Synthesis and Characterization of PH-and Salt-Sensitive Hydrogel Based on Chemically Modified Poultry Feather Protein Isolate

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ABSTRACT: Hydrogels were synthesized from poultry feather protein by crosslinking ethylene diamine tetraacetic dianhydride (EDTAD)-modified feather protein isolate (FPI) with glutaraldehyde (Glu). Different molar ratios of EDTAD/FPI were used to obtain FPI of different degrees of acylate modification. Differential scanning calorimeter measurements of glass transition temperature suggested that hydrogel formation was based on the hydrogen bond between EDTAD-modified FPI segments. The swelling properties of modified FPI hydrogel were investigated in deionized water and in solutions of different salt contents (i.e., ionic strengths) and pH. An optimal swelling ratio (SR) of 63 g/g was obtained when molar ratios of EDTAD/FPI and Glu/FPI were 0.12 and 0.008, respectively. SR decreased substantially with increase in ionic strength, and at a given ionic strength, SR increased with solution pH in 4.0 to 10.0 range. The water transport mechanism of the hydrogel was also pH dependent and was controlled by Fickian diffusion and polymer relaxation. At higher pH value, the water transport profile became more dependent on polymer relaxation than at lower pH. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 116: 602–609, 2010

Key words: feather protein; hydrogel; modification; swelling behavior; pH-dependence

INTRODUCTION

Hydrogels are structurally crosslinked hydrophilic polymers that have the ability to absorb large amounts of water or aqueous fluids in relatively short time. They are useful as biomedical materials, superabsorbents, sensors, etc.[1–3] Hydrogels may be grouped according to their origin as natural and synthetic. Considerable effort has been made to synthesize and characterize acrylate-based ionic hydrogels. These synthetic hydrogels, however, are not biodegradable by either hydrolytic or enzymatic means. As a result, acrylate systems are limited in their potential for biodegradable applications. To overcome this limitation, a range of natural polymers has been used to prepare crosslinked hydrogel networks. For example, pH-sensitive hydrogels based on polypeptides, proteins, and polysaccharides have been produced.[4–6]

Feathers are available in large quantities, an estimated two to four billion pounds each year in United States alone,[7] as a waste by-product from poultry processing plants. The current options for the industry are either to pay for their disposal in landfills or to sell them for low price to producers of animal feed.[8–10] Feather proteins, which are almost pure keratin (90% or more), contain proportionately more cysteine than most tissue proteins and food proteins.[11] Cystine is a crystalline, sulfur-containing amino acid, formed from two molecules of the amino acid cysteine (Fig. 1). Cystines play a valuable role by crosslinking proteins, which increase the molecular stability in the harsh extracellular environment and also help confer proteolytic resistance. Intracellularly, disulfide bridges between cysteines within polypeptide support the protein's secondary structure. These properties make feather a valuable resource for varied uses. However, usefulness of feather proteins for value-added noon-feed applications has not received much attention.

Hydrogels from various synthetic polymers have been widely investigated. At present, biodegradable hydrogels, such as those of protein origin, are being actively researched due to the increased demands for biocompatibility and environmental protection.[12] Hydrogels made from feather protein are opaque and do not swell sufficiently to be useful for

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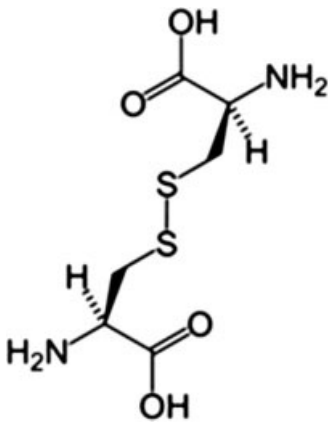

Figure 1 Structure of cysteine.

biocompatible applications. However, feather protein contains several reactive side groups, including amino, hydroxyl, sulfhydryl, and carboxyl moieties, which can be used as sites of chemical modification and crosslinking to produce novel polymeric hydrogels with improved functionalities.

The objectives of this study were to: (1) chemically modify the cysteine residues of feather protein isolate (FPI), (2) optimize the synthesis of ethylenediaminetetraacetic dianhydride (EDTAD)-FPI hydrogels, (3) characterize the swelling behavior of the hydrogels in water, salt, and buffer solutions, and (4) investigate the swelling kinetics at various pH values.

