolves to become a sol owing to the detachment of polymer chains from Con A as a result of competitive binding of free glucose to Con A. The sol can become a gel again upon removal of free glucose.

Experimental

Synthesis of allylglucose

Allylglucose (AG) was synthesized by the modified Fischer reaction (Horejsi and Kocourek, 1974; Tally *et al.*, 1945). A 100 g amount of α-D-glucose (MW of 180.16) (Aldrich)

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CCC 0952–3499/96/060549–09

Received 25 May 1995
Accepted (revised) 24 August 1995

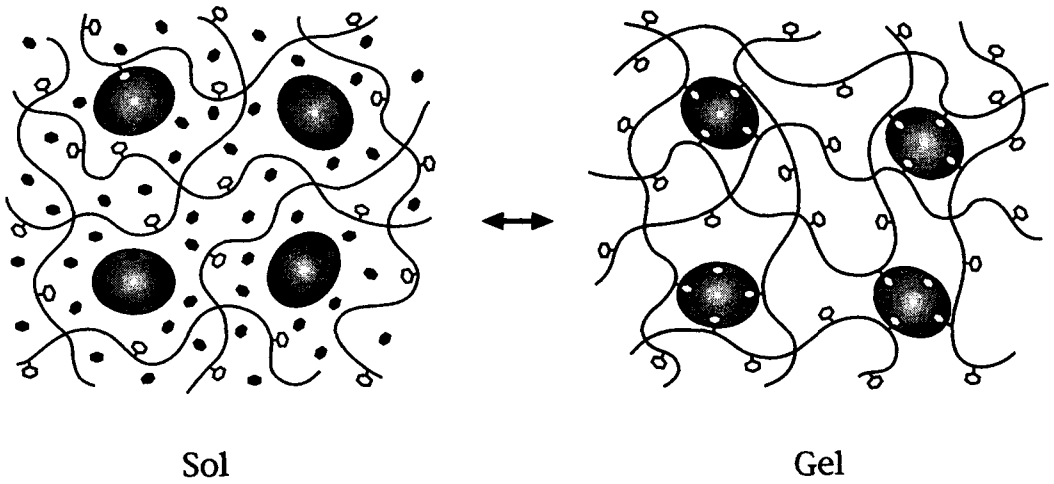

Figure 1. Pictorial representation of the sol–gel phase transition. Large circles represent Con A molecules, small open hexagons represent glucose attached to the polymer chain and small closed hexagons represent free glucose.

was ground in a mortar to reduce the particle size. Dry HCl gas (Aldrich) was dissolved in 200 mL of allyl alcohol (Aldrich) to make 3% (w/w) HCl. The HCl was added by bubbling the gas through allyl alcohol using poly(vinyl chloride) (PVC) tubing (Aldrich). The allyl alcohol–HCl mixture was added to the ground glucose and reacted in a three-necked 1000 mL flask at 80°C for 4 h with reflux and stirring. Openings were plugged using Drierite to prevent moisture from entering during the reaction. After the reaction, the solution was allowed to cool. The HCl was neutralized by addition of ammonia solution. Unreacted allyl alcohol was evaporated under reduced pressure and heat. AG was then extracted with dry acetone (Fisher). Finally, AG was crystallized from the concentrated extract. The crystals were washed with clean, dry acetone several times and dried overnight under vacuum.

Synthesis of allylglucose-containing copolymers

Copolymers of AG and 1-vinyl-2-pyrrolidinone (VP) (Aldrich) were synthesized by free radical polymerization. The copolymers were synthesized from a solution of VP and AG using an initiator, 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (Wako) in the presence of *N,N,N′,N′*-tetramethylethylenediamine (Bio-Rad). The contents were placed in a test-tube and polymerization was carried out at 60°C for 1 h. The copolymer solution was then dialyzed extensively against distilled, deionized water. A dialysis tube with a molecular weight cut-off of 6000–8000 was used. The copolymers were then separated by precipitation using dry acetone. The precipitate was filtered using a glass Buchner funnel. The separated copolymer was dried overnight under vacuum. The proposed structure of AG–VP copolymer is shown in Fig. 2.

Formation of hydrogel

Con A (MW 102 000) (Sigma) was dissolved in 2× phosphate-buffered saline (PBS, pH 7.4) solution with 1 mM $CaCl_2$ and 1 mM $MnCl_2$ to make a stock solution of 0.30 g/mL. The copolymer solutions were also prepared using the same buffer solution. The copolymer concentration was varied from 22.5 to 180 mg/mL for each copolymer. Equal volumes of Con A and copolymer solutions were mixed to form the physical gels. The gels were formed immediately after mixing. The fast gel formation may have introduced inhomogeneities in the network structure owing to inefficient mixing. Visual inspection, however, showed no macroscopic inhomogeneities in the turbidity of the prepared gels.

