Comparison of Poly(aspartic acid) Hydrogel and Poly(aspartic acid)/Gelatin Complex for Entrapment and pH-Sensitive Release of Protein Drugs

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ABSTRACT: Biodegradable poly(aspartic acid) (PASP) hydrogel and PASP/gelatin complex were prepared to evaluate their potential application as pH-sensitive matrices for controlled protein release. Entrapment of myoglobin (Mb) and its release were compared between the two types of carriers. It was found that incorporation of Mb into PASP hydrogel strongly depended on the medium pH and NaCl concentration, and was time-consuming. However, complete entrapment of Mb into PASP/gelatin complex was found within pH ranged from 2.5 to 4.0, which was concomitant with the formation of PASP/gelatin complex. By adjusting Mb feed ratio, Mb entrapment in the complex can be up to 31.54% (by weight) with high loading efficiency (96.2%). Gradual release of Mb from PASP hydrogel was observed within pH 2.0–7.4, while Mb release from PASP/gelatin complex was negligible within pH 2.0–4.2 for 4 days. In addition, pulsatile Mb release can be achieved by combining polyanhydride with pH-sensitive PASP/gelatin complex, while the device composed of polyanhydride and PASP hydrogel is mechanically unstable. PASP/gelatin complex formed by electrostatic interactions is superior to the single-component PASP hydrogel synthesized by chemical cross-linking as pH-sensitive matrices for controlled protein release when entrapment of proteins and pH-sensitivity of protein release are concerned. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 2320–2329, 2006

Key words: poly(aspartic acid); hydrogel; polyelectrolyte complex; pH-sensitive release; controlled protein release

INTRODUCTION

Controlled protein release plays important roles in both drug therapy and tissue engineering.[1,2] Hydrophobic biodegradable polymers, such as poly(lactide-*co*-glycolide) (PLGA) and polyanhydrides, and hydrogels are widely used as matrices for protein delivery.[3] The former has an advantage of in vivo absorbability and adjustable degradation rate. However, the hydrophobic surface of these polymers, together with their inner acidic microclimates generated during degradation, would denature most of the proteins.[4] In contrast, hydrophilic hydrogels represent a class of biomaterials that are more compatible with protein drugs and may retain protein bioactivities.[5] Unfortunately, the release duration of proteins from hydrogels was always found to be very short.[3] To take advantages and circumvent the limitations of both hydrophobic biodegradable polymers and hydrogels, it is essential to combine them together so that proteins can be released from the composite matrices in an active state for a reasonable period of time.[6] Although protein stability was always found to be enhanced by this method, severe burst release was frequently reported, which may be due to the swelling of the incorporating hydrogels after water ingresses the composite device.[7,8] Since acidic microclimate is generally observed during degradation of PLGA or polyanhydrides and the pH value inside the polymers can be as low as 2.0 for PLGA and 5.0 for polyanhydrides,[9,10] the protein release behavior may be improved by incorporating pH-sensitive gels, which should have low swelling degree and retain the loaded proteins at low pH (<5.0), into the hydrophobic biodegradable polymers.[11]

Polyelectrolytes were commonly used to prepare pH-sensitive gels by either chemical cross-linking or physical complexation.[12,13] Swelling of chemically cross-linked polyelectrolyte hydrogels is dependent on medium pH because of protonation and dissociation of ionic groups at various pH. For example, polyanion hydrogels swell extensively at neutral condition because of the repulsive interactions between anionic groups, whereas deswell at low pH resulting from the protonation of dissociated residues. The com-

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plexation between polyanion and polyampholytes also strongly depends on pH, which makes polyanion/polyampholyte complexes useful as pH-sensitive gels for controlled protein release.[14] Since connective tissues are mainly composed of hyaluronic acid and collagen, which form three-dimensional networks,[15] polyanion/polyampholyte complexes mimic in vivo environment of proteins and may be benign for protein drugs.[16] In addition, numerous polyanion/polyampholyte pairs can form insoluble complexes by electrostatic interaction, so that protein release behavior can be finely tailored by selecting suitable polyelectrolytes.

