A Mechanistic Assessment of Enzyme-Induced Degradation of Albumin-Crosslinked Hydrogels

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ABSTRACT: Pepsin-induced degradation of albumin-crosslinked hydrogels was studied as a function of the degree of albumin incorporation in the network and the concentration of pepsin. The degree of albumin incorporation, which represents the sum of chemical crosslinks and physical entanglements in the network, was controlled by changing the concentration of initiator in the monomer solution and the degree of vinylic functionality on albumin. Swelling characterization studies showed that the degree of hydrogel swelling decreased as the concentration of chemical initiator for the polymerization increased or as the degree of vinylic functionality on albumin increased. This indicated that the degree of albumin incorporation in the network increased by raising either the concentration of chemical initiator or the degree of albumin functionality. The rate and mechanism of gel degradation was also dependent on the degree of albumin incorporation in the network. A low degree of albumin incorporation resulted in a predominance of surface degradation while a high degree of albumin incorporation resulted in a predominance of bulk degradation. The transition from surface degradation to bulk degradation occurred at lower concentrations of chemical initiator when the degree of vinylic functionality on albumin was high. However, when the degree of vinylic functionality on albumin was low, the transition from surface degradation to bulk degradation was observed at higher concentrations of chemical initiator. The rate of gel degradation became slower as the concentration of pepsin was reduced. The results suggest that the rate and mechanism of hydrogel degradation was dependent on the steric constraints imposed by polymer chains of the network and on the conformational constraints of the albumin crosslinker.

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INTRODUCTION

The enzymatic degradation of polymer systems has received considerable attention in recent years [1–9]. The use of hydrogels as drug delivery systems has been investigated for both the parenteral and oral routes of administration [10–17]. Polymer networks with oligopeptide crosslinks have been investigated by Kopecek et al. [18]. It was shown that the digestibility of the oligopeptide sequences by α-chymotrypsin was related to the steric hindrances of the polymer chains and the structure of the oligopeptide crosslinker [18–21]. Larger oligopeptide sequences were more susceptible to cleavage if suitable subsite-substrate interactions were present during the formation of the enzyme-substrate complexes. Cleavage of shorter oligopeptide sequences, however, was more dependent on the steric hindrances from the polymer chains as the site of cleavage moved closer to the polymer backbone.

The effect of network structure on the enzymatic degradation of modified dextran microparticles has been studied [12,22,23]. It was shown that the degree of dextran modification determined the digestibility of the network in the presence of dextranase or lysosomal enzymes. An increase in the crosslinking density of the network, a function of dextran modification, increased the resistance of microparticles to dextranase-catalyzed hydrolysis and prolonged the residence time of the microparticles in the body. The change in the rate of enzymatic degradation as a function of crosslinking density has also been observed with oligopeptide-crosslinked hydrogels [21].

The effect of enzyme concentration on the degradation of partially deacetylated chitin films was studied by Pangburn et al. [24]. They showed that an increase in the concentration of lysozyme reduced the time required for complete dissolution of the network. It is now generally accepted that the kinetics of enzyme-catalyzed degradation of polymer networks can be influenced by several structural and enzyme-related factors.

In this laboratory, enzyme-digestible swelling hydrogels have been developed as long-term oral drug delivery systems [25–30]. The degradation of hydrogels was achieved by constructing a polymer network with functionalized human serum albumin (FA) as the crosslinking agent [25]. Albumin-crosslinked networks were shown to be susceptible to proteolytic degradation by various enzymes, such as pepsin, trypsin, and chymotrypsin [25,26].

The rate and mechanism of hydrogel degradation was dependent on the degree of albumin modification [26]. The rate of hydrogel degradation by pepsin decreased as the degree of albumin modification in-

creased. A predominance of surface degradation was observed when the degree of albumin modification was below 27%. When the degree of albumin modification was 27% or greater, a predominance of bulk degradation was observed. Polyacrylamide gel electrophoresis of digested samples of FA (functionalized albumin) and native human serum albumin showed that the presence of vinylic pendant groups on the FA slowed its digestion by pepsin. The electrophoresis study suggested that the digestibility of albumin-crosslinked hydrogels was also influenced by the mere presence of unreacted, vinylic pendant groups on the incorporated FA [26].

To better understand the nature of hydrogel degradation, we synthesized albumin-crosslinked hydrogels using different concentrations of chemical initiator in the monomer solution. This was intended to vary the degree of albumin incorporation in the networks prepared with the same degree of albumin modification. Hydrogel degradation was then studied as a function of initiator content and pepsin concentration. A mechanistic assessment discussing important factors that influence the rate and mechanism of degradation for albumin-crosslinked hydrogels is presented.