MATERIALS

Sodium hydroxide (NaOH), EDTAD, glutaraldehyde (Glu), sodium bisulfite ($NaHSO_3$), polyethylene glycol (PEG) were obtained from Alfa Aesar (Johnson Mattey Co., MA); Feather fiber, potassium biphthalate buffer, potassium phosphate monobasic-sodium hydroxide buffer, potassium carbonate-potassium hydroxide buffer were purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents were of analytical grade. Deionized water was used to prepare all the solutions.

METHODS

Preparation of feather protein isolate

Measured quantity of feather fiber was dissolved in 10% (w/w) $NaHSO_3$ solution for 24 h to open the double sulfide linkage in the cysteine and break the original three-dimensional network structure in the feather protein. Then it was hydrolyzed with 30% NaOH solution for 24 h, filtered, and treated with 1*N* HCl to precipitate the dissolved protein to yield FPI.

EDTAD modification of FPI

Ten grams of 3% (w/v) FPI was first adjusted to pH 12 by adding NaOH, then it was acylated by stepwise addition of 0.5 to 5 g solid EDTAD while stirring the solution at 35°C for 3 h. At the end of the reaction, the pH of the protein solution was adjusted to 5 by adding HCl to precipitate the protein. After filtering, the suspension was centrifuged at 1500 rpm for 10 min; the acylated protein precipitate was obtained by filtering again. The final precipitate was re-dissolved in deionized water at pH 7.0 and the acylated protein solution was concentrated by adding PEG to solid content 15% (w/v) without additional purification. The acylate modification degree (AMD) of FPI was quantified by the 4-chloro-7-nitrobenzofurazan spectrophotometric method[13] to determine the cysteine content of unmodified and modified protein.

FPI Hydrogel preparation

Fifteen percent (w/v) dispersion of the modified FPI was prepared in water at pH10, stirring the solution for 20 min. To this 25% Glu solution, pre-adjusted to pH10, was added at different Glu/FPI molar ratios of 0.004, 0.008, and 0.012. The mixture was stirred for about 20 min and allowed to cure overnight at room temperature to form hydrogel. The cured hydrogel was dried in an oven at 50°C until constant mass. Hydrogels were also prepared with unmodified FPI in the same manner and used as controls. After complete drying, the gels were ground to particle size less than 1.0 mm and used for further experiments.

FTIR analysis

The dry FPI and FPI resin (EDTAD/FPI and Glu/FPI molar ratios of 0.12 and 0.008, respectively) were analyzed using an FTIR spectrophotometer (Perkin Elmer Spectrum 100, Waltham, MA). The products were dried overnight under 50°C in oven until constant weight. The dried samples were ground into fine powder, mixed with dried KBr powder and compressed into disks. The scanning wave numbers ranged from 4000 to 1000 cm^{-1}.

Scanning electron microscope (SEM) observation

The surface morphology of dry FPI and FPI resin (EDTAD/FPI and Glu/FPI molar ratios of 0.12 and 0.008, respectively) samples were determined using a scanning electron microscope (SEM) (Jeol GSM6100, Japan). The dry sample was ground into powder, mounted on a metal stub, and coated with gold. The surfaces of samples were observed and photographed by SEM.

Glass transition temperature

Glass transition temperature (T_g) of the dried hydrogel resins was determined by a DSC (TA Instruments, Inc. DSC 2920, New Castle, DE) in nitrogen atmosphere. About 10 mg of the dried resin sample was placed in an aluminum pan with a pinhole. The sample chamber was purged with nitrogen to obtain uniform and stable thermal environment. The samples were scanned twice. The first heating scan, which was conducted to eliminate the residual water and solvent, was carried out at 20°C/min from room temperature up to 120°C followed by cooling to 25°C at a rate of 20°C/min. The second heating scan was performed at 10°C/min from 25 to 150°C followed by cooling to 25°C at a rate of 10°C/min. At the end of each heating scan, the sample was held at the final temperature for 5 min.

