In Vitro Degradation and Protein Release of Semi-IPN Hydrogels Consisted of Poly(acrylic acid-acrylamide-methacrylate) and Amylose

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ABSTRACT: The enzymatic degradation mechanism of semi-interpenetrating network (semi-IPN) hydrogel of poly (acrylic acid-acrylamide-methacrylate) crosslinked by azo-compound and amylose *in vitro* was investigated in the presence of Fungamyl 800L (α-amylase) and rat cecum content (cecum bacteria). The degradation mechanism involves degradable competition, i.e., reduction of azo crosslinkage is dominant in the earlier period of degradation. Subsequently, the degradation of gels is continued by combination of reduction of azo crosslinkage and hydrolysis of amylose. The cumulative release ratios of Bovine serum albumin (BSA, as a model drug) loaded semi-IPN gels are 25% in pH 2.2 buffer solutions and 74% in pH 7.4 buffer solutions within 48 h. Moreover, the release behavior of BSA from the semi-IPN gels indicates that it follows Fickian diffusion mechanism in pH 2.2 media and non-Fickian diffusion and polymer chains relaxation mechanism in pH 7.4 media. The results indicate that the release of BSA from the semi-IPN gels was controlled via a combined mechanism of pH dependent swelling and specificity to enzymatic degradation. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 3432–3438, 2007

Key words: semi-IPN hydrogel; *in vitro* degradation; protein release

INTRODUCTION

In recent years, the delivery of drug to the colon has received more and more attention because the colon is an effective site for treating special diseases such as ulcerative colitis, Crohn's diseases, inflammatory bowel diseases, infectious diseases, and colon cancer.[1] In addition, colonic drug delivery is also useful for systemic absorption of protein and peptide drugs because of the low activity of proteolytic enzymes in the colon.[2]

The systems developed for colon-specific drug delivery mainly include time-dependent release, pH-dependent and enzymatically-controlled delivery systems. However, the time-dependent release systems are unable to sense any variation in the upper gastrointestinal tract (GIT) transit time, the pH difference between the small intestine and the large intestine not being very pronounced.[3,4] To further promote selective degradation in the colonic environment, many hydrogels were designed containing both pH-sensitive acidic monomers and enzymatically degradable azocompound crosslinkers.[5–9] Bajpai and Saxena reported a semi-IPN hydrogel of poly(acrylic acid) and starch.[10] In our previous study, we synthesized semi-IPN hydrogels consisted of poly(acrylic acid-acrylamide-alkyl methacrylate) crosslinked by azocompound and amylose and discussed their swelling behavior.[11] This type of semi-IPN hydrogel swells hard at pH 2.2 (the pH value in the stomach) and swells easily at pH 7.4 (the pH value in the colon) because of the ionization of carboxyl groups and expand of amylose chains. Amylose is a natural and nontoxic polysaccharide, which can be enzymatically degraded[10,12] together with the azo crosslinker by colonic enzymes.[5–9] Moreover, the presence of amylose is expected to enhance the mechanical strength of the hydrogels and the colonic specificity of the hydrogels.

The present work evaluates the enzymatic degradability of the semi-IPN hydrogels by Fungamyl 800L and rat cecum content. The release behavior of bovine serum albumin (BSA) from the gels was investigated in phosphate buffer solution of pH 2.2 or 7.4 (with or without rat cecum content) at 37°C.

EXPERIMENTAL

Materials

Acrylic acid (AA), methyl methacrylate (MMA), ethyl methacrylate (EMA), and butyl methacrylate

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TABLE I
The Feed Composition of Semi-IPN hydrogels

Sample (nx − y)	AA (mol %)	AM (mol %)	n-alkyl MA (mol %)	BMAAB (mol %)	AIBN (mg)	DMSO (g)	amylose (mL)[a]
n1 − 1	49.9	40	10 (methyl)	0.1	30	7	1
n2 − 1	49.9	40	10 (ethyl)	0.1	30	7	1
n4 − 0	49.9	40	10 (n-butyl)	0.1	30	7	0
n4 − 1	49.9	40	10 (n-butyl)	0.1	30	7	1
n4 − 2	49.9	40	10 (n-butyl)	0.1	30	7	2
n4 − 3	49.9	40	10 (n-butyl)	0.1	30	7	3

[a] The aqueous amylose solutions (1.1 wt %).