In our previous works, we prepared three types of polyanion/gelatin complexes and investigated the pH-sensitivity of the complex dissociation and protein release. The transition of both complex dissociation and protein release was found to occur within a rather narrow pH range.[14] Furthermore, by coating PMAA/gelatin complex disc with hydrophobic membrane and leaving one-end open, protein can be released in a pseudo zero order for up to 20 days.[17] However, it is preferred to use biodegradable polyanions to prepare the complexes. In this work, we prepared biodegradable poly(aspartic acid) (PASP) hydrogel and PASP/gelatin complexes, and compared the entrapment and pH-sensitive release of myoglobin (Mb) from the two types of matrices. The feasibility of combining pH-sensitive PASP/gelatin complex with biodegradable hydrophobic polymer for finely modulating Mb release was also primarily investigated.

EXPERIMENTAL

Materials

D,L-Aspartic acid, gelatin (type B, isoelectric point (IEP) is 4.9), and ethylenediamine were purchased from Shanghai Chemical Co. (Shanghai, China). Myoglobin (Mb) (from horse heart) was supplied by Sigma Chemical Co. (St. Louis, MO). Poly(succinimide) (PSI) and PASP were synthesized according to literature.[18] Poly(trimellitylimidoglycine-*co*-sebacic anhydride)-*b*-polyethylene glycol (P(TMA-gly-*co*-SA)-*b*-PEG, 50:30:20 by mole) tricopolymer was synthesized by melt-condensation copolymerization.[19] Poly(lactide-*co*-trimethyl carbonate) (PLTMC, 30:70 by mole) was synthesized by ring-opening copolymerization of lactide and trimethyl carbonate using $Sn(Oct)_2$ as catalyst in our laboratory.[20]

Preparation of PASP hydrogel

To 20 mL of PSI solution in dried DMF (0.25 g/mL) was added 0.6 g of ethylenediamine. The mixture was emulsified into 100 mL of silicon oil, and then stirred overnight at room temperature under nitrogen atmosphere. The precipitates generated during the reaction were collected by centrifugation, filtrated, washed with acetone, and dried in vacuum to give 4.2 g of flow powders, which were then immersed in 40 mL of NaOH solution (0.1 g/mL). After the suspension was stirred for 1 h at room temperature, the medium pH was adjusted to neutral by adding 35% aqueous HCl. The swelling particles were immersed in an excess amount of distilled water for >1 week to remove any soluble moieties, then collected by filtration, washed with ethanol, and dried in vacuum. The complete conversion of succinimide units to aspartic residues was confirmed by IR analysis.

Swelling degree of PASP hydrogel

The swelling degree of PASP hydrogel was determined gravimetrically. The dried hydrogel was immersed in 5 mL of 0.1*M* buffer at 37°C until equilibrium was reached. Then, the swollen hydrogel was taken out of the medium, blotted by wax paper, and weighed. The swelling degree was calculated by the following equation:

$$\text{Swelling degree} = (W_s - W_d)/W_d$$

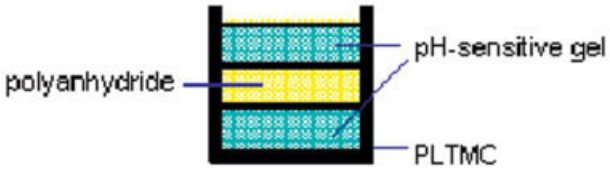
where W_s and W_d are the weight of the hydrogel in the swollen and the dry state.

Loading of Mb into PASP hydrogel

Mb was dissolved in distilled water with certain pH and NaCl amount at a concentration of 10 mg/mL, and the hydrogel in the swollen state was immersed in the solution for 3 days at 37°C. Then, the hydrogel was taken out and washed extensively with distilled water to remove any loosely-bound Mb. The pH of the distilled water for rinsing hydrogel was adjusted to the same value of the loading medium with diluted HCl. The amount of Mb loaded in the hydrogel was monitored by UV–Vis spectrophotometer at 420 nm (Cary-100, Varian).