Hydrogel swelling ratio

The swelling ratio (SR) of the hydrogel samples was measured at 25°C by tea-bag method.[14] The tea bags, made of 300-mesh nylon net, were 40 cm in diameter. The dry hydrogel ($\sim$ 0.5 g) and nylon bag were weighed and recorded as W_o and W_n, respectively. The hydrogel samples were placed in tea bags and fully submerged in immersing solutions. The tea bags were removed after 24 h and their surfaces were carefully wiped off with soft tissue paper to remove the excess surface water. The total mass of the tea bag together with the swollen hydrogel was measured and recorded as W_t. SR was calculated as:

$$\text{SR (g/g)} = \frac{W_t - W_o - W_n}{W_o} \quad (1)$$

Equilibrium SR of modified FPI hydrogels was also determined as a function of ionic strength and pH. Salt (NaCl) solutions of concentrations ranging from 0.01 to 0.25 mol/kg were prepared to investigate the effect of ionic strength of the swelling medium on hydrogel SR. Different buffer solutions at 25°C were used, holding ionic strength constant at 0.05 mol/ kg, to determine the pH-sensitivity. The following buffers were used: pH 4, potassium biphthalate buffer; pH 7, potassium phosphate monobasic-sodium hydroxide buffer; and pH 10, potassium carbonate-potassium hydroxide buffer.

Kinetic experiments

Buffer solutions of pH 4.0, 7.0, and 10.0 were selected as swelling media to investigate the diffusion mechanism of FPI hydrogels. The mass of hydrogels was recorded during the course of swelling periodically at about 10 to 20 min intervals until equilibrium as described earlier. The kinetics of hydrogel diffusion was analyzed using the following semi-empirical equation for the condition $\frac{M_t}{M_\infty} < 0.6$[15]:

$$\frac{M_t}{M_\infty} = Kt^n \quad (2)$$

Where, M_t and M_∞ are mass of water absorbed at time t and when $t \to \infty$, respectively; K is a characteristic constant of the gel microsphere; and n is a characteristic exponent related to the mode of penetrant transport.

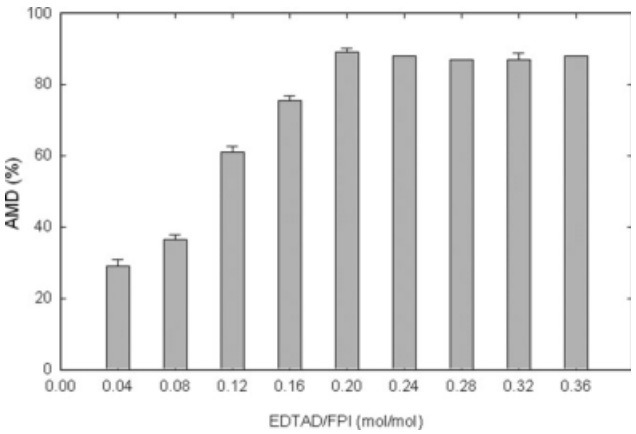

Figure 2 Acylate modification degree (AMD) of FPI as a function of $m_{\text{EDTAD}}/m_{\text{FPI}}$ ratio.

RESULTS AND DISCUSSION

Modification of cysteine residue

As shown in Figure 2, AMD increased with increasing EDTAD/FPI molar ratio. That is, as more ammonia groups are modified, more carboxyl groups are introduced into the protein structure. AMD reached a maximum value of 86% when EDTAD/FPI molar ratio was 0.20 and remained fairly stable thereafter when the amount of EDTAD was further increased. On the basis of the reaction condition, EDTAD has introduced about two or three carboxylic groups for each cysteine residue as depicted in Figure 3[16]; this reaction yields a protein hydrogel having higher SR and greater overall anionic charge than the hydrogel made from unmodified FPI. As ammonia groups are also required for crosslinking of the modified protein with Glu, the maximum AMD was limited to 60%, which was obtained at EDTAD/FPI molar ratio of 0.12.

FTIR analysis

Figure 4 shows the infrared FTIR spectra of the FPI and FPI resin. The carbonyl group exhibits a peak at

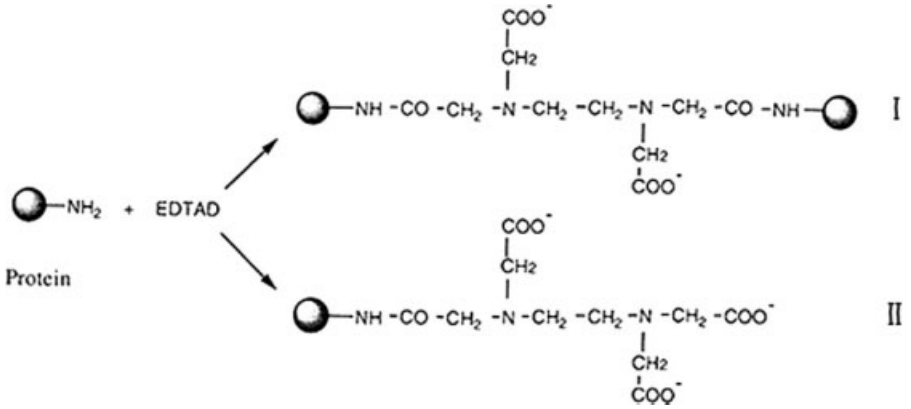

Figure 3 Reaction routes of protein with EDTAD.