Determination of glucose concentration

The glucose incorporation into the copolymer was quantitated by the phenol–sulfuric acid assay (Dubois *et al.*, 1956). A calibration graph was constructed using known

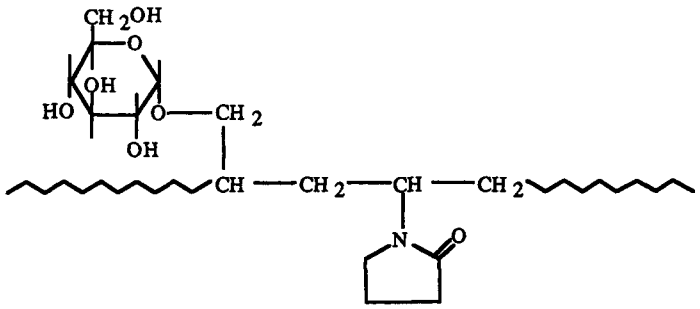

Figure 2. Representative structure of a copolymer made from allylglucose and vinylpyrrolidinone monomers.

concentrations of α-D-glucose. A 0.1 mL volume of phenol and 5 mL of concentrated sulfuric acid were added to glucose standard solutions or to copolymer solutions totalling 2 mL each. After 30 min of incubation at 30°C, the solutions were read on Beckman DU-7 spectrophotometer at 490 nm.

Elemental analysis

The elemental analysis of the copolymers was performed at the Purdue University Microanalysis Center using a Perkin-Elmer 240C elemental analyzer. The copolymer composition was determined using the percentage nitrogen values.

Determination of reactivity ratios

For the measurement of reactivity ratios, polymerization was carried out for several minutes to obtain a conversion of less than 10% of the total monomers. Polymerization was stopped by pouring the reaction mixture into an excess amount (more than 200-fold by volume) of acetone with rapid stirring. The copolymers were separated by filtering through a glass Buchner funnel. The copolymer was dried overnight under vacuum. After the determination of the copolymer composition, the reactivity ratio was calculated using the graphical method of Fineman and Ross (1950).

Nuclear magnetic resonance (NMR)

The proton and carbon NMR spectra of AG monomer and copolymer were obtained on a Bruker ARX300 instrument using D_2O (Cambridge Isotope Laboratories) as the solvent. For carbon spectra, sodium 3-(trimethylsilyl)-1-propane-sulfonate (Aldrich) was used as the internal standard. Solutions of copolymer and AG were prepared at the concentration of 20% (w/v). The spectra were obtained by taking 8 and 80 scans for proton and carbon NMR, respectively.

Turbidity measurements

To each well of a 96-well micro test plate were added 100 μL of copolymer solution and 100 μL of Con A solution while stirring. The final concentration of the copolymer ranged from 4.5 to 90.4 mg/mL and that of Con A from 0.05 to 0.15 g/mL. The absorbance value of each well was read at 630 nm on EL311 Microplate Autoreader (Bio-Tek Instruments).

Determination of phase transition and reversibility

The sol–gel phase reversibility was tested using a dialysis tube with a molecular weight cut-off of 6000–8000. The hydrogel was prepared inside a dialysis tube. The dialysis tube containing the hydrogel was placed in a beaker containing 300 mL of buffer solution (2×PBS with 1 mM $CaCl_2$ and 1 mM $MnCl_2$). Glucose was added to the buffer solution in the beaker. The glucose concentration at which the hydrogel completely dissolved was determined. The gel was cycled between the buffer solution with and without glucose to test the phase reversibility.

Results

Synthesis of copolymers

The synthesis of AG was confirmed by proton NMR (Fig. 3). The peaks at 5.2 and 5.8 ppm are indicative of the double bond, while the group of peaks at around 3.5 ppm indicate the presence of glucose. AG was mixed with VP to synthesize copolymers. The concentration of VP was fixed at 8%, 12% or 16% and at each VP concentration the concentration of AG in the feed was varied, the molar ratio of AG to VP varying from 0.10 to 0.72. The carbon NMR spectrum of the synthesized copolymer is shown in Fig. 4. The copolymer spectrum contains the peaks resulting from the VP moiety at 36, 47 and 180 ppm. The other peaks between 55 and 105 ppm are due to the glucose moiety in the copolymer. As the AG concentration increased in the feed, the heights of the peaks between 55 and 105 ppm in the carbon NMR spectrum increased. The carbon NMR data clearly show the presence of both glucose and VP moieties in the copolymer.

The compositions of glucose and VP moieties in the synthesized copolymers are given in Table 1. In general, as the initial feed ratio of AG increased, more glucose was incorporated into the copolymer. This was also indicated by a lower nitrogen content in the copolymer. The increase in the glucose concentration in the copolymer, however, was not a linear function of the AG concentration in the feed. In Fig. 5 the [AG]/[VP] molar ratio in feed is plotted against the [AG]/[VP] molar ratio in copolymer. When the molar ratio was small, all of the AG was incorporated into the copolymer. As the molar ratio in the feed increased, however, less AG was incorporated into the copolymer.

To examine the distribution of glucose in the synthesized copolymer, the reactivity ratios of AG and VP were determined. In the reactivity ratio calculation, VP was chosen as M_1. The reactivity ratios obtained were $r_1 = 1.1$ and $r_2 = -0.08$. The r_2 value was essentially zero. These two values did not clearly indicate what type of copolymer was formed, but suggested that the synthesized copolymer was not a purely random or block copolymer. Thus, it appeared that an alternating copolymer was formed.