MATERIALS AND METHODS

Functionalization of Albumin

Human serum albumin (Sigma, Fraction V) was alkylated using glycidyl acrylate (Aldrich) as described previously [25]. The extent of alkylation, which is the degree of modification, was determined using the method described by Snyder and Sobocinski [31]. The alkylation reaction was carried out for 12 h, 19 h, and 49 h. The resulting degree of modification on the FA was 27%, 50%, and 90%, respectively [26]. Here, 100% modification indicates that all of the reactive amine groups on albumin have been alkylated.

Hydrogel Preparation

Albumin-crosslinked polyvinylpyrrolidone (PVP) hydrogels were prepared by free radical polymerization using 1-vinyl-2-pyrrolidinone (Aldrich) and functionalized albumin (FA) as the crosslinking agent, in the presence of 2,2-azobis[2-methylpropionitrile] (ABMP, Eastman Kodak) as the initiator [26]. The concentration of 1-vinyl-2-pyrrolidinone and FA were held constant at 36% (w/v) and 3.6% (w/v), respectively. The degree of modification on the FA was either 27%, 50%

or 90%. To alter the degree of albumin incorporation in the network, the concentration of ABMP used was varied from 0.01% to 1.0% (w/w) of the monomer. The monomer solution was degassed and purged with nitrogen followed by polymerization at 60°C for 18 h under nitrogen. Once polymerization was complete, the gels were removed, cut into discs (5 mm diameter by 3 mm length), washed over a 3-day period in distilled deionized water, and dried as described previously [26].

Swelling Characterization Studies

The effect of the ABMP concentration on hydrogel synthesis was examined through dynamic swelling studies. Dried hydrogels were weighed and then placed in pepsin-free simulated gastric fluid (SGF) at 37°C [32]. At specific timed intervals, the swelling gels were removed, weighed, and returned to their original solution. The swelling ratio (Q) was determined from the following relationship:

$$Q = W^*/W$$

where W^* and W are the weights of the swollen and dry gels, respectively. The dynamic swelling studies were carried out until equilibrium was reached. The degree of equilibrium swelling was used to assess the degree of albumin incorporation in the network. A low degree of swelling indicates a high degree of albumin incorporation in the network while a high degree of swelling indicates otherwise. Here, the degree of albumin incorporation in the network is broadly defined as the combination of chemical crosslinking and physical entanglements between FA and PVP chains.

Enzymatic Digestion Studies

The degradable properties associated with albumin-crosslinked PVP hydrogels were examined as a function of the ABMP concentration in the monomer solution. Dried hydrogels were weighed and then placed in pepsin-containing SGF at 37°C. The concentration of pepsin from porcine stomach mucosa (Sigma, 2500 units/mg) was 250 units/mL. At timed intervals the degrading gels were removed, weighed, and returned to their original solution. As in previous studies [26,29], Q was used to characterize the rate and mechanism of hydrogel degradation. A predominance of surface degradation was characterized by the following observations: (1) at times exceeding 1 h of exposure to pepsin, the swelling ratios of the degrading gels became significantly lower

than that of the nondegrading control samples indicating a loss of polymer chains from the gel; (2) the value of Q decreased to as low as 2 before complete dissolution of the gel occurs; and (3) the integrity of surface degrading gels, while being reduced in size, was comparable to that of nondegrading control samples. A predominance of bulk degradation was characterized by the following observations: (1) at times exceeding 8 h of exposure to pepsin, the swelling ratio and the size of the degrading gels became significantly larger than that of the nondegrading control samples indicating cleavage of the FA throughout the gel; (2) the release of PVP from the degrading network was delayed [28]; and (3) the integrity of the hydrogel was reduced over time leading to complete gel disruption. When gel disruption occurred, the gel could no longer be handled due to its loss of mechanical integrity [25].

Effect of Enzyme Concentration on Hydrogel Degradation

The effect of pepsin concentration on the rate and mechanism of hydrogel degradation was studied in SGF (simulated gastric fluid) using 7.75 units/mL, 31.25 units/mL, 62.5 units/mL, 125 units/mL, and 250 units/mL of enzyme. At timed intervals, Q was calculated over time and the mechanism of hydrogel degradation was assessed as described above.