1627.67 cm^{-1} for FPI but a peak at 1631.96 cm^{-1} for FPI resin. This negative shift suggests intermolecular bond formation between carbonyl groups. The stretching vibration of $-COO^{-1}$ shifted from 1514.78 cm^{-1} in FPI to 1519.86 cm^{-1} in FPI resin, which could be correlated with increased aggregation and stiffening of the links between the proteins. This also means the formation of intermolecular hydrogen bond during FPI hydrogel formation.

SEM studies

Figure 5(a,b) shows scanning electron micrographs of FPI powder and FPI resin, respectively. The presence of a macrospores structure in FPI rein is obvious [Fig. 5(b)]. This porous structure allows water to enter the dried resin matrix rapidly by capillary action; consequently, the SR of modified FPI resin is very high. On the other hand, the structure of FPI [Fig. 5(a)] is rough with very few small pores, which explains the much lower SR of FPI.

Glass transition temperature

Modified FPI gel is a crosslinked polymer. Therefore, the dried gel should show obvious glass transition temperature (T_g). The formation of hydrogen bond, which would retard the movement of polymer segment, will tends to increase T_g.[17,18] The thermograms of different modified FPI hydrogels at pH 7.0 are displayed in Figure 6. The T_g values for FPI were 66.7, 67.5, 69.8, 73.4, 74.1, and 75.6°C, respectively corresponding to EDTAD/FPI molar ratios of 000, 0.08, 0.12, 0.16, 0.20, and 0.24; the Glu/FPI molar ratio was kept at 0.008. These T_g values indicate that more carboxyls and amidos are incorporated into FPI and more hydrogen bonds are formed in FPI. At the same time, EDTAD is incorporated into FPI and the side chains of EDTAD occupy the

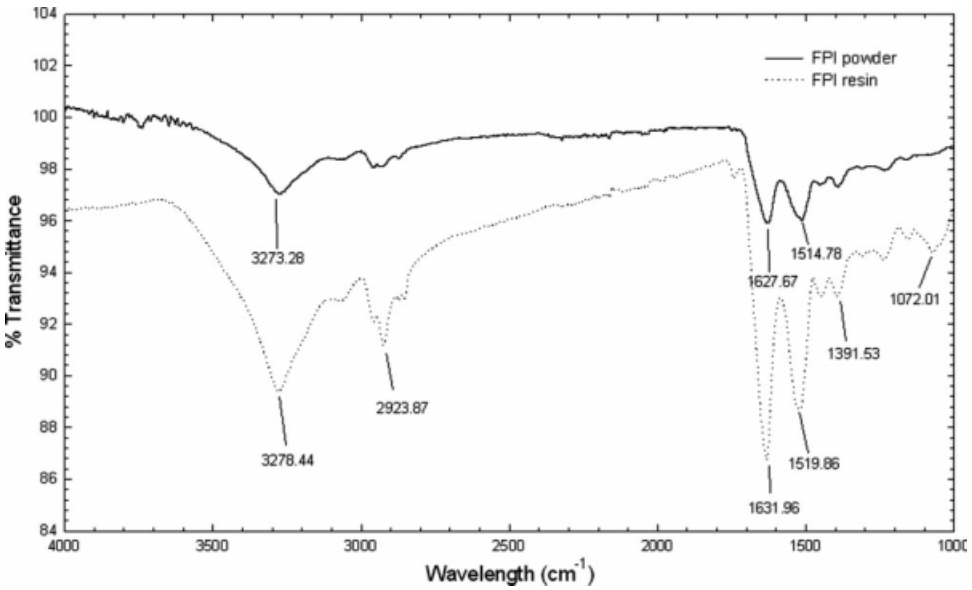

Figure 4 DSC thermograms of FPI hydrogels modified using EDTAD at different m_{EDTAD}/m_{FPI} ratios.