Hydrogel formation

The formation of hydrogel by copolymer and Con A was examined using the copolymers listed in Table 1. Initially we were interested in whether the mixture of copolymer and Con A can form a hydrogen or not. Thus, 0.2 mL of the copolymer solution and 0.2 mL of Con A solution were placed in a test-tube and the mixture was examined visually.

The copolymer concentrations used were 22.5, 45, 90, 135 and 180 mg/mL. The Con A concentration was fixed at 0.3 g/mL. The final concentrations of the copolymer and Con A were reduced to half of the initial concentration after mixing. When 180 mg/mL of copolymer was mixed with Con A, no hydrogel was formed and no appreciable change in the viscosity of the solution was observed. Apparently, the copolymer and Con A did not interact to form a cross-linked network. As the copolymer concentration decreased, the viscosity of the mixture increased and the mixture became more difficult to flow. The system, however, still did not produce a hydrogel. When the copolymer at a concentration of 22.5 mg/mL was mixed with Con A, a homogeneous hydrogel was formed. The formation of the gel was almost instantaneous upon mixing. Since the concentration of the copolymer appeared to be important in hydrogel formation, the copolymer concentration was reduced to 4.5 mg/mL.

To examine the hydrogel formation in a quantitative manner, we measured the turbidity of the copolymer–Con A mixture at 630 nm. When a hydrogel was formed, the turbidity of the system increased dramatically. Since the precipitation process also resulted in an increase in turbidity, we always made sure that the hydrogel was formed. The gel, which could be separated from the container as one solid piece, was easily distinguished from the precipitates by visual inspection. Figure 6 shows the effect of the final copolymer concentration on the formation of gel. The copolymers used in Fig. 6 were those synthesized with 12% VP in the feed in Table 1. The final Con A concentration was varied from 0.05 g/mL (5%) to 0.15 g/mL (15%). The general trend in the figure is that as the copolymer concentration increases, the absorbance value decreases, indicating the lack of gel formation. The gel was formed when the copolymer concentration was $\leq$22.5 mg/mL. As the concentration of copolymer decreased while the concentration of Con A was fixed, the molar ratio of Con A to copolymer increased. Since Con A functioned as a cross-linking agent, it appeared that as the concentration of a cross-linking agent increased, the hydrogel formation became easier.

The importance of the concentration of Con A relative to the copolymer concentration in the three-dimensional network formation is readily seen in Fig. 7. The copolymers used in Fig. 7 were those synthesized with 16% VP in the feed in Table 1. When the copolymer concentration was low (e.g. 4.52 mg/mL), hydrogels were formed readily. When the copolymer concentration was high (e.g. 90.4 mg/mL), however, the turbidity of the mixture increased as the concentration of Con A increased from 5% to 15%. This