(BMA) were purchased from Tianjin Chemical Group, China, and were distilled before use. Amylose, acrylamide (AM), 2,2′-azobis(isobutyronitrie) (AIBN), dimethyl sulfoxide (DMSO) (purchased from Shanghai Chemical Group, China) were of analytical reagent grade and used without further purification. Fungamyl 800L (a-amylase, liquid, 800 FAU/mL) were kindly provided by Novozyme, China. The freeze-dried caecum content suspension for degradation of hydrogels was prepared as follows: the caecum contents of male Sprague-Dawley rats (about 250 g) were isolated and suspended in distilled water (1 g in 10 mL) prebubbled with nitrogen. The suspension was filtered through glass wool and freeze dried. The freeze-dried caecum was kept at −20°C before use. Bovine serum albumin (BSA) was purchased from Roche, Switzerland. 4, 4′-bis(methacryloylamino)azobenzene (BMAAB) was prepared as described previously.[13] yield: 30%, m.p: 268–269°C, ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.10 (s, 6H, —CH_3), δ 5.52, 5.83 (d, 4H,—CH_3C=CH_2), δ 7.64 (s, 2H, —NH—), δ 7.72–7.75, 7.81– 7.84 (m, 8H, Ar—H).

Preparation of semi-IPN hydrogels

The hydrogels were prepared through the free-radical copolymerization of AA, AM, n-alkyl MA (MMA, EMA, BMA) in the aqueous amylose solutions (1.1 wt %) as described previously.[14] Briefly, various amounts of aqueous amylose solutions and AIBN were added in the mixture solutions of total monomers. The total amount of monomer and aqueous amylose solution are 3 g and 1–3 mL, respectively. The solution was bubbled with nitrogen to discharge oxygen for about 30 min. The free radical copolymerization was carried out at 80°C for 48 h. The polymer gel slab was cut into circular disks using punches. The samples were immersed in absolute ethanol for 3 days, then in 60% ethanol/deionized water for 6 days and gradually transferred into deionized water to remove the unreacted monomers and solvent. Finally, the samples were dried in vacuum at 50°C to a constant weight and stored for further use. The feed composition for the preparation of hydrogels is listed in Table I. The samples are designated as nx − y, where x is the carbon number of the n-alkyl group and y denotes the amount of amylose (mL).

In vitro degradation of semi-IPN hydrogels by Fungamyl 800L (α-amylase) and rat cecum content (cecum bacteria)

Enzymatic degradation of the semi-IPN hydrogels was performed according to a modified method described elsewhere.[15] Briefly, dried hydrogels of known weight (W_{d1}) were allowed to equilibrium swell in deionized water at 37°C and the weight of the swollen hydrogels (W_{s1}) was recorded after blotting surface water with tissue paper. The swollen hydrogels were subsequently further equilibrated at the same temperature in a vial containing buffer solution of pH 7.4 and fungamyl 800L. After bubbling with nitrogen for 5 min to obtain anaerobic solution, the vials were closed and tightly sealed. The vials were incubated in a thermostatic shaker with speed of 50 rpm for 24, 48, 96, and 120 h. The gels were washed with deionized water to inactivate and remove the enzyme and allowed to equilibrium swell again (W_{s2}) in deionized water at 37°C. The hydrogels were dried in vacuum at 50°C to a constant weight (W_{d2}). The equilibrium swelling ratio (SR) was calculated before and after incubation by the following equation:

$$\mathrm{SR} = \frac{W_s}{W_d} \tag{1}$$

Degradation of hydrogels in the presence of rat cecum content was performed according to the similar above method. Dried hydrogels were swollen in deionized water at 37°C, then immersed in a cell suspension mixture consisting of 30 mg freeze dried rat cecum content, 1×10^{-3} mol/L neutral red, 20 wt % α-D-glucose, and 20 mL buffer solution of pH 7.4. After bubbling with nitrogen for 5 min to obtain anaerobic solution, the vials were closed and tightly sealed. The latter procedure is the same as

TABLE II
Standard Equations of BSA Curves in Different pH Buffer Solutions

pH	Standard equations of curves	Correlative coefficient (R^2)
2.2	$y = 0.608x + 0.041$	0.999
5	$y = 0.6317x + 0.0124$	0.998
7.4	$y = 0.972x - 0.223$	0.999

the degradation of hydrogels in the presence of amylose.