Preparation of Mb-loaded PASP/gelatin complex

A mixed solution of gelatin and Mb was titrated with PASP aqueous solution till end point of titration approached (the maximum turbidity). The pH of the gelatin/Mb-mixed solution was adjusted to the same value with that of PASP solution before titration. The complex precipitate was centrifuged, washed with double-distilled water, and dried in vacuum at room temperature.



Scheme 1 Structure of the laminated device for pulsatile Mb release. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Determination of Mb entrapment in PASP/gelatin complex

Mb entrapment in complexes was determined by the following procedures. After the end point of titration was approached, the sample was centrifuged at 4000 $\times$ *g* for 15 min. The protein concentration in the supernatant was measured with HPLC (Shimadzu LC-3A). A calibration curve obtained by plotting the absorbency of the standard Mb solution (pH was adjusted to the same value with that of the supernatant) against the protein concentration was employed. The protein entrapment in complexes was calculated from the differences between the initial added protein and the remaining in the supernatant.

Preparation of the laminated device

The laminated device composed of one P(TMA-gly-*co*-SA)-*b*-PEG layer and two PASP hydrogel (or PASP/gelatin complex) layers was prepared according to the procedure reported previously[11] (Scheme 1). In brief, each layer was individually prepared (2.8 mm in diameter) by compression molding of the P(TMA-gly-*co*-SA)-*b*-PEG or PASP hydrogel (or PASP/gelatin complex) powder (<50 μm) with a homemade apparatus at 100 kg/cm^2 and room temperature for 5 min, then compressed together into an alternate cylindrical preparation by compression molding at 100 kg/cm^2 and room temperature for 10 min, and finally coated in PLTMC film with one open-end left.

Mb release

PASP hydrogel in the dried state was sieved (600–1000 μm) and immersed in 5 mL of buffer solution with various pH and ionic strength at 37°C. Mb release was monitored at 420 nm.

The complexes were ground with a mortar and pestle and sized by use of sieves (600–1000 μm). The blank or protein-loaded particles were immersed in 5 mL of buffer solution with various pH and ionic strength at 37°C. Dissolution fluid was removed and fresh solution was added back periodically. The amount of gelatin released from the blank complexes was determined by a bichinchoninic acid protein microassay. Mb release from the complexes was monitored at 420 nm.

Mb assay

A capillary electrophoresis apparatus (Spectra, Model 1000-CE00, Thermo Separation products, San Jose, CA) was employed for the analysis of Mb conformation, using phosphate buffer (pH 10.4) as a mobile phase. A UV detector (wavelength, 200 nm) was used to detect Mb. Mb soret band was obtained by UV–Vis spectrophotometer (Cary-100, Varian).

RESULTS AND DISCUSSION

Swelling of PASP hydrogel and complexation of PASP/gelatin

Figure 1 shows the swelling of PASP hydrogels in 0.1*M* buffer solution with various pH. It can be seen that the swelling degree of PASP hydrogel increases with buffer pH, which can be ascribed to the dissociation of carboxylic groups at high pH and repulsion of the ionic groups. The swelling transition of PASP hydrogel is very broad (about 3 unit), which is similar to that of other weak polyanion hydrogels.[21,22]

Figure 2 represents the dependence of PASP/gelatin complexation on pH, which reveal that PASP/gelatin complex can be formed between pH 2.0 and 4.5. The interaction of PASP and gelatin is mainly based on the electrostatic forces, since at high NaCl concentration no precipitates were generated. The complexation behavior is analogous to that of other polyanion/gelatin pairs, such as poly(acrylic acid)/gelatin and heparin/gelatin.[14] Mattison et al. investigated the dependence of the interactions of numerous polyelectrolyte/protein pairs on medium pH using turbidimetry and found that all the pairs showed sharp transition of turbidity with pH.[23] The complexation behavior of PASP/gelatin pair is also similar to those reported in literatures. The complexes were collected and dried. Swelling degree was measured by similar method as PASP hydrogel. It was observed that the pH has little effect on complex swelling degree (about 1.8) within the pH range from 2.5 to 4.0, which may be attributed to the neutralization of ionic groups by electrostatic interactions.