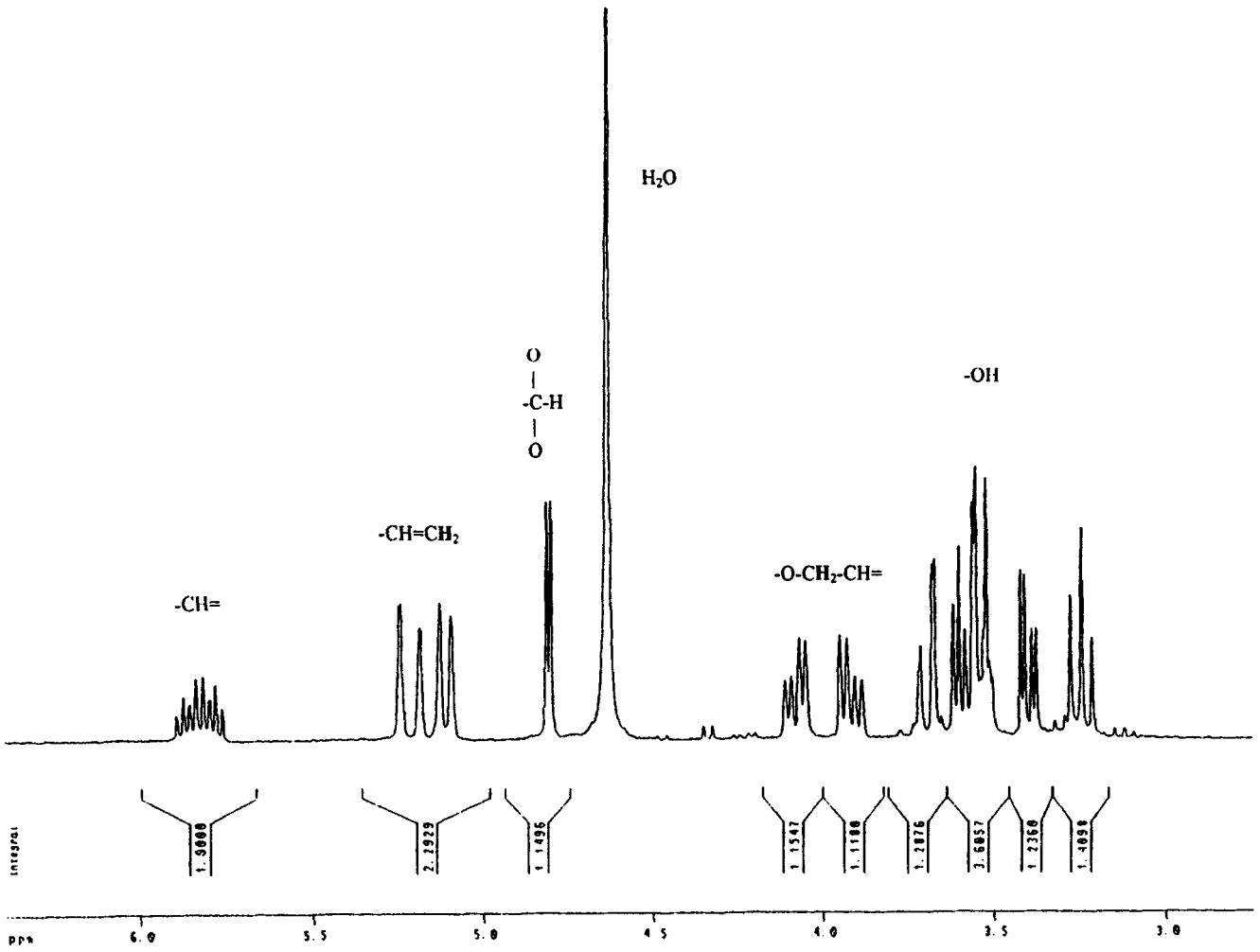

Figure 3. Proton NMR spectrum of allylglucose in D_2O.

trend was observed when the [AG]/[VP] ratio was ≤ 0.28. The overall trend observed in Figs 6 and 7 is that a hydrogel was formed more readily with a lower copolymer concentration and/or a higher Con A concentration.

The changes in copolymer concentration have corresponding changes in the glucose concentration in the copolymer. The effect of the glucose concentration in the copolymer on the hydrogel formation is shown in Fig. 8.