Morphology of the fractured hydrogels before or after degradation

The morphology of hydrogels before or after degradation was examined using a field emission scanning electron microscopy (Sirion 200, FEI). The equilibrium swollen gel was frozen at −80°C for 12 h in refrigerator and then freeze-dried. The fractured specimens were covered with gold vapor. The acceleration voltage was 10 kV.

In vitro release of bovine serum albumin from the gels

Accurately weighted BSA was separately dissolved in 250 mL buffer solutions of pH 2.2, 5.0, and 7.4. The absorbance (A) of a series of BSA concentration (C) at 280 nm was determined with UV–Vis spectrophotometer (UV-2102 PC, UNICO). The standard equations of absorbance and concentration of BSA in different buffer solutions are listed in Table II.

The dried gel samples (about 25 mg) were immersed in the buffer solutions containing BSA at room temperature and allowed to equilibrium swell. The gel samples were wiped by tissue paper to remove surface water and dried in vacuum at 50°C to a constant weight.

In vitro release of BSA from hydrogels was carried out by a modified method described elsewhere.[15] Briefly, dried BSA-loaded hydrogels were immersed at 37°C in vials containing 20 mL of phosphate buffer solution of pH 7.4 with or without rat cecum content. After bubbling with nitrogen for 5 min to obtain anaerobic solutions, the closed vials were incubated in a thermostatic shaker with speed of 50 rpm. At designed interval of time, the solution of 1 mL was pipetted out and replaced with equal volume of the same dissolution medium. The solution was centrifuged and the released BSA was analyzed at 280 nm with a UV spectrophotometer. Then the weight of the released BSA was calculated by the standard equations.

RESULTS AND DISCUSSION

In vitro degradation of hydrogels

In vitro degradation of the semi-IPN hydrogels was performed by incubation in the presence of fungamyl 800L or rat cecum content. It can be characterized through determination of the equilibrium swelling ratio (SR) of hydrogels before and after degradation.[16] Figures 1 and 2 show the increments of SR of the gels as a function of incubation time within 5 days. Apparently, all SR of the gels increased after degradation. It could be attributed to an enzyme-induced reaction of degradation resulting in the decrease of polymer network density. Figure 1 (left plot) shows the effect of the side chain length of *n*-alkyl MA on the degradation. The increments of SR for the gel n2 − 1 are much more than that of the gel n1 − 1. This mainly resulted from the easier relaxation of polymer chains for n2 − 1 comparing with n1 − 1. However, the increments of SR for the gel n4 − 1 are less than other gels. This could be owing to the faster swelling and partial erosion of gel n4 − 1. Figure 1 (right plot) shows the effect of the content of amylose on the degradation of gels within 5 days. The increments of their SR depend on the content of amylose. The higher content of

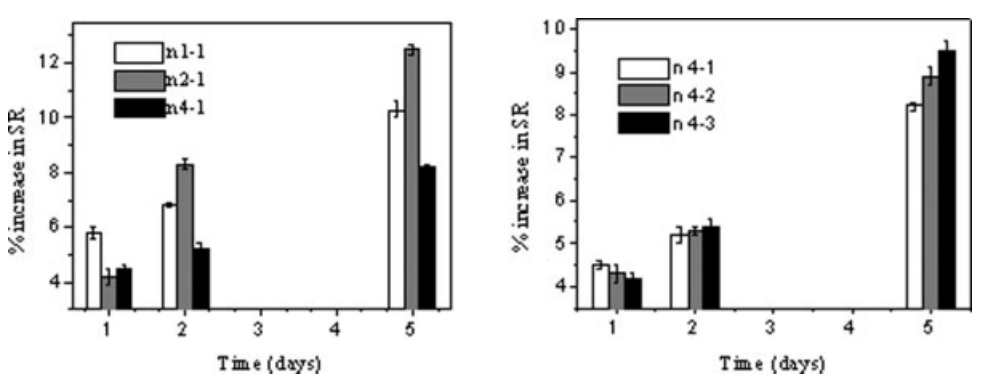

Figure 1 Percentage increments of equilibrium swelling ratio (SR) of the gels as a function of incubation time in the presence of fungamyl 800L. (20 mg dried gel, 1mL fungamyl 800L AU/mL, 20 mL buffer solution of pH 7.4).