RESULTS

Swelling Characterization Studies

Varying the concentration of ABMP in the monomer solution altered the degree of albumin incorporation in the network. The degree of hydrogel swelling in pepsin-free simulated gastric fluid (SGF) was inversely related to the concentration of ABMP present during polymerization. As the ABMP concentration increased, the degree of swelling decreased for gels crosslinked with the 27%-modified albumin (Figure 1). The swelling ratio (Q) of gels prepared with 0.01% ABMP was more than twice that of gels prepared with 1% ABMP during dynamic swelling. At equilibrium, Q ranged from 30 with 1% ABMP to 65 with 0.01% ABMP. The inverse relationship between the degree of hydrogel swelling and the concentration of ABMP was also observed with gels synthesized with the 50%- and 90%-modified albumin. The equilibrium swelling ratio of gels prepared with the 50%-modified albumin ranged from 26 with 1% ABMP to 48 with 0.01% ABMP. A similar result was obtained with the gels prepared with 90%-modified

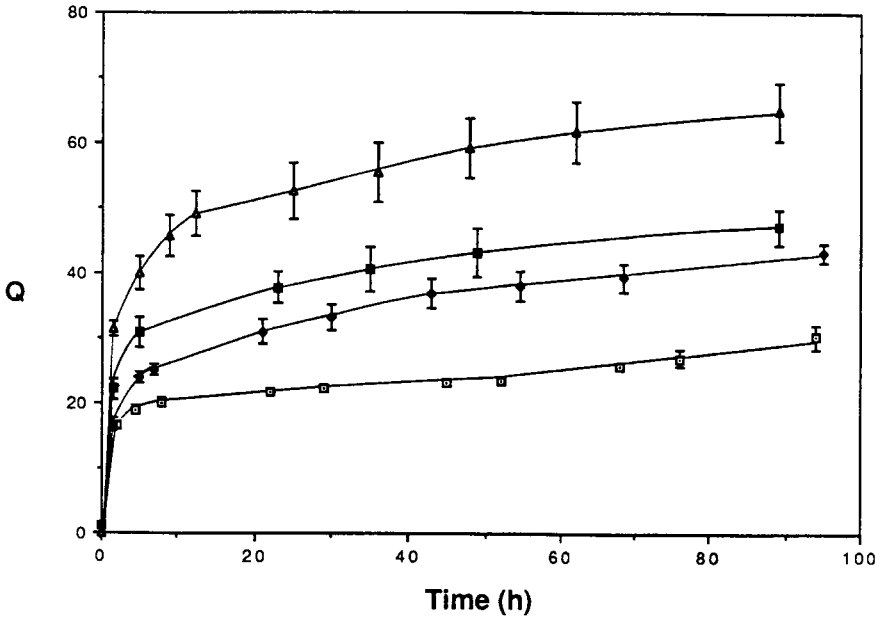

Figure 1. Dynamic swelling of albumin-crosslinked hydrogels in pepsin-free SGF as a function of time. The degree of functionality on FA was 27%. The concentrations of ABMP were 0.01% (Δ), 0.1% (■), 0.3% (♦), and 1% (⊡) (w/w) of the monomer (n = 4).

albumin. The swelling studies indicate that the initiator concentration in the monomer solution affects the degree of albumin incorporation in the network by influencing the degree of chemical crosslinking and physical entanglements between FA and PVP chains.

Enzymatic Digestion Studies

The degradable properties associated with albumin-crosslinked PVP hydrogels were studied as a function of the ABMP concentration in the monomer solution. The distinction in the swelling profiles between surface degrading and bulk degrading hydrogels are illustrated in Figures 2a and 2b.

It was observed that the surface degrading systems undergo a transient maximum followed by a rapid decrease in Q (Figure 2a). The decrease occurred more slowly as the ABMP concentration increased from 0.01% to 0.1%. Compared to nondegrading control samples, Q for surface degrading systems was significantly smaller and resulted in Q values as low as 2 before complete dissolution of the gel occurred. The

reduction in Q coincided with a marked reduction in gel size over time. Furthermore, no apparent loss in the structural integrity of the gel was observed during the terminal decline in Q. Shown in Figure 2b are the swelling properties for bulk degrading hydrogels. After 8 h of exposure to pepsin, bulk degrading hydrogels swelled to a greater extent than control samples. Bulk degradation was characterized by the gradual loss in gel integrity followed by gel disruption. In Figure 2b, the final data points measured in the presence of pepsin represent the last data points obtained prior to gel disruption. As the ABMP concentration increased from 0.1% to 1%, the rate of bulk degradation decreased and the gel disruption point (GDP) was prolonged from 30 h to 92 h (Figure 2b).