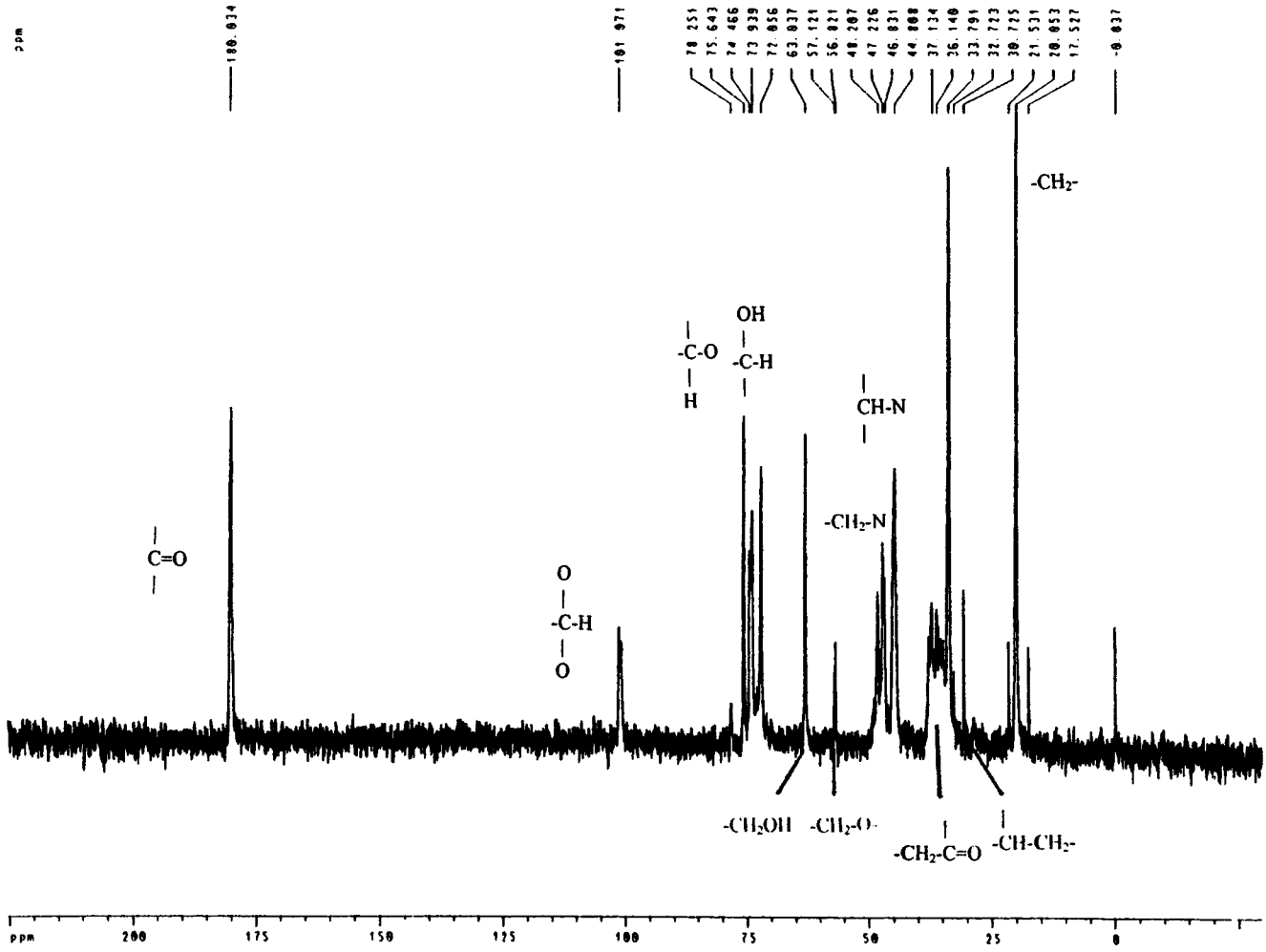

Figure 4. Carbon NMR spectrum of allylglucose (AG)–vinylpyrrolidinone (VP) copolymer in D_2O. The VP concentration in the feed was 8% and the [AG]/[VP] molar ratio in the copolymer was 0.13.

Table 1. Copolymers synthesized with AG and VP in different ratios

Concentration of VP in feed	[AG]/[VP] molar ratio in feed	Nitrogen content (%)	Copolymer composition (mol%) [AG]	[VP]	[AG]/[VP] molar ratio in copolymer
8	0.19	10.1	11.2	88.9	0.13
	0.36	8.6	19.2	80.8	0.24
	0.51	7.3	26.9	73.1	0.37
	0.72	6.0	35.6	63.4	0.56
12	0.13	10.3	10.3	89.7	0.11
	0.16	9.27	15.3	84.7	0.18
	0.32	7.8	23.6	76.4	0.31
	0.48	6.6	31.5	68.5	0.46
16	0.10	10.5	9.1	90.9	0.10
	0.15	9.3	15.4	84.6	0.18
	0.30	8.1	22.1	77.9	0.28
	0.45	7.6	25.2	74.8	0.34

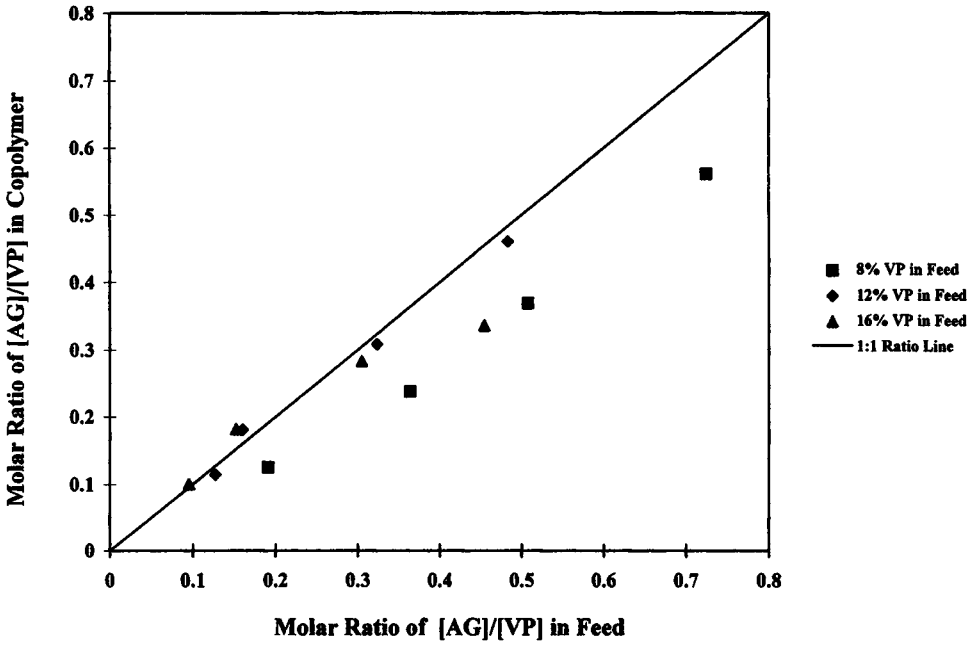

Figure 5. [AG]/[VP] molar ratio in the copolymer as a function of that in the feed.

The copolymers used in Fig. 8 were those synthesized with 8% VP in the feed in Table 1. As the [AG]/[VP] ratio incresed to 0.37, a hydrogel was formed despite the high concentration of copolymers. The inability of hydrogel formation by copolymers with [AG]/[VP] ratios of 0.13 and 0.24 at high copolymer concentrations indicates that copolymers with a higher content of glucose form hydrogels more readily than copolymers with a lower content of glucose. Copolymers with higher glucose contents are expected to interact with Con A more readily than those with lower glucose contents.