Entrapment of Mb into PASP hydrogel and PASP/gelatin complex

Loading of proteins into polyelectrolyte hydrogels is based on the absorption of proteins to hydrogel matrices mainly by electrostatic interactions, which is always time-consuming. Figure 3 shows the effect of NaCl concentration in the loading medium on Mb

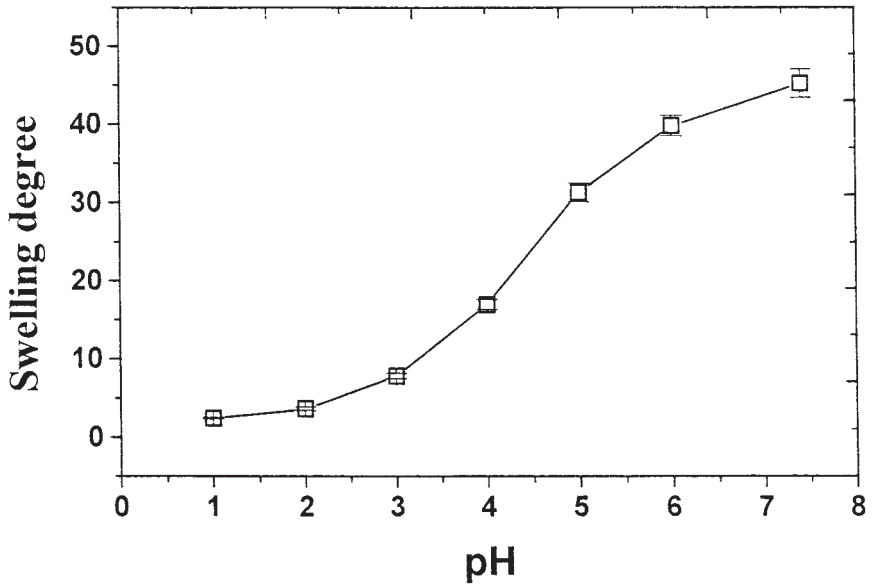


Figure 1 Equilibrium swelling degree of PASP hydrogel at various pH.

absorption to PASP hydrogel (pH 5.0). It can be seen that the amount of Mb absorbed to PASP hydrogel decreases with an increase in NaCl concentration, indicating that Mb is entrapped into PASP hydrogel *via* electrostatic interactions between carboxylic groups carried by PASP and cationic residues in Mb. At pH 5.0, Mb presents positive net charges (IEP_{Mb} was 6.9) and can interact with PASP by ionic bonds. The dependence of Mb absorption on the pH of loading medium is illustrated in Figure 4. At pH 4.0 and 5.0, most of the Mb was absorbed to PASP hydrogel within 30 h (>60%), while large amount of Mb was still detectable in the loading medium (>80%) at pH 7.4, 6.0, and 3.0. In more detail, the amount of absorbed Mb decreases in the order of 5.0, 4.0, 3.0, 6.0, and 7.4. At pH 7.4, there are no electrostatic interactions between Mb and PASP and the loading efficiency is the lowest. It seems that maximal interaction between Mb and PASP occurs at pH 5.0 because of the high charge density of both PASP and Mb.