Illustrated in Figure 3 are the differences in the rate and the change in mechanism of hydrogel degradation as a function of ABMP concentration. For gels formed by FA with 27%, 50%, and 90% modification, hydrogel degradation underwent a transition from a predominance of surface degradation to a predominance of bulk degradation as the con-

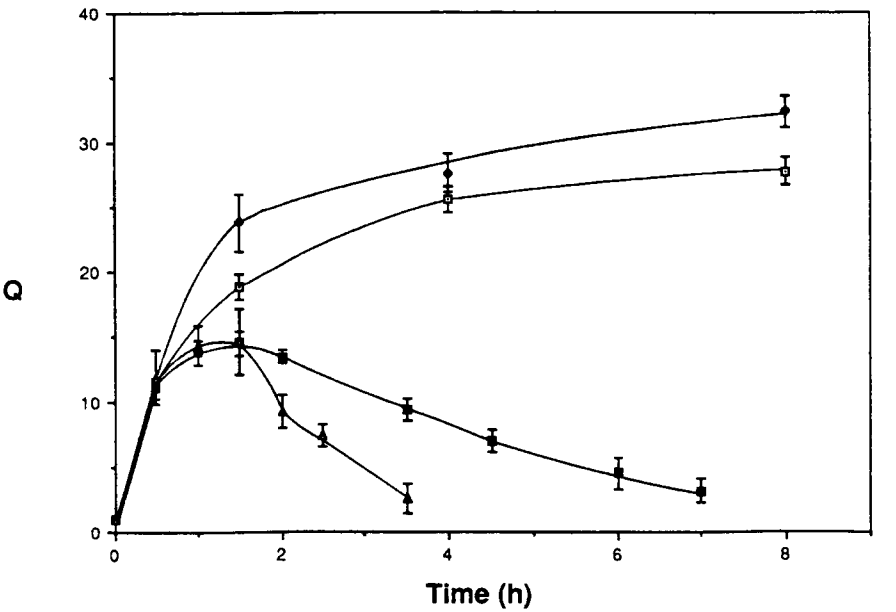

Figure 2a. Dynamic swelling of albumin-crosslinked hydrogels as a function of time. The degree of functionality on FA was 50%. The concentrations of ABMP were 0.1% (⊡,■) and 0.01% (♦,Δ) (w/w) of the monomer. Swelling was measured in the absence (♦,⊡) and in the presence (Δ,■) of pepsin in SGF (n = 4).

centration of ABMP increased. Gels formed by FA with 27% modification degraded by surface degradation when the concentration of ABMP ranged from 0.01% to 0.1% (Figure 3a). The rate of surface degradation, as indicated by the reduction in Q, decreased as the concentration of ABMP increased. The transition to bulk degradation occurred when the concentration of ABMP was 0.3% (Figure 3a). The rate of bulk degradation, similar to the rate of surface degradation, decreased as the concentration of ABMP increased from 0.3% to 1.0%. The GDP was prolonged from 13.5 h with 0.3% ABMP to 30 h with 1.0% ABMP. An increase in the degree of albumin incorporation in the network influenced both the rate and the mechanism of hydrogel degradation as shown in Figures 1 and 3a.

The mode of hydrogel degradation as a function of ABMP concentration remained the same for gels formed with 50% (Figure 3b) and 90% (Figure 3c) albumin modification. A summary of the degradation mechanisms obtained from the swelling data in Figure 3 is presented in Table 1. The data in Table 1 shows that the degradation mechanism