The results in Figs 6–8 indicate that the hydrogel formation by glucose-containing copolymers and Con A becomes more favorable if the glucose content on a copolymer is increased and/or the Con A concentration is increased. As the Con A concentration increases (i.e. as the concentration of a cross-linking agent increases), more copolymers can be cross-linked to form a three-dimensional

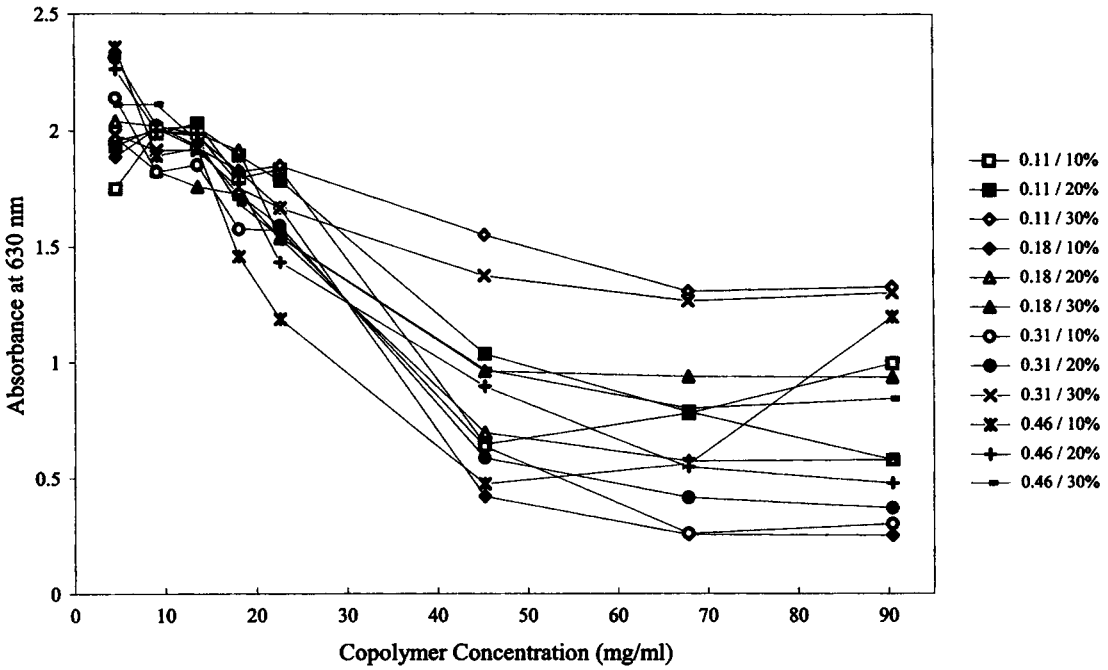

Figure 6. Absorbance at 630 nm of mixtures of copolymer and Con A as a function of copolymer concentration. As the hydrogel formed the absorbance increased. The left-hand number (e.g. 0.11) in the legend indicates the [AG]/[VP] molar ratio in the copolymer and the right-hand number (e.g. 10%) indicates the final concentration of Con A.