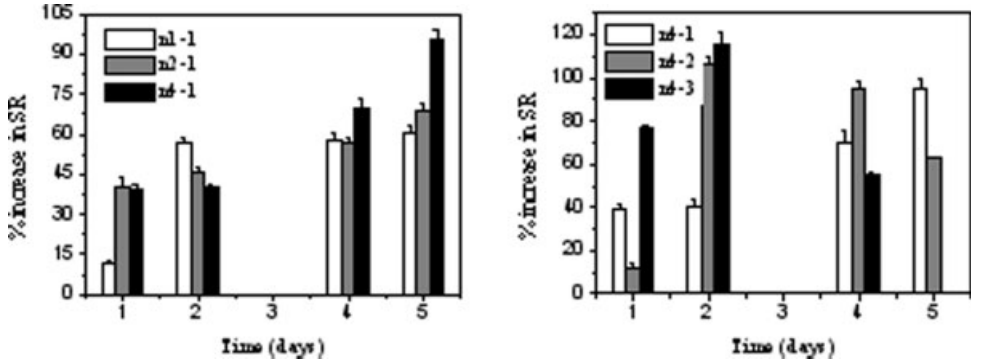

Figure 2 Percentage increments of equilibrium swelling ratio (SR) of hydrogels as a function of incubation time in the presence of rat cecum content. (20 mg dried gel, 30 mg freeze dried rat cecum content, 0.5 mL 1 × 10^{-3} mol/L neutral red, 0.5 mL 20 wt % α-D-glucose, 20 mL buffer solution of pH 7.4).

amylose in the gels, the more completely degradation of the gels.

In comparison with Figure 1, the increments of SR for the gels are much higher in the presence of rat cecum content as shown in Figure 2. This is mainly attributed to that both azo crosslinkage and amylose could be degraded by cecum bacterial. The dual degradations for the gels greatly reduced gel network density which led to higher swelling. The effect of side chain length and content of amylose on the SR in the presence of cecum bacterial is similar to that in the presence of fungamyl 800 as shown in Figure 1. It should be noted that the increments of SR for the gel n4 − 3 deceased on the 4th day, which resulted from the degradation of larger quantity of amylose by cecum bacterial and causes partial mass loss of gels. To confirm that, degradation was also assessed by measuring the weight loss ratio, which was defined as the following equation:

$$\text{Weight loss ratio } (\%) = \frac{100(W_1 - W_2)}{W_1} \qquad (2)$$

where W_1 and W_2 are the weights of the gel before and after degradation, respectively.

As shown in Table III, the weight loss ratios of gels at designed intervals of time increase with the increase of amylose content. This is mainly due to the degradation of amylose by enzyme.

TABLE III
The Weight Loss Ratios of semi-IPN Hydrogels During the Degradation

	The weight loss ratios (%)			
Sample	Control[a]	1st day	2nd day	5th day
n4 − 1	0	9.2	17.2	28.1
n4 − 2	0	11.3	19.1	30.7
n4 − 3	0	15.4	24.5	35.6

[a] Degradation medium without rat cecum contents.