The entrapment of Mb into complexes is associated with the formation of PASP/gelatin complex. Table I shows the effect of pH on the complex yield and Mb

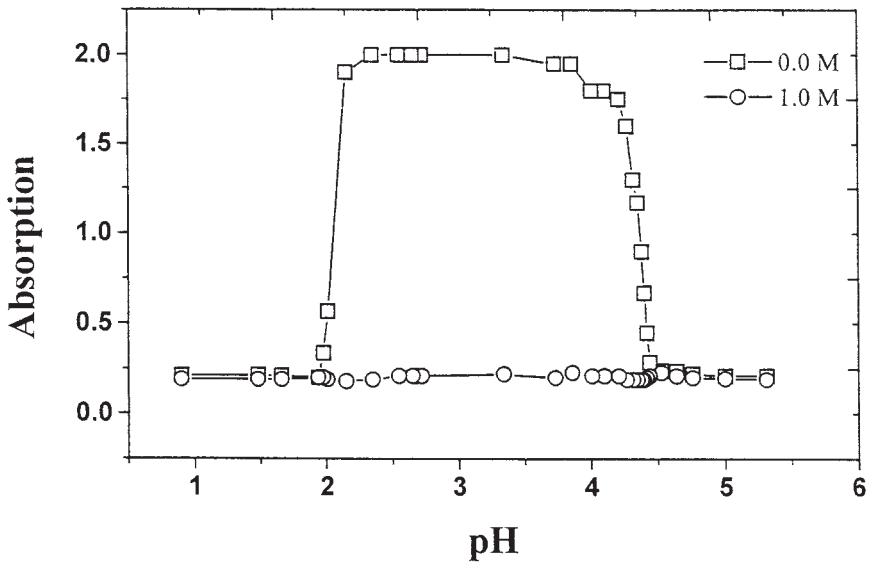


Figure 2 Turbidity changes of PASP/gelatin systems *vs.* pH, titrated with 0.1*M* HCl solution. Gelatin concentration: 8 mg/mL. PASP/gelatin = 1.0.

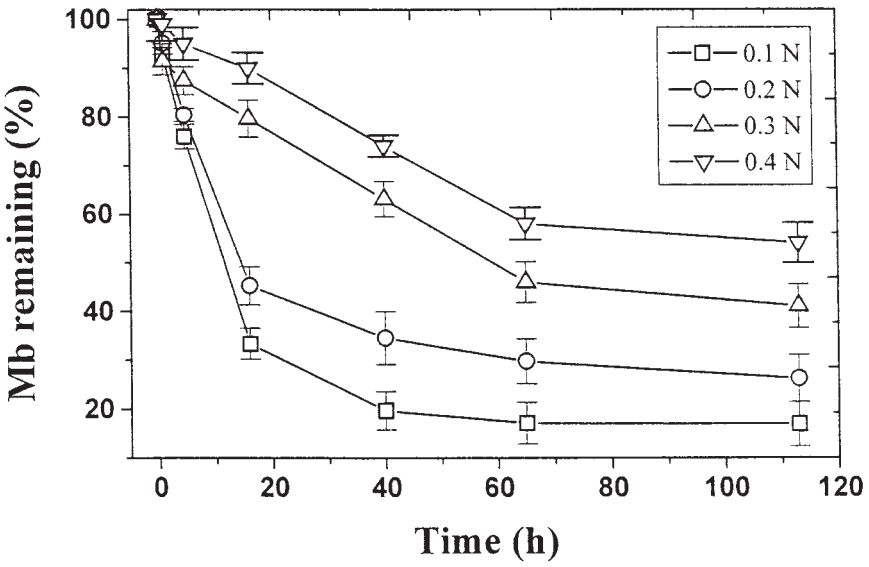


Figure 3 Dependence of Mb absorption into PASP hydrogel on NaCl concentration at pH 5.0. Mb/PASP ratio (in weight) was 0.1 ($n = 3$).