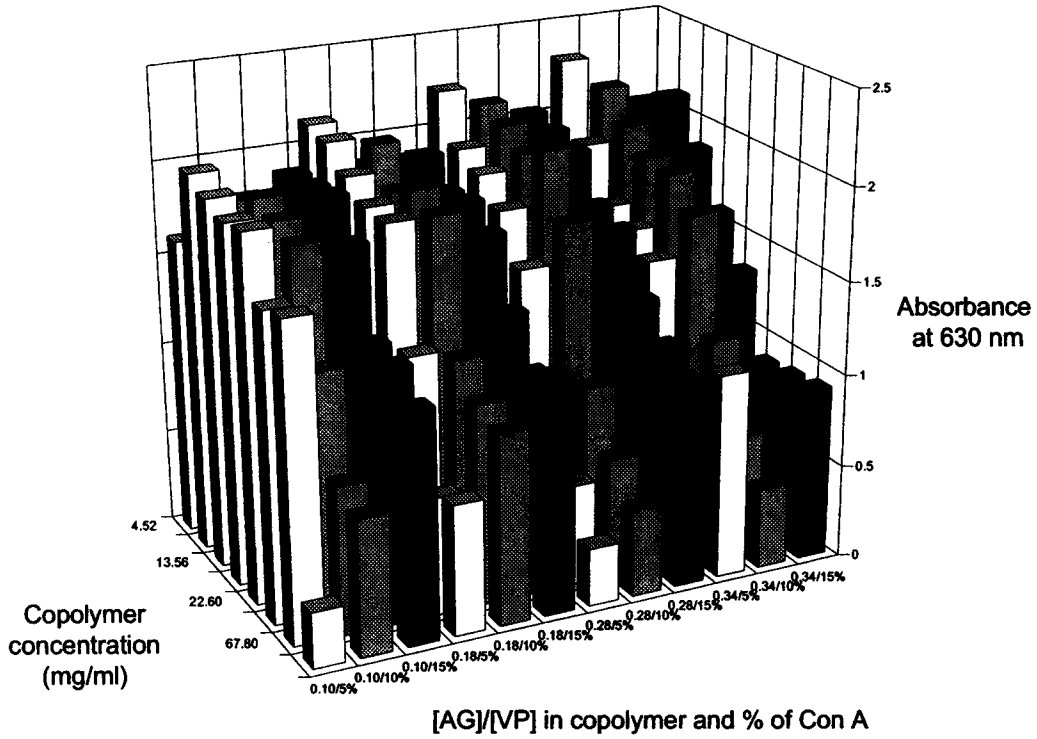

Figure 7. Absorbance at 630 nm of mixtures of copolymer and Con A as a function of copolymer concentration. As the hydrogel formed the absorbance increased. The left-hand numbers (0.10, 0.18, 0.28 and 0.34) on the *x*-axis indicate the [AG]/[VP] molar ratio in the copolymer and the right-hand numbers (5%, 10%, and 15%) indicate the final concentration of Con A. The copolymer concentrations were 4.52, 9.04, 13.56, 18.08, 22.60, 45.20, 677.80 and 90.40 mg/mL.

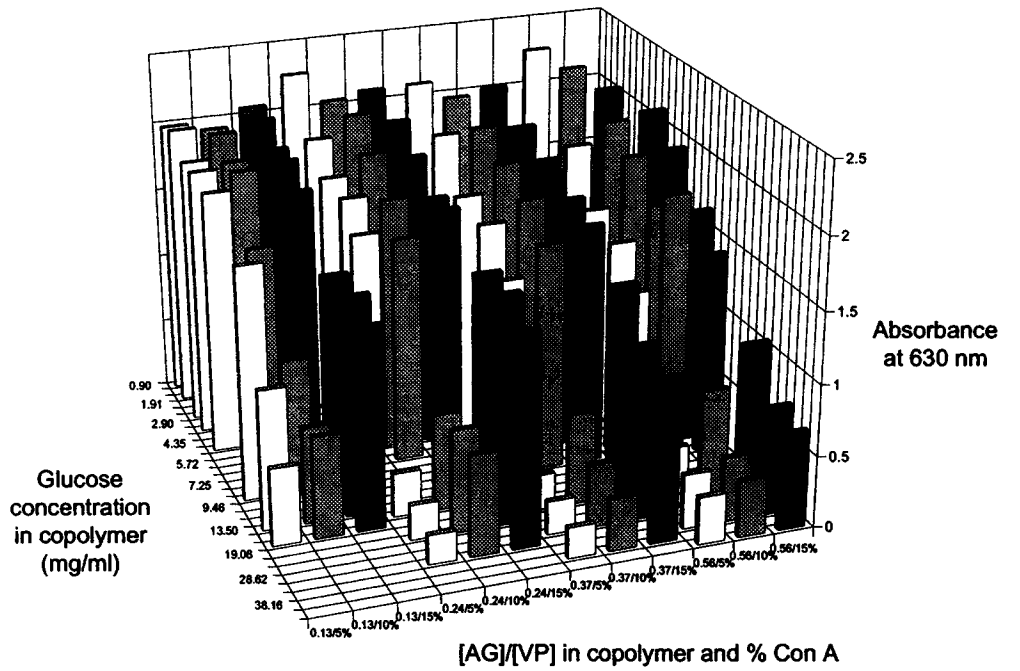

Figure 8. Absorbance at 630 nm of mixtures of copolymer and Con A as a function of glucose concentration in the copolymer. As the hydrogel formed the absorbance increased. The left-hand numbers (0.13, 0.24, 0.37 and 0.56) on the *x*-axis indicate the [AG]/[VP] molar ratio in the copolymer and the right-hand numbers (5%, 10% and 15%) indicate the final concentration of Con A. The glucose concentrations in the copolymers were 0.90, 1.45, 1.80, 1.91, 2.37, 2.70, 2.90, 3.60, 3.82, 4.35, 4.50, 4.73, 5.73, 5.80, 7.09, 7.25, 7.63, 9.00, 9.46, 9.54, 11.82, 13.50, 14.50, 18.00, 19.08, 21.75, 23.64, 28.62, 29.00, 35.46, 38.16 and 47.27 mg/mL.

Table 2. Concentration of free glucose necessary for gel to sol phase transition

Glucose concentration in copolymer (mg/mL)	Glucose concentration in medium (mg/mL)
0.46	2
2.97	13
8.6	38
10.1	45

network. As the copolymer concentration decreases at a fixed Con A concentration, the relative Con A concentration increases and this makes hydrogel formation easier.

Sol–gel transition

When the hydrogel formed was placed in a solution with excess glucose, the gel became a sol, i.e. the hydrogel dissolved. The environmental glucose concentration at which the phase transition occurs was tested. To keep the copolymer and Con A together through the phase-transition period, the gel was made inside the dialysis tube. Table 2 gives the environmental glucose concentration necessary to dissolve the hydrogel. Different hydrogels were formed using copolymers having different concentrations of glucose on their polymer backbone. The glucose concentrations in the copolymer was varied from 0.46 to 10.1 mg/mL. As shown in Table 2, for the four hydrogels tested, the concentration of free glucose in the medium to transform the hydrogels into the sol phase was about four times the glucose concentration in the copolymer.