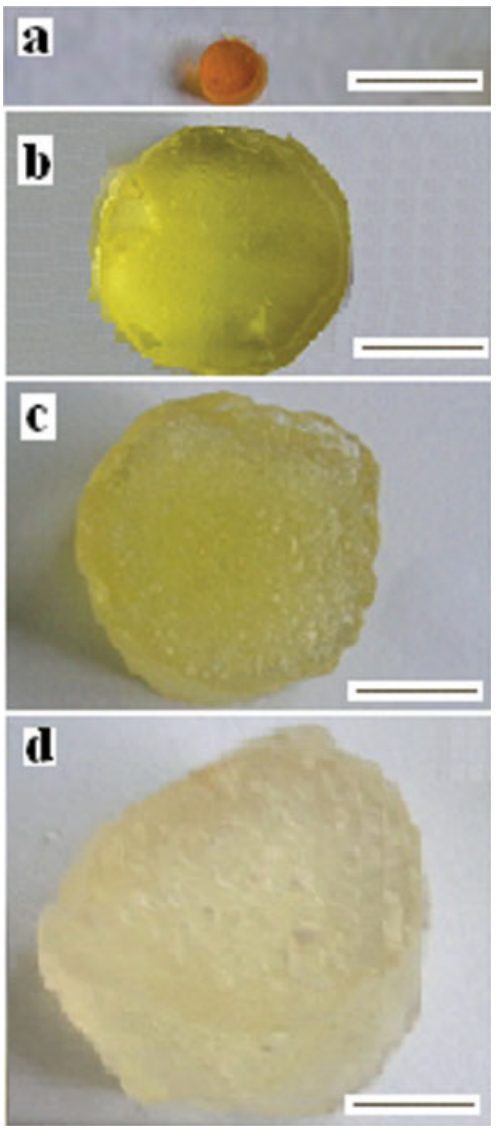

Figure 3 Photos of (a) dried n4 − 1gel. (b) equilibrium swollen n4 − 1gel. (c) degraded n4 − 1gel by fungamyl 800L on the day 5 and (d) degraded n4 − 1gel by rat cecum content on the day 5. The bar indicates 1 cm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

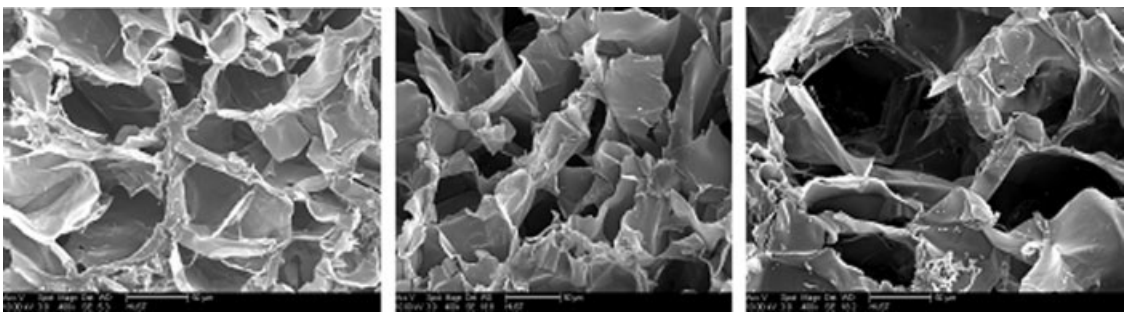

Figure 4 SEM images of the gel n4 − 1 (left: 400×) before degradation. Degraded gel by fungamyl on the day 3 (middle: 400×). Degraded gel by rat cecum content on the day 3 (right: 400×).

Figure 3 shows the changes of color and shape of gels during the degradation process by fungamyl 800L or rat cecum content. For the degraded hydrogels, rat cecum content makes gel colorless and porous on the 5th day. However fungmyl 800L makes gel keeping light yellow color on the 5th day. This phenomenon further confirms that both amylose and azo crosslinkage in the gel can be degraded by rat cecum content.

Figure 4 shows SEM images of cross section of the gel n4 − 1 before and after degradation by fungamyl or rat cecum content. Before degradation, the pore size is small and the pore wall is thicker (left image in Fig. 4) in comparison with that after degradation (middle and right images in Fig. 4). Furthermore, the degradation of gels by rat cecum content was more completed in comparison with that by fungamyl on 3rd day. In other words, these results indicate that the effect of rat cecum content on the degradation of the gel is outstanding in comparison with that of fungamyl. It implies a potential application of the gels as a colon-specific delivery carrier.