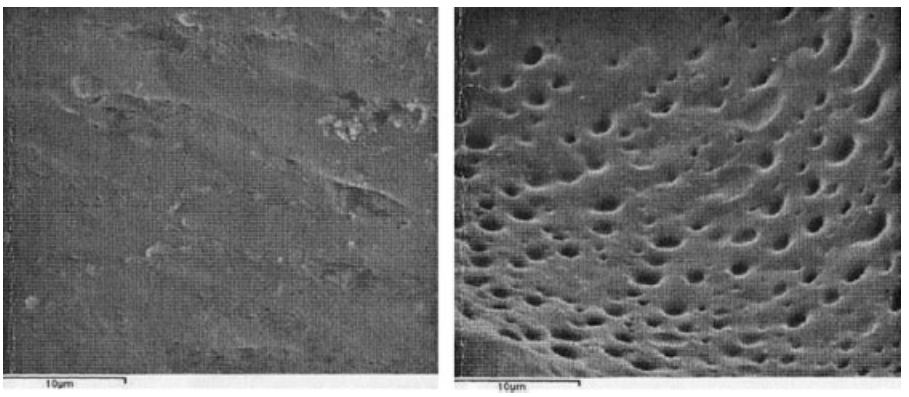

Figure 5 Swelling ratio (SR) of modified FPI hydrogels as a function of m_{EDTAD}/m_{FPI} and m_{Glu}/m_{PFI} ratios at 25°C.

space around the protein molecules, which are difficult to rotate or twirl when temperature is increased. As the amount EDTAD introduced is increased, it occupies more space and exerts increased resistance to protein molecules to move about. The result also supports the fact that EDTAD has interacted with FPI (the EDTAD molecules may behave as steric spacers between the protein molecules), and the increased net negative charge enhances protein–protein electrostatic repulsion.

Effect of EDTAD/FPI on swelling ratio

Figure 7 shows SR of FPI hydrogels in deionized water. SR of the unmodified FPI hydrogel was around 5 to 7 g/g, whereas SR of the modified FPI hydrogels range from 27 to 63 g/g depending on the amount of EDTAD used for modification. Clearly, introduction of carboxyl groups at cysteine residue in the protein enabled the protein network to imbibe large amount of water. It is quite likely that, in addition to increasing the total negative charges of FPI or because of it, the added EDTAD carboxyl groups may cause extensive unfolding of the protein and expose additional polar groups for binding water.[19]

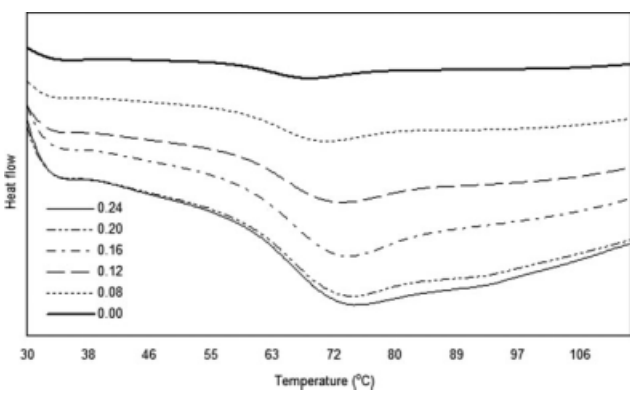

Figure 6 Swelling ratio (SR) of modified FPI hydrogels as a function of ionic strength of the swelling medium at m_{EDTAD}/m_{FPI} and m_{Glu}/m_{PFI} being 0.3 and 0.02, respectively.

Chemical modification with EDTAD causes extensive conformational changes in protein. As proteins with loose structures have better water binding capacity, the dependence of water uptake properties on the extent of modification might be related to the degree of randomization of the protein structure. The randomization of the protein structure is due primarily to intramolecular electrostatic repulsion and also hydration repulsion. We observed a decrease in SR when EDTAD/FPI molar ratio was over 0.12. This behavior may be due to the large number of water-binding sites of EDTAD and macroporous structure of the EDTAD-FPI complexation network. Higher amount of EDTAD may have produced additional crosslink points in the protein network resulting in a tighter structure and hence the lower SR.