entrapment efficiency. It can be seen that the PASP/gelatin ratio in the complex increases with a decrease in pH, while complex yield and Mb entrapment efficiency remain nearly unaffected (>90%). It is well known that charge densities of both PASP and gelatin depend on pH because of their weak polyelectrolyte nature, decreasing as pH progresses toward low value for PASP and increasing for gelatin. So, when pH value decreases, more PASP is needed to neutralize gelatin molecules. It is interesting to observe that Mb entrapment efficiency in the complex at low pH (pH 3.0, 2.5, and 2.0) is still very high, although the electrostatic interactions between PASP and Mb become very weak, revealed by absorption of Mb to PASP hydrogels (Fig. 4). Such results may be resulted from either the induced dissociation of carboxylic groups carried by PASP in the presence of cationic gelatin at low pH[24] or the interactions between gelatin and Mb by hydrogen bond and hydrophobic forces. Table II represents the effect of Mb/gelatin ratio in the initial gelatin/Mb-mixed solution on Mb entrapment efficiency and entrapment. It can be seen that the needed

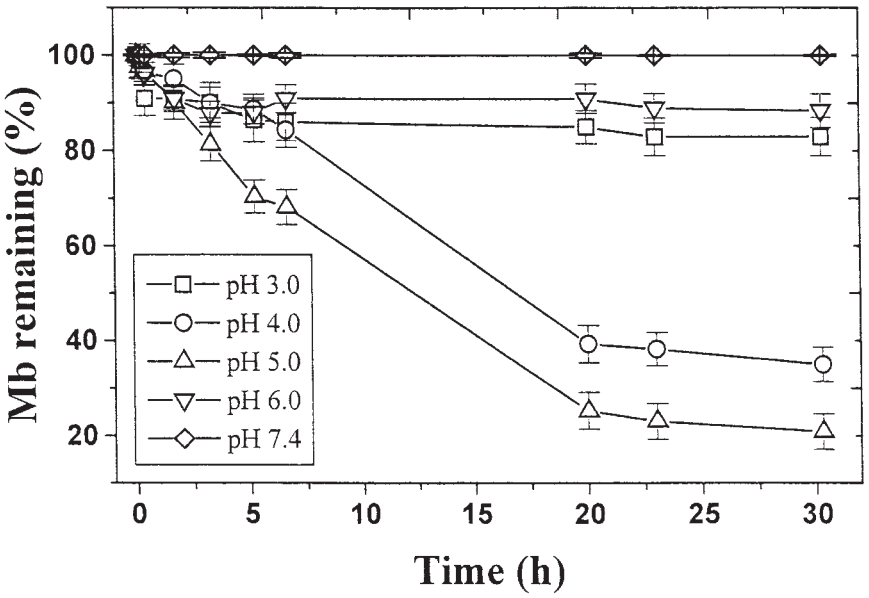


Figure 4 Dependence of Mb absorption into PASP hydrogel on pH in 0.1*M* buffer solution. Mb/PASP ratio (in weight) was 0.1 ($n = 3$).

TABLE I
The Effect of pH on PASP/Gelatin Yield and Mb Entrapment Efficiency[a]

pH	PASP/gelatin ratio[b]	Complex yield (%)	Entrapment efficiency (%)	Entrapment (%, w/w)[c]
2.5	4.95	95	96 ± 0.8	3.23
3.0	2.11	96	97 ± 1.2	6.24
3.5	0.74	94	92 ± 1.5	10.57
4.0	0.28	92	94 ± 2.1	14.69

[a] PASP concentration was 2 mg/mL, gelatin concentration was 0.4 mg/mL, and Mb/gelatin ratio (by weight) was 0.2.
[b] Determined by colloid titration method (see experimental section).
[c] Calculated by the actual amount of Mb entrapped/the amount of complex.

PASP amount at the end point of titration increases with an increase in Mb/gelatin ratio. In addition, the entrapment of Mb in PASP/gelatin complex can be up to 31.54% with rather high entrapment efficiency (about 96%).