When the dissolved hydrogel (i.e. the mixture of copolymer and Con A) in the dialysis tube was placed back in a buffer solution without free glucose, hydrogel was formed again on removal of free glucose by dialysis. It took about 30 min for the sol phase to turn into the gel phase again. The time for the sol–gel phase transition can be adjusted by controlling the dimensions of the system, which affect the diffusion of free glucose molecules. The gel in a dialysis tube was cycled through buffer solutions with and without the appropriate glucose concentration necessary for phase transition. The sol–gel phase transition was repeated more than 10 times without any problems. The exact limit of the repeatability has not yet been determined.

Discussion

We have synthesized sol–gel phase-reversible hydrogels which are sensitive to glucose using glucose-containing VP polymer and Con A. Glucose-sensitive phase-reversible hydrogels have been synthesized before by Kitano *et al.* (1992), but the system employed a phenylboronic acid moiety on a copolymer as a glucose-sensing moiety, thus the system is not really glucose-specific. To make the hydrogel system more glucose-specific, we used Con A, a glucose-binding protein. The specific interaction between glucose and Con A has also been used before for application in glucose sensing (Morris *et al.*, 1993; Nakamae *et al.*, 1994). In those studies, however, the glucose-containing polymer and Con A did not result in hydrogel formation; only precipitates were formed instead of hydrogels. In our study, we were able to form hydrogels using a similar system composed of glucose-containing polymer and Con A. As our study showed, the concentration of copolymers and the concentration of the copolymer relative to that of Con A were important in obtaining hydrogels rather than precipitates. Under our experimental conditions, the copolymers at concentrations <22 mg/mL resulted in hydrogels which readily undergo sol–gel phase transition.

The glucose moiety on the copolymer needs to preserve free hydroxyl groups at the C-3, C-4 and C-6 positions to interact with Con A (Goldstein and Hayes, 1978). We therefore attached an allyl group to the C-1 position of glucose. AG has been utilized to synthesize *O*-glycosyl polyacrylamide gel for use as an affinity chromatographic material to separate Con A (Horejsi and Kocourek, 1974; Tichà and Kocourek, 1991). This gel chromatographic matrix performed well in the separation of Con A. In our study, the modified glucose also bound to Con A to form hydrogels.

When the glucose-containing copolymers are mixed with Con A, the formation of hydrogel was almost instantaneous. The dissolution of the hydrogel into the sol phase, however, takes time. It depends on the diffusion of free glucose into the hydrogel and subsequent displacement of the polymer-bound glucose from the glucose binding sites of Con A. Hence the response time can be adjusted by controlling the dimensions of the hydrogel. The ability to respond fast to changes in environmental glucose concentration is important if the sol–gel phase-reversible hydrogel system is to be used in either glucose sensing or insulin delivery in the future.

The sol–gel phase-reversible hydrogels synthesized in this study need much more characterization and improvement. Although we have empirically observed that the concentration of free glucose necessary to dissolve hydrogels is approximately four times that of the glucose moiety in the copolymer, it is not clear whether this factor of four is universal or not. For now, however, we can use this information to prepare hydrogels which undergo sol–gel phase transition at around the physiological glucose concentration, i.e. 1–3 mg/mL. For example, hydrogels made of copolymers with 0.46 mg/mL of glucose (see Table 2) can undergo phase transition in that glucose range. The measurement of the affinity between glucose on the copolymer and Con A will help us elucidate why more free glucose is necessary for the dissociation of polymer-attached glucose from Con A and whether the factor of four has any meaning or not. The use of copolymers made of monomers other than VP will also help in understanding the interaction between glucose-containing copolymer and Con A.

Con A requires both Ca^{2+} and Mn^{2+} for glucose binding. We used 1 mM Ca^{2+} and Mn^{2+} (Bhattacharyya *et al.*, 1988). Con A is known to form dimers and develops turbidity in a time-dependent manner at pH $\geq$ 7 when the ionic strength of the medium is 0.1 (Mckenzie and Sawyer, 1973). This time-dependent turbidity is eliminated at pH 7 by either an increase in ionic strength to values $\geq$0.3 or by the addition of glucose. Since Con A molecules exist as a tetramer when the ionic strength is $\geq$0.3, we used twice concentrated phosphate buffer to make the ionic strength >0.3. The

requirement for an ionic strength larger than that of the physiological value may cause some difficulty in the *in vivo* application of the glucose–Con A system.

In this study, the specific interaction between glucose and Con A was utilized to form the sol–gel phase-reversible hydrogel. Other specific interactions, such as antigen–antibody and avidin–biotin interactions, also occur in nature. These interactions can also be utilized to form sol–gel phase-reversible hydrogels specific to certain ligands. It is commonly observed that antigen–antibody reaction results in precipitate formation rather than gel formation. As shown in this study, the choice of the correct concentrations of polymers and receptors (such as Con A in our study) is important in obtaining stable hydrogels. This type of ligand-specific sol–gel phase-reversible hydrogels is expected to find various applications in the areas of biosensors (Morris *et al.*, 1993; Nakamae *et al.*, 1994) and controlled drug delivery (Taylor and Adams, 1995).

Acknowledgment

The authors gratefully acknowledge support from the American Diabetes Association, Indiana Affiliates.

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