Glutaraldehdye is an excellent crosslinking agent to form protein hydrogel. It crosslinks protein by reacting with amino groups, which results in a

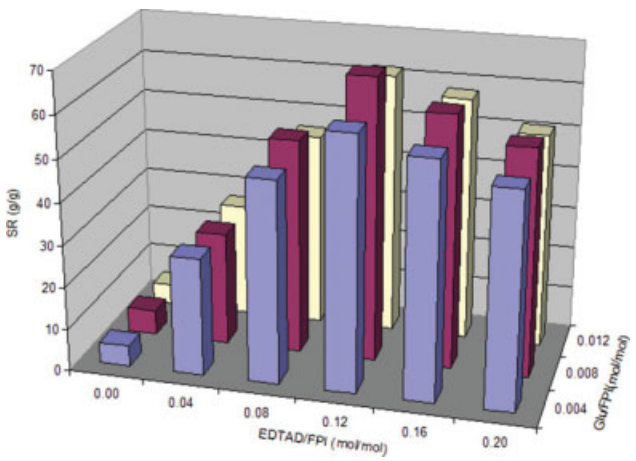

Figure 7 Swelling ratio (SR) as a function of time of modified FPI hydrogels (m_{EDTAD}/m_{FPI} = 0.1, 0.2, 0.3) swollen in different pH buffer solutions ionic strength adjusted to 0.05 mol/kg (4.0(●), 7.0(■), 10.0(▲), respectively and m_{Glu}/m_{PFI} = 0.02). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

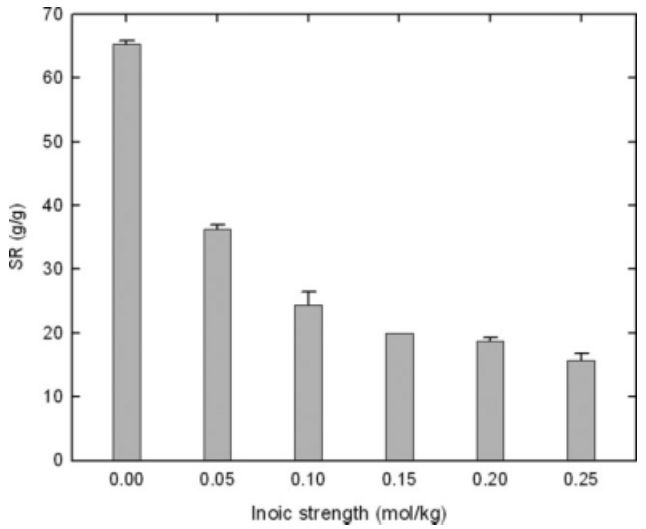

Figure 8 Swelling kinetics of FPI hydrogels in different pH buffer solutions with ionic strength adjusted to 0.05 mol/kg at $m_{EDTAD}/m_{FPI} = 0.3$ and $m_{Glu}/m_{PFI} = 0.02$: pH 4.0(●,y_1, x_1), pH 7.0 (■ y_2, x_2) and pH 10.0(▲, y_3, x_3).

decreased number of amino groups and an increased number of hydrophobic groups in the protein. However, higher Glu concentration could induce more conformation and structure changes that might not be favorable for SR. The mechanism and chemistry involved in Glu crosslinking reaction is not fully understood.[20] Our results show that the maximum SR was 63 g/g at EDTAD/FPI and Glu/FPI molar ratios of 0.12 and 0.008, respectively.

Effect of ionic strength on swelling ratio

The hydrogels swelled appreciably less in saline solutions than in deionized water. This well-known phenomenon, commonly observed in the swelling of ionizable hydrogels,[21] is often attributed to the charge-screening effect of added cations. As shown in Figure 8, when salt concentration (C_{NaCl}) was 0.00, 0.01, 0.05, 0.10, 0.15, 0.20, and 0.25 mol/kg, the average SR values were 65.1, 50.1, 36.1, 23.2, 20.6, 17.1, and 16.1 g/g, respectively. The increase in the net anionic charge of the acylated protein, which aids in unfolding of the protein structure, and the osmosis of the gel network is higher than that of the surrounding medium. Water automatically diffuses into the hydrogel network to balance the osmosis; this should enhance water uptake of the gel. When NaCl was added to a polyelectrolyte solution, some of the mobile electrolytes diffuse into the polyelectrolyte network. The electrostatic screening of carboxyl groups by sodium ions causes a marked reduction in the repulsive potential between charges in the network, which leads to network contraction and decreases porosity of the gel network.