Based on the above results, a possible mechanism of degradation can be proposed. At the earlier period of degradation, relaxation of polymer chains in the outer surface of gel occurs due to the water diffusion resulting in the repulsion between the charged COO^- groups. A colorless edge and yellow core of the gel imply that the degradation only occurs in the outer surfaces of gel. With the undergoing of degradation, more cecum bacteria are allowed to permeate into the gel and cause the reduction of azo groups and hydrolysis of amylose. The hydrolyzed product of amylose, α-D-glucose[17] can promote the reduction of azo groups.[18] Finally, the gels become fragile and collapsed networks (right image in Fig. 4). Therefore, degradation mechanism involves degradable competition, that is, the reduction of azo groups is dominant in the earlier period of degradation. Subsequently, the degradation of gels is continued by both reduction of azo groups and hydrolysis of amylose.

Loading and release of BSA in the hydrogels

The left plot in Figure 5 depicts the loading capacity of gels in pH 5 buffer solution. The loading amount of BSA decreases with an increase of amylose content. Gel n4 − 1 exhibits larger loading capacity than the others. This mainly results from the fact that the increasing of amylose led to entanglements of amylose chains, which inhibits diffusion of BSA into the gel network.

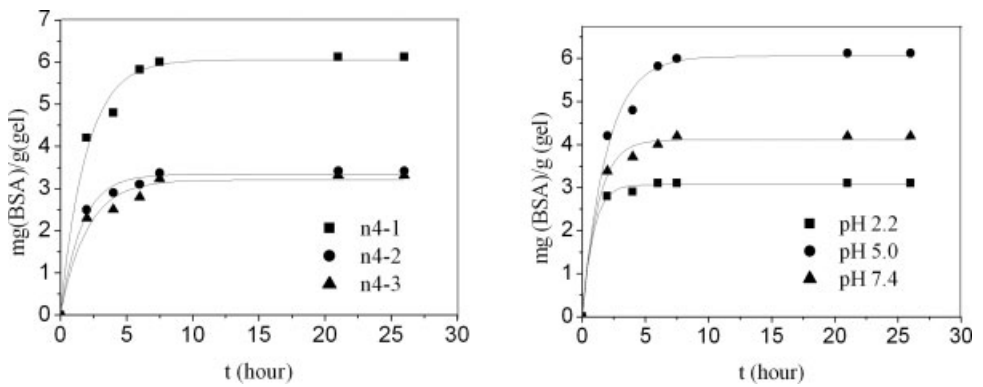

Figure 5 BSA adsorption kinetics for the gels in pH 5 buffer solutions (left plot) and BSA adsorption kinetics for the gel n4 − 1 in varied pH buffer solutions (right plot).

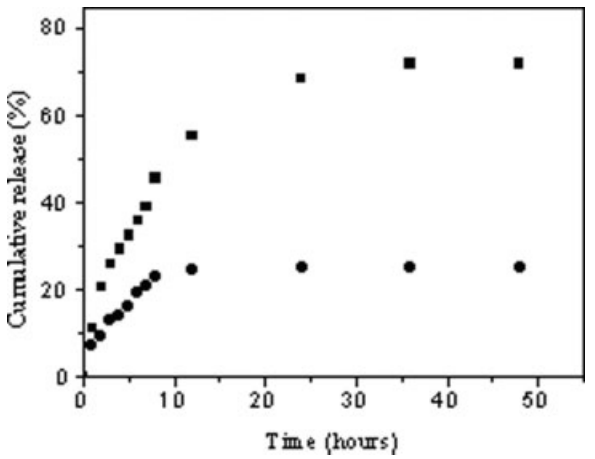

Figure 6 The release profiles of BSA from the gel n4 − 1 in pH 7.4 (■) and pH 2.2 (●) buffer solutions.

The right plot in Figure 5 shows the effect of pH of buffer solutions on the loading of BSA. In the pH 5 buffer solution, the loading amount of BSA in the gels was 6.12 mg per a gram of dried gel, while in the pH 2.2 and 7.4 buffer solutions, the loading amounts of BSA were 3.12 and 4.19 mg per a gram of dried gel, respectively. This difference could be explained by the interactions between hydrogel and the protein. As is well known, the isoelectric point (pI) of BSA is at the pH 4.7[19] close to pH 5. Therefore, almost no electrostatic interactions occur between polymer and BSA with zero charge in the pH 5 buffer solution. BSA molecules can easily diffuse into the gel network. However, positive charged BSA hard diffuse into the gel network in pH 2.2 buffer solution due to the electrostatic interactions between positive charged BSA and the negative charged COO^- groups in the gel.[20] A similar explanation can be used for that in the pH 7.4 buffer solution.