Mb release from PASP hydrogel and PASP/gelatin complex

Mb release from PASP hydrogel was evaluated at various NaCl concentration and pH. Figure 5 represents the effect of NaCl concentration in the dissolution fluid at pH 5.0 on Mb release. It can be seen that Mb release rate from the hydrogel increases with NaCl concentration and essentially none is released when the hydrogel was immersed in distilled water. Such results are similar to those reported in literatures.[25] As the salt concentration increases, screening of ionized groups predominates, which impairs the electrostatic interactions between PASP and Mb and results in the acceleration of Mb release. Since no Mb release was observed in distilled water, it can be concluded that Mb is liberated from the hydrogel at pH 5.0 *via* an ionic exchange-driven release mechanism proposed by Inoue *et al*.[26] The effect of medium pH on Mb release from PASP hydrogel is shown in Figure 6. Generally, release rate of Mb decreases as pH approaches lower value except that pH is 4.0. Protein release from polyelectrolyte hydrogels is governed by two factors: swelling rate of the hydrogel and the electrostatic interactions between protein and hydrogel matrices. Since electrostatic interactions between Mb and PASP are weak at pH 7.4, 6.0, 3.0, and 2.0 (Fig. 4), Mb release from PASP hydrogel is mainly governed by the hydrogel swelling, which follows the order of hydrogel swelling (Fig. 1). At pH 5.0 and 4.0, both hydrogel swelling and dissociation of salt-bonds formed between Mb and PASP affect Mb release behavior.

Mb release behavior from PASP/gelatin complex is dictated in Figures 7 and 8. High pH-sensitive release of Mb from the complex can be observed (Fig. 7). At pH 7.4–5.0, Mb is delivered completely within 3 h. However, negligible Mb was detected in the dissolution fluid with pH ranged from 2.0 to 4.0. Only 2.1% Mb releases from PASP/gelatin complex at pH 4.0 for 4 days (results not shown). Below pH 2.0, Mb is rapidly liberated from the complex. The formation of PASP/gelatin complex takes place within pH 2.0–4.5 (Fig. 2). In addition, there are specific interactions between Mb and the complex matrices, revealed by the entrapment experiments (Table I). Therefore, Mb is retained in the complex when pH of the dissolution fluid ranges from 2.0 to 4.0. The dissociation of PASP/gelatin at pH $>$ 4.0 and $<$2.0 results in the rapid release of the incorporated Mb. The inset graph in Figure 7 shows dependence of Mb release from hydrogel and complex on pH. It can be apparently observed that Mb release from PASP/gelatin complex is more pH-sensitive than that from PASP hydrogel. The dependence of Mb release from PASP/gelatin complex on NaCl concentration in the dissolution fluid at

TABLE II
The Effect of Mb/Gelatin Ratio on Complex Yield and Mb Entrapment Efficiency[a]

Mb/gelatin ratio	PASP/gelatin ratio[b]	Complex yield (%)	Entrapment efficiency (%)	Entrapment (%, w/w)[c]
0.2	0.74	98.0	95.1 ± 0.5	10.93
0.4	0.79	97.8	98.9 ± 0.4	22.10
0.6	0.83	98.1	96.2 ± 0.6	31.54

[a] PASP concentration was 2 mg/ml, gelatin concentration was 0.4 mg/mL, and the pH was fixed at 3.5.
[b] Determined by colloid titration method (see experimental section).
[c] Calculated by the actual amount of Mb entrapped/the amount of complex.