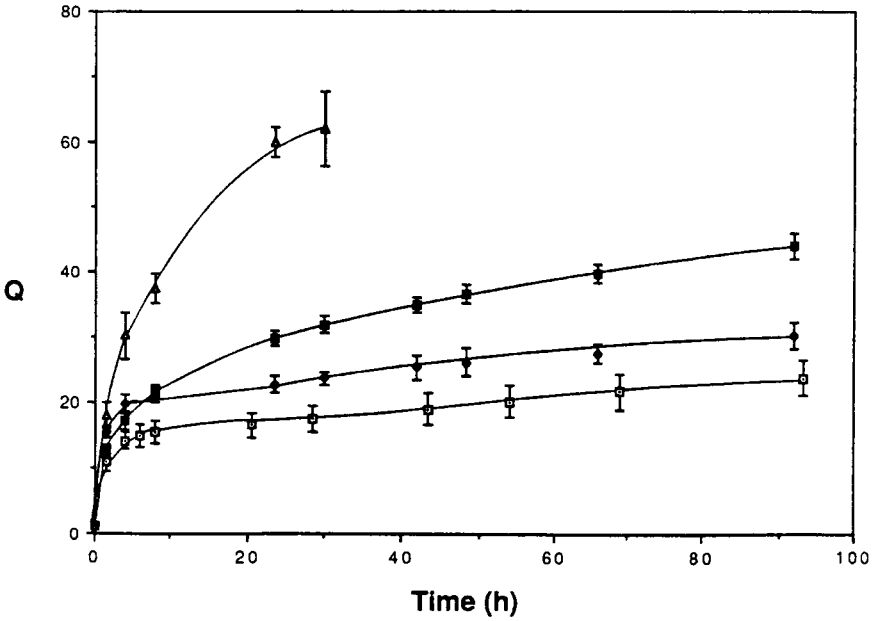

Figure 2b. Dynamic swelling of albumin-crosslinked hydrogels as a function of time. The degree of functionality on FA was 90%. The concentrations of ABMP were 1% (⊡,■) and 0.1% (♦,Δ) (w/w) of the monomer. Swelling was measured in the absence (♦,⊡) and in the presence (Δ,■) of pepsin in SGF (n = 4).

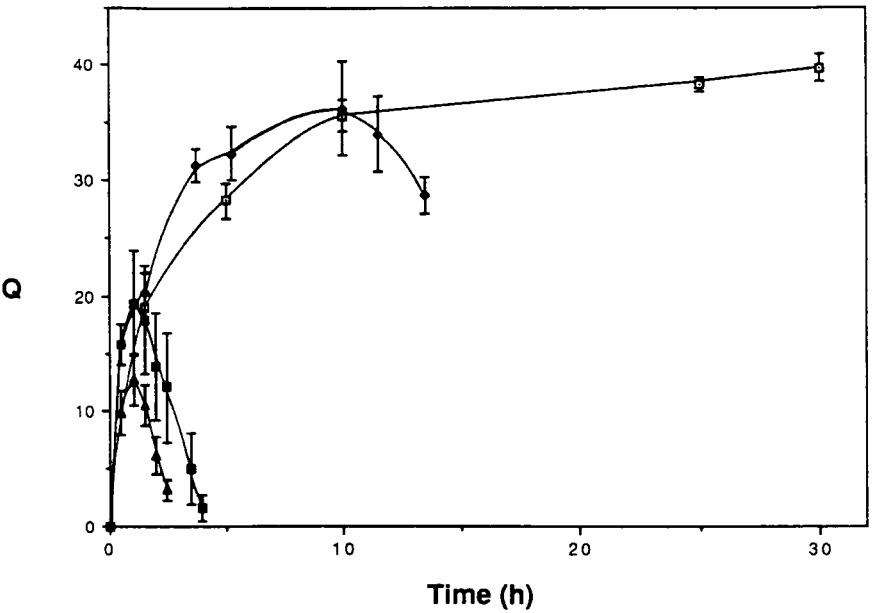

Figure 3a. Dynamic swelling of albumin-crosslinked hydrogels in pepsin-containing SGF as a function of time. The degree of functionality on FA was 27%. The concentrations of ABMP were 0.01% (Δ), 0.1% (■), 0.3% (♦), and 1% (⊡) (w/w) of the monomer (n = 4).

depends largely on the initiator concentration. Even with 90% albumin modification, surface degradation still occurred when the concentration of ABMP was very low. Surface degradation dominated below 0.1% ABMP because the low concentration of ABMP in the monomer solution resulted in a low degree of albumin incorporation in the network. The data in Table 1 illustrates the relationship between the gel disruption point (GDP) and the concentration of ABMP for bulk degrading hydrogels. The GDP was prolonged as the concentration of ABMP increased from 0.1% to 1%. For gels crosslinked by FA with 50% modification, the GDP ranged from 36 h with 0.3% ABMP to 58 h with 1.0% ABMP. The GDP for gels crosslinked by FA with 90% modification, however, was much greater ranging from 66 h with 0.3% ABMP to 92 h with 1.0% ABMP.

Effect of Enzyme Concentration on Hydrogel Degradation

Changes in the rate and mechanism of hydrogel degradation as a function of pepsin concentration in SGF were also studied. It was

observed (Figure 4) that the rate of surface degradation, as indicated by the decline in Q, decreased as the concentration of pepsin was reduced from 250 units/mL to 7.75 units/mL. At a pepsin concentration of 250 units/mL, only 4 h was required for the swelling ratio to fall below 3. When the concentration of pepsin was 7.75 units/mL, however, 12.5 h was required for the swelling ratio to fall below 3. As the concentration of pepsin decreased, the initial phase of gel swelling was significantly higher. The increase in swelling and slow decline in Q suggests that the cleavage of albumin crosslinks and the subsequent release of polymer chains from the gel surface was slower as the concentration of pepsin decreased. The gel disruption point for bulk degrading hydrogels was prolonged at lower concentrations of pepsin (Table 2). As the concentration of pepsin decreased from 250 units/mL to 7.75 units/mL the GDP prolonged from 13.5 h to 27 h. Pepsin concentrations greater than 250 units/mL were not studied since it has been observed previously that the rate of hydrogel degradation is independent of enzyme concentration at pepsin levels greater than 125 units/mL [33]. Only the rate of hydrogel degradation was affected by varying the concentration