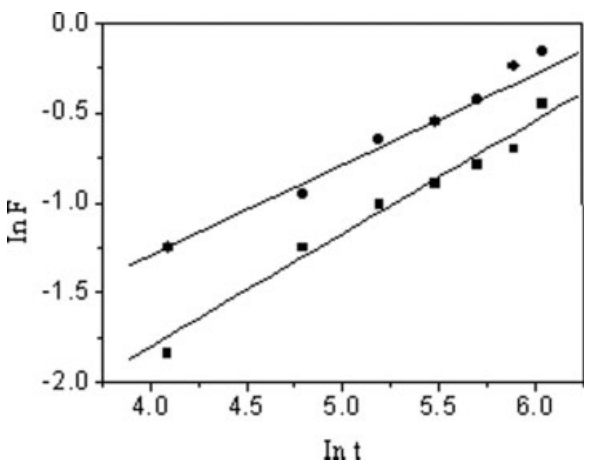

Figure 7 Plot of ln t versus ln F for the gel n4 − 1 in pH 7.4 (■) and pH 2.2 (●) buffer solutions.

As reported in our previous work, the semi-IPN hydrogels were in the deswelling (or collapsed) status in the pH 2.2 media and swelling status in pH 7.4 media.[11] It implies that the drug-loaded semi-IPN hydrogel can be protected from the gastric enzyme before its entry into the colon. Figure 6 shows the release kinetics of BSA-loaded gel n4 − 1 in pH 2.2 and 7.4 buffer solutions. Approximately 25% (1.53 mg) of the BSA (cumulative release ratio) were released in the pH 2.2 buffer solution within 48 h. However, ~74% (4.51 mg) of BSA were released in the pH 7.4 buffer solution within 48 h. This difference is apparently related to the gel status in different pH media as mentioned above.

The release mechanism of BSA from the gel matrices was simulated using an equation:[21]

$$F = \frac{M_t}{M_\infty} = kt^n \tag{3}$$

where M_t is the released amount of BSA at time t, M_∞ the total released amount of BSA, k the release rate constant, and n the exponent. The exponent n expresses the diffusion mechanism of BSA. For cylindrical hydrogels, when n is 0.5, it signifies a Fickian diffusion mechanism.[18] When n is 1, it signifies that polymer chains relaxation is predominant and controlled diffusion in the swelling process. When n is between 0.5 and 1.0, the diffusion mechanism is non-Fickian, where both the diffusion and polymer chains relaxation control the swelling process. Figure 7 shows a plot of ln t versus ln F for the gel n4 − 1. The values of the exponent n calculated according to above equation were found to be 0.51 and 0.63 in the pH 2.2 and pH 7.4 media, respectively. Apparently, the release behavior of the BSA-loaded gels

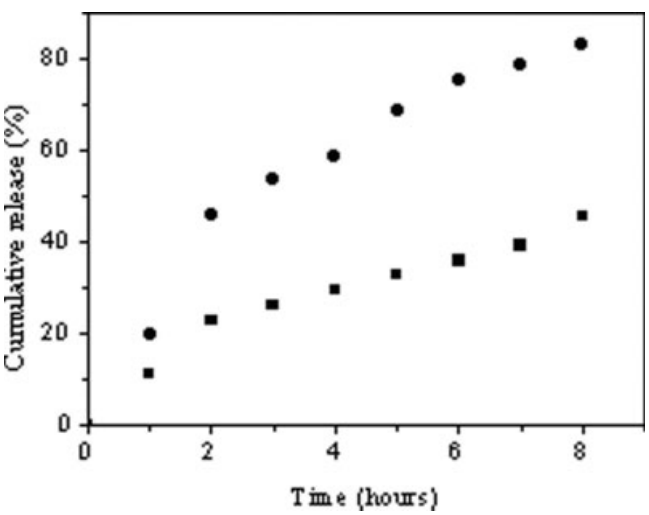

Figure 8 The release profiles of BSA for the gel n4 − 1 in the presence (●) and absence (■) of rat cecum contents in pH 7.4 buffer solutions.