Effect of ph on swelling ratio

SR of the modified FPI hydrogels evaluated at pH 4.0, 7.0, and 10.0 (ionic strength 0.05 mol/kg) are shown in Figure 9. It is evident that SR increased with increase in the surrounding pH. FPI is a kind of polyelectrolyte based on natural amino acid, and there are many carboxylic and carboxamide groups

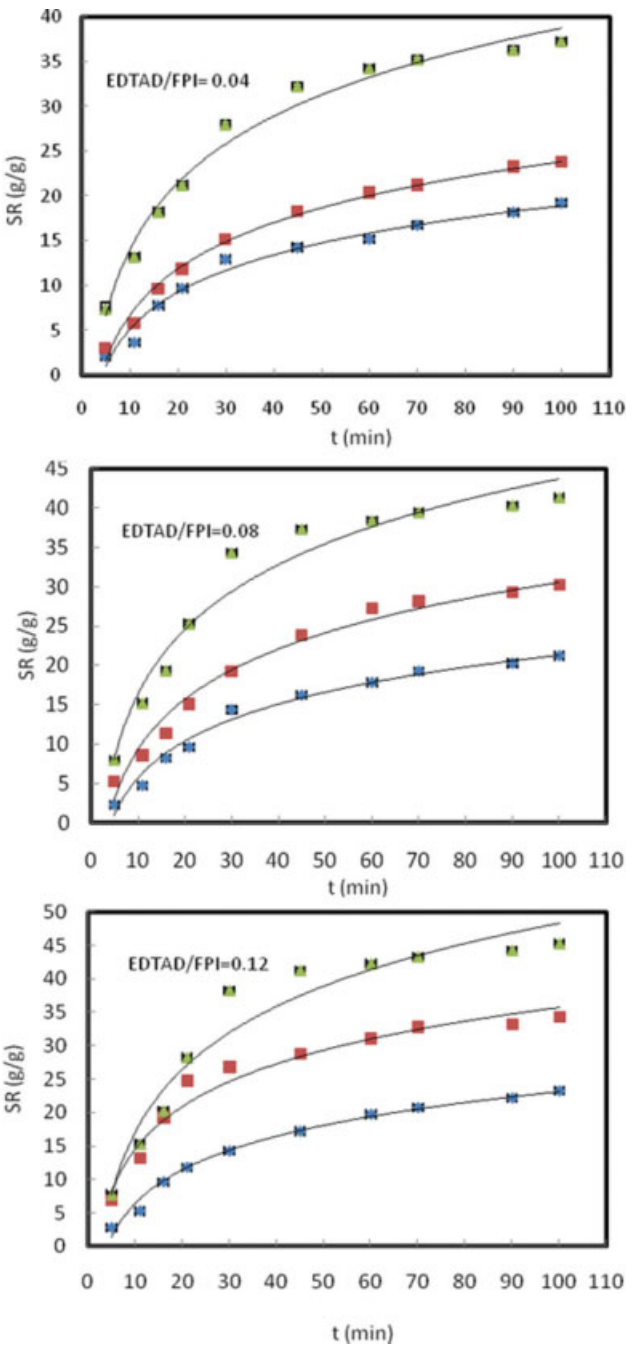

Figure 9 Swelling ratio (SR) as a function of time of modified FPI hydrogels swollen in different pH buffer solutions [4.0 (◆), 7.0 (■), 10.0 (▲)]; ionic strength adjusted to 0.05 mol/kg and Glu/FPI = 0.02. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in its molecular chain whose protonation degree is closely related to the pH value of the medium. This is the reason for pH-dependence of FIP hydrogels.

In all cases, SR is larger at higher pH values and this fact is easy to interpret. When pH is increased, most of hydroxyl groups are in the form of negative charge and this leads to a higher ionic strength of the aqueous medium. At higher ionic strength, the negatively charged ionic backbone of the protein is more expanded and therefore, at later stages, this expanded form makes the penetration of water molecules into the network structure easier. On the contrary, at low pH, the hydroxyl groups are mostly in protonated form, and show a less polar character and therefore, result in a polymer with lower water compatibility. The polymers formed at low pH are thus a less expanded.

At given pH, SR is strongly dependent on EDTAD content in the network: the higher the EDTAD content (EDTAD/FPI $\leq$ 0.12), the higher the SR. It is believed that the acylating agent EDTAD, by reacting with cysteine residues of the protein, leads to extensive unfolding of protein molecules via intramolecular electrostatic repulsion caused by the carboxylic acid substituent on the acylating agent. This converts the rigid, globular feather protein into a random-coil type, polyanionic polymer. The substantial polyanionic character, which the carboxylic acid moieties impart to feather protein, provides numerous sites for water binding.