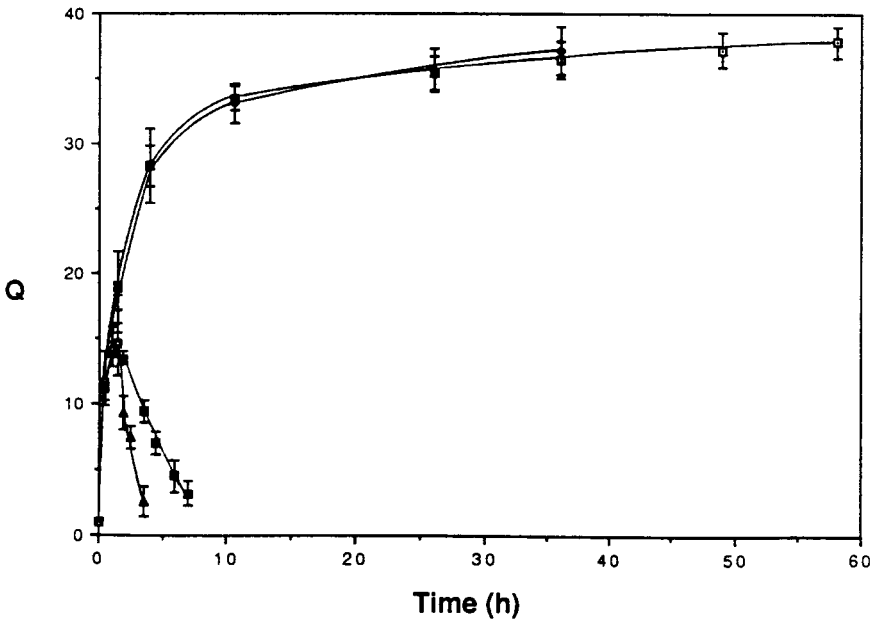

Figure 3b. Dynamic swelling of albumin-crosslinked hydrogels in pepsin-containing SGF as a function of time. The degree of functionality on FA was 50%. The concentrations of ABMP were 0.01% (Δ), 0.1% (■), 0.3% (♦), and 1% (⊡) (w/w) of the monomer (n = 4).

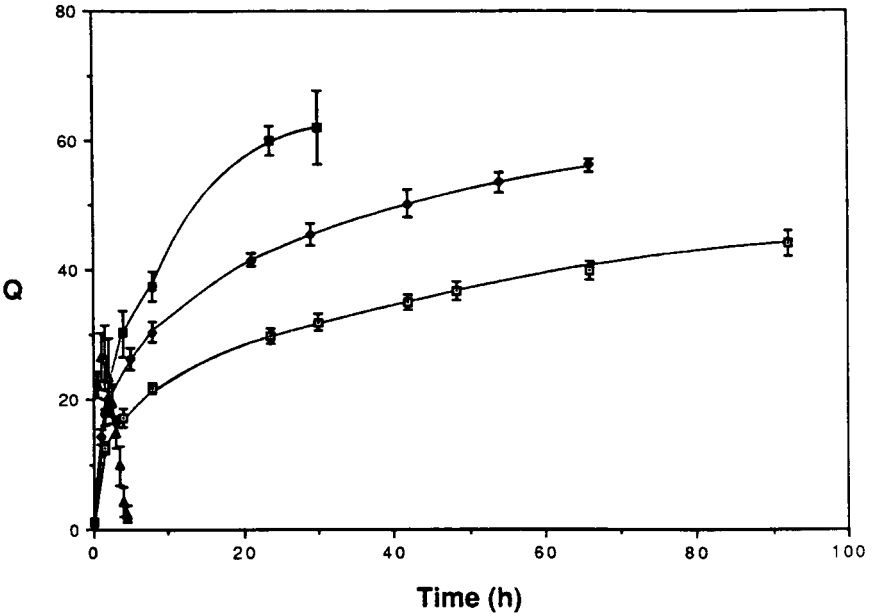

Figure 3c. Dynamic swelling of albumin-crosslinked hydrogels in pepsin-containing SGF as a function of time. The degree of functionality on FA was 90%. The concentrations of ABMP were 0.01% (Δ), 0.1% (■), 0.3% (♦), and 1% (⊡) (w/w) of the monomer (n = 4).

of pepsin. There was no indication, based on the control samples, that the mechanism of degradation was affected by the change in the concentration of pepsin.