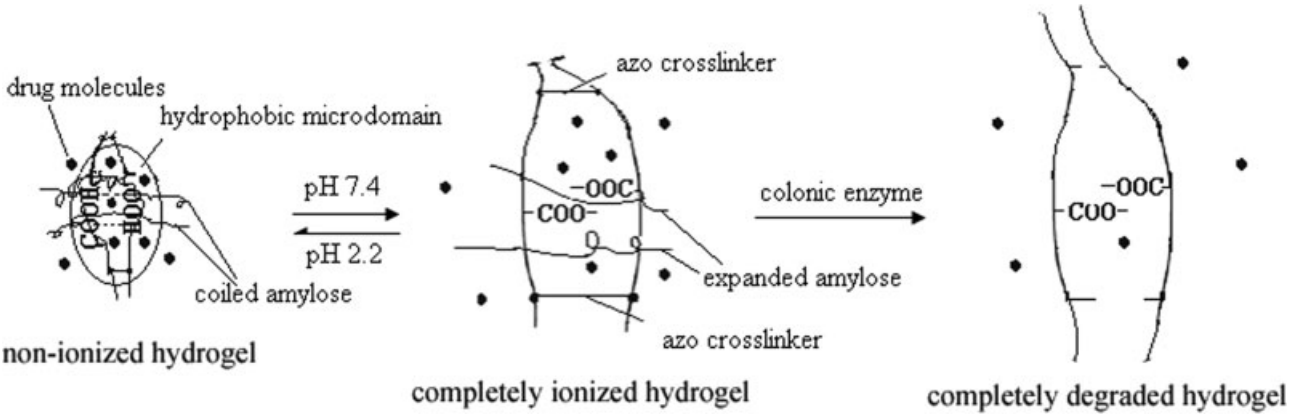

Figure 9 Schematic illustrations for the mechanism of BSA release and enzymatic degradation of semi-IPN hydrogel.

follows Fickian diffusion mechanism in pH 2.2 media. Oppositely, the n value of 0.63 indicates that the release behavior of the BSA-loaded gels follows non-Fickian diffusion and polymer chains relaxation controlled release mechanism in pH 7.4 media.[18]

Figure 8 shows release behavior of the BSA-loaded gel n4 − 1 in the presence and absence of rat cecum bacterial in the pH 7.4 buffer solutions. Approximately 46% (2.8 mg) of the BSA were released from the gel in the absence of rat cecum bacterial and 83% (5.1 mg) of the BSA were released in the presence of rat cecum bacterial within 8 h. Large release of BSA is attributed to the degradation of glucuronic linkage in amylose and azo crosslinkage in the semi-IPN gel network by rat cecum bacterial.

Therefore, a combined release mechanism of BSA-loaded hydrogel could be proposed, that is, both pH dependent swelling and specific enzymatic degradation. This release mechanism could be significant for a specific-colon drug delivery system. When a drug-loaded semi-IPN gel reaches the stomach with low pH values (ca. pH 2.2), drug molecules are protected from being digested by gastric and pancreatic enzymes because it is captured in the collapsed gel. Once gels arrive at the colon with high pH values (~7.4), cecum bacteria are allowed to permeate into the swollen gels. Both the azo crosslinkage and α-D-glucopypyranose bonds are degraded by cecum bacteria, which results in the release of entrapped drug. The mechanism of BSA release and enzymatic degradation is schematically represented in Figure 9.

CONCLUSIONS

The enzymatic degradability of the semi-IPN hydrogels containing amylose was investigated by using Fungamyl 800L (α-amylase) and rat cecum content (cecum bacteria). The results indicate that the swelling ratios of the semi-IPN gels increased after degradation resulting in the decrease of polymer network density. There is a degradable competition between the reduction of azo crosslinkages and hydrolysis of amylose in the earlier period of degradation. The morphology of the gels before and after degradation indicates that the degradation was more completed by rat cecum content comparing with that by fungamyl 800 L. The release behavior of BSA in the gels indicate that it follows Fickian diffusion controlled mechanism in pH 2.2 media and non-Fickian diffusion and polymer chains relaxation controlled mechanism in pH 7.4 media.

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