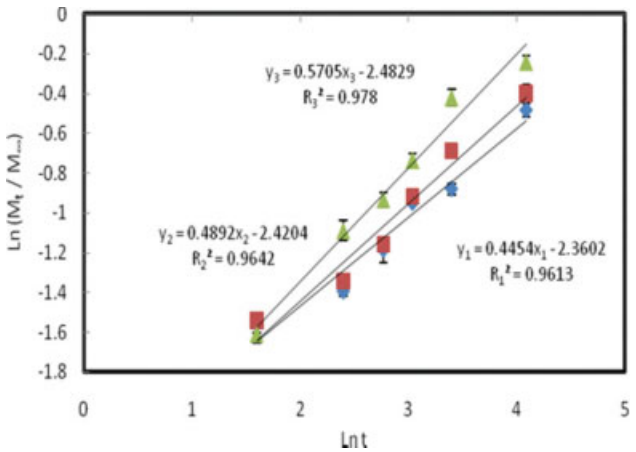

Figure 10 Swelling kinetics of FPI hydrogels in different pH buffer solutions [4.0 (◆y_1, x_1), 7.0 (■y_2, x_2), 10.0 (▲y_3, x_3)] with ionic strength adjusted to 0.05 mol/kg at EDTAD/FPI = 0.12 and Glu/FPI = 0.008. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Swelling kinetics of modified FPI hydrogels

The rate and extent of hydrogel swelling are governed by the rate of diffusion of water into the hydrogel and the rate and extent of relaxation of the polymer network in response to water diffusion. In general hydrogel swelling kinetics depends on the rates of diffusion of three species in the hydrogel structure.[22]

1. Water must enter or leave the gel.
2. Acid or base must diffuse into the gel to spark its swelling or collapse.
3. Solutes which are not excluded from the gel will diffuse into the gel.

Therefore, the swelling kinetics is complicated because usually all three of these mass transfer processes occur simultaneously. Data in Figure 9 show that the rate of water uptake by the dry hydrogel is rapid during the first 30 min, and decreases slowly thereafter. The initial rapid phase might be related to diffusion of water into the hydration of the charged groups in the polymer network. During this phase, in addition to hydrating the ionic groups, water may tend to disrupt polar protein–protein interactions in the hydrogel network. This should enhance the relaxation rate of the polymer network. The decrease in the hydrogel swelling rate after the first hour indicates that although protein was denatured, its rate of structural relaxation in the gel network is not comparable to that of a truly random-coil polymer.[23] It has been shown that even after exposure of modified FPI gel to the above denaturing conditions, the protein regained a significant amount of alpha helix and beta sheet structures when the conditions were reversed back to normal temperature.[24] These folded secondary structures in the crosslinked protein network might oppose relaxation of the gel network as water diffuses into the network.

According to literature[25] n values from eq. (2) are given as follows: $n < 0.45$ for Fickian diffusion (Case I), $0.45 < n < 0.89$ for non-Fickian diffusion (or anomalous), $n = 0.89$ for zero order (Case II), and $n > 0.89$ for super Case II type of penetrating mechanism. The anomalous case is considered as the summation of Fickian diffusion (Case III) and non-Fickian diffusion (Case II). Figure 10 shows the ln (M_t/ M_∞) versus ln (t) curve for pH value of 4.0, 7.0 and 10.0. The n value of the gel was <0.45 at pH 4.0 and >0.45 at pH 7.0 and 10.0, which indicates that the water absorption mechanism of the modified FPI hydrogel is governed by Fickian diffusion at pH 4.0 and gradually anomalous transport when pH is raised to neutral and alkaline values. Increase in pH of the surrounding liquid from 4.0 to 10.0 leads an increase in the n values. At higher pH values, SR becomes more dependent on the polymer relaxation. This effect is attributed to the increased ionization of —COOH groups of EDTAD segment of the hydrogel at high pH. An increase in the number of fixed

ionized groups within the hydrogel structure gives rise to major polymer relaxation.

CONCLUSIONS

pH-sensitive and salt-sensitive hydrogels were prepared using EDTAD-modified FPI via glutaraldehyde crosslinking. An optimal swelling ratio (SR) of 63 g/g was obtained in deionized water. SR decreased steadily and appreciably with increasing ionic strength of the surrounding medium. At a given ionic strength, SR increased with solution pH in the range of 4.0 to 10.0. The water diffusion mechanism of the modified FPI hydrogel was also pH dependent. Therefore, it is possible to control both the degree of swelling and the swelling kinetics of FPI hydrogels by altering the surrounding environmental conditions.

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