DISCUSSION

The objective of this work was to examine how the rate and mechanism of hydrogel degradation was influenced by the degree of albumin incorporation in the network and the enzyme concentration. The degree of albumin incorporation in the network was effectively altered by controlling the concentration of initiator (ABMP) in the monomer solution and the degree of albumin modification. We observed (Figure 1) that when the concentration of ABMP was high, the degree of hydrogel swelling was low. The reduction in the swelling ratio was attributed to the increase in chemical crosslinking and physical entanglements between FA and PVP chains. The degree of albumin incorporation in the network was directly related to the number of propagating units that interacted with the FA during polymerization.

Table 1. The mode of hydrogel degradation and the gel disruption point as a function of the initiator concentration.

Concentration of ABMP (%)	Albumin Functionality (27%)	(50%)	(90%)
0.01	S	S	S
0.10	S	S	B(30.0)*
0.30	B(13.5)	B(36.0)	B(66.0)
0.45	B(23 0)	B(49 0)	B(78.0)
1.00	B(30.0)	B(58.0)	B(92.0)

* The gel disruption point in hours
S. surface degradation
B bulk degradation

High levels of ABMP increased the number of propagating units during polymerization [34], and therefore increased the probability of interaction between the FA and the growing PVP chains. The resulting network had a higher crosslinking density and a lower molecular weight between crosslinks (M_c). A similar effect between the amount of chemical initiator and M_c has been observed by Stjarnkvist et al. [35] and Laakso et al. [36].

For gels formed with FA of the same degree of modification, the rate of hydrogel degradation decreased as the degree of albumin incorporation in the network increased. As a result, a transition from a predominance of surface degradation to a predominance of bulk degradation was observed as the concentration of ABMP in the monomer solution increased (Figure 3). Gels formed by FA with higher degrees of modification were more resistant to enzymatic degradation since the degree of albumin incorporation in the network was also dependent on

Table 2. The gel disruption point (GDP) for bulk degrading hydrogels as a function of the pepsin concentration.

Pepsin (units/mL)	GDP (h)
7.75	27.00
31.25	25.00
62.50	23.25
125.00	23.00
250.00	13.50

Functionality of FA: 27%.
Concentration of ABMP: 0.3% (w/w) of the monomer.

the degree of albumin modification; a parameter which controls the extent of chemical crosslinking. Thus, it appears that hydrogels formed with a high degree of albumin modification will undergo surface degradation only if the concentration of ABMP is low enough to sufficiently reduce the degree of albumin incorporation in the resulting network (Table 1).

The rate of hydrogel degradation was also influenced by the concentration of pepsin. Based on the data in Figure 4 and Table 2, as the concentration of pepsin decreased over 30 fold, gel degradation was prolonged. Lowering the concentration of pepsin increased the time required for a sufficient number of crosslinks to be cleaved. Because of this, the rate of hydrogel degradation was slower. It was initially expected that gels which underwent surface degradation at high concentrations of pepsin would undergo bulk degradation at low pepsin concentrations if the mechanism of gel degradation was concentration dependent. The data, however, suggest that the pepsin-catalyzed cleavage of FA and the subsequent release of PVP chains from the gel