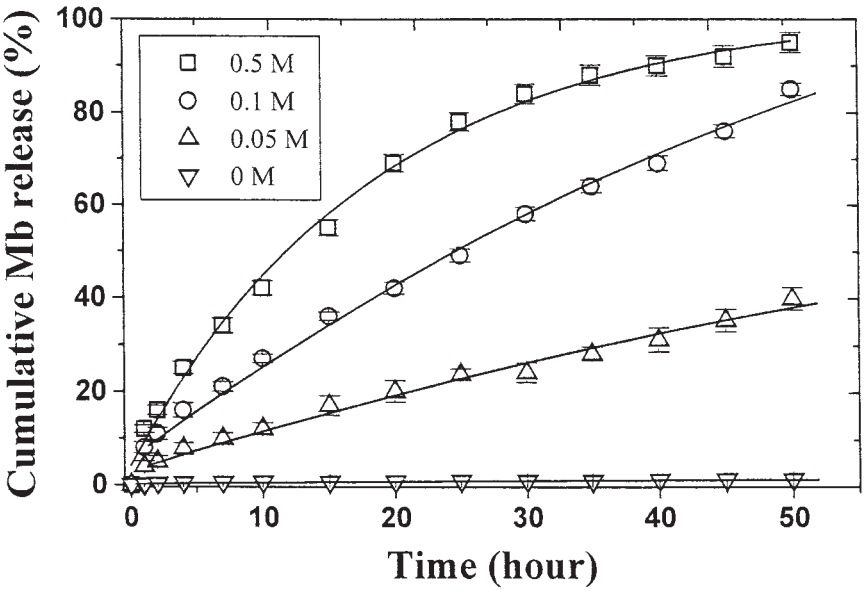


Figure 5 The effect of NaCl concentration on Mb release from PASP hydrogel at pH 5.0. Mb loading percent was 7.4% (n = 3).

pH 4.0 is shown in Figure 8. In contrast to that from PASP hydrogel, NaCl concentration has little effect on Mb release within the evaluated concentration. Mb can hardly be liberated from the complex when NaCl concentration ranges from 0.1 to 0.3*M*. Such results may be attributed to the occurrence of specific interactions between Mb and gelatin, such as hydrogen bond and hydrophobic interactions, which are insensitive to the salt concentration.

Pulsatile Mb release from the laminated device

During Mb release test, the laminated device composed of P(TMA-gly-*co*-SA)-*b*-PEG and PASP hydrogel deformed and the outer coating membrane ruptured because of the extensive swelling of PASP hydrogel; therefore, no Mb release data can be obtained. However, the device composed of polyanhydride and PASP/gelatin complex maintains its shape within the testing period. Figure 9 shows Mb release profile from the laminated device composed of polyanhydride and PASP/gelatin complex. Two separated pulses (each pulse about 12 h) after that followed by a lag time of 18 h with little Mb release can be observed. The release behavior is similar to that from a device composed of P(TMA-gly-*co*-SA)-*b*-PEG and poly(methacylic acid)/poly(ethoxazoline) complex and can also be attributed to the syn-

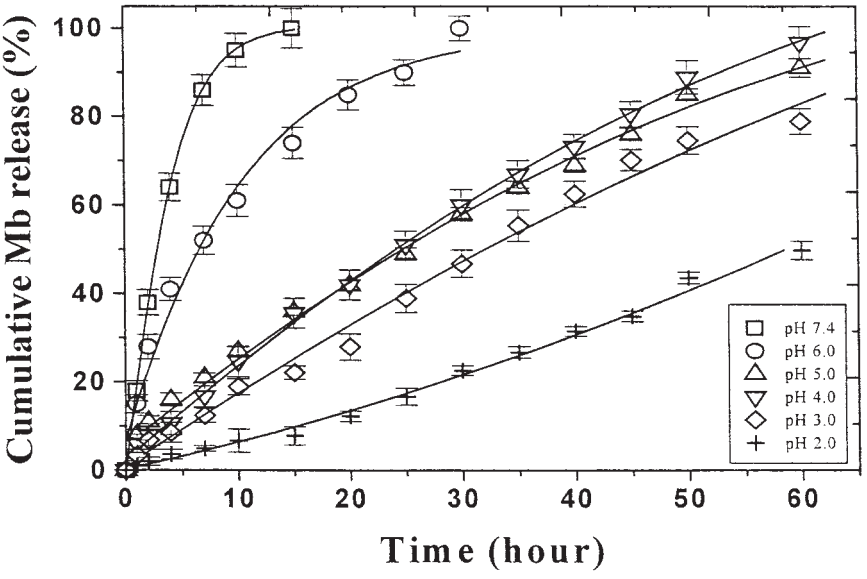


Figure 6 The effect of pH on Mb release from PASP hydrogel in 0.1*M* buffer solution. Mb loading percent was 7.4% (n = 3).