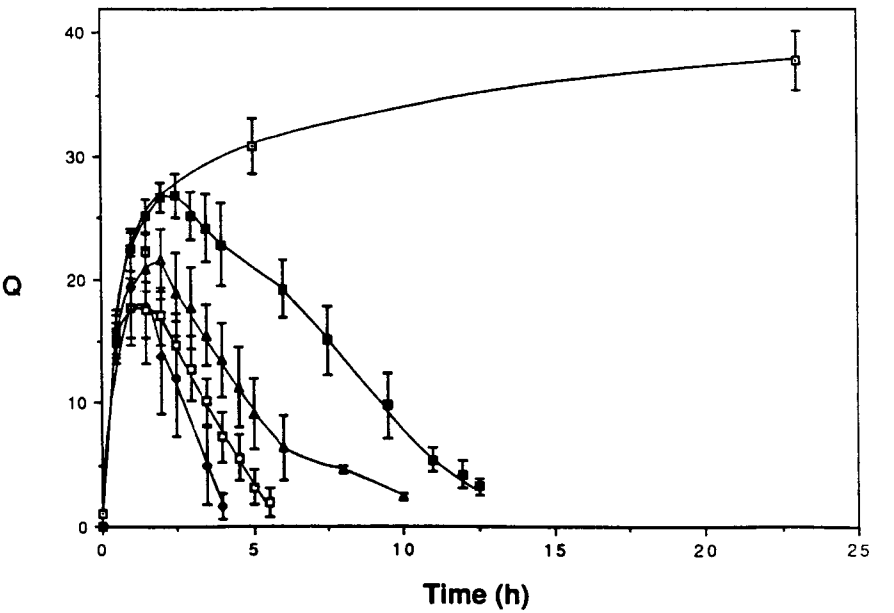

Figure 4. Dynamic swelling of albumin-crosslinked hydrogels in pepsin-containing SGF as a function of time. The degree of functionality on FA was 27% and the concentration of ABMP was 0.1% (w/w) of the monomer. The concentration of pepsin was 0 units/ml (⊡), 7.75 units/ml (■), 31.25 units/ml (Δ), 125 units/ml (□), and 250 units/ml (♦) (n = 4).

surface was quite rapid, and hence no transition from surface degradation to bulk degradation was observed in the range of concentrations studied. In the development of albumin-crosslinked hydrogels for oral drug delivery, this consistency in the degradation mechanism would be helpful when one considers the variations in the levels of pepsin found in the stomach [37,38]. By utilizing a system which can undergo surface or bulk degradation over a wide range of enzyme concentrations, the rate of drug release from the system can be effectively controlled.

Pepsin-induced degradation of albumin-crosslinked hydrogels was dependent on the steric constraints from the polymer chains of the network and the conformational constraints of the incorporated FA. When a dry, glassy hydrogel swells in pepsin-containing SGF, it is conceivable that two penetration fronts arise. The first front is the pepsin-free swelling front which is responsible for the initial swelling of the gel. The second front is the pepsin-containing degrading front. The two penetration fronts arise due to the steric constraints from the polymer chains of the network which restrict the diffusion of pepsin into the rubbery phase of the gel. Mesh-size dependent restrictions on enzyme penetration have been observed with similar polymeric devices as a function of pore size [12] and solvent content [39]. At the surface of the gel, the polymer chains will limit the formation of enzyme-substrate complexes due to conformational and orientational restrictions on the pepsin molecule [40,41]. As the degree of swelling increases, pepsin will be able to diffuse into the network. With the penetration of the degrading front into the gel, the activity of pepsin will be restricted by steric constraints from the polymer chains which give rise to a "sieving" effect that will limit the movement of pepsin within the network and control its ability to achieve suitable orientations near cleavage sites [42,43]. However, steric constraints from polymer chains are reduced with further gel swelling. As a result, the "sieving" effect on pepsin diffusion decreases and the ability of pepsin to form enzyme-substrate complexes within the network is enhanced. Therefore, as the hydrogel approaches equilibrium swelling, steric constraints from the polymer chains will contribute less and less to the inhibition of enzyme-catalyzed degradation. The second factor that influences pepsin-induced degradation of albumin-crosslinked hydrogels is the conformational constraints of the incorporated FA. Conformational constraints arise as a function of chemical crosslinking and physical entanglements between FA and PVP chains. For the oligopeptide sequences of FA that exist between polymer chains and chemical crosslinks, conformational constraints will restrict the number of conformations that will permit the formation of enzyme-substrate complexes. Therefore, as the degree of

albumin incorporation increases with higher levels of ABMP, the average chain length and mobility of the oligopeptide segments decreases. The resulting increase in conformational constraints decreases the rate of enzyme-catalyzed degradation. With a low degree of albumin incorporation in the network, the digestibility of FA by pepsin is enhanced due to an increase in the average chain length and mobility of the oligopeptide segments between polymer chains and chemical crosslinks. If the rate of albumin cleavage and the subsequent loss of polymer chains is rapid during degradation, surface degradation dominates. This explanation is consistent with Figure 3 which shows that the rate of gel degradation increased as the concentration of ABMP decreased. In contrast, high degrees of albumin incorporation in the network will increase the conformational constraints on the FA and restrict the formation of enzyme substrate complexes. As a result, bulk degradation is observed because the network is more resistant to degradation and will permit the penetration of the degrading front into the gel upon further reduction of steric constraints from the polymer chains.

In summary, steric constraints from the polymer chains will control the penetration of pepsin (i.e., degrading front) into the gel and limit the activity of pepsin through conformational and orientational restrictions. The degree of albumin incorporation in the network will control the magnitude of conformational constraints of the FA, and hence control the rate of enzyme-catalyzed degradation. The data presented in this paper support the above hypothesis regarding factors important to pepsin-induced degradation of albumin-crosslinked hydrogels. Future experiments will examine how the steric constraints from the polymer chains influence enzyme penetration into the hydrogel and hydrolytic activity toward FA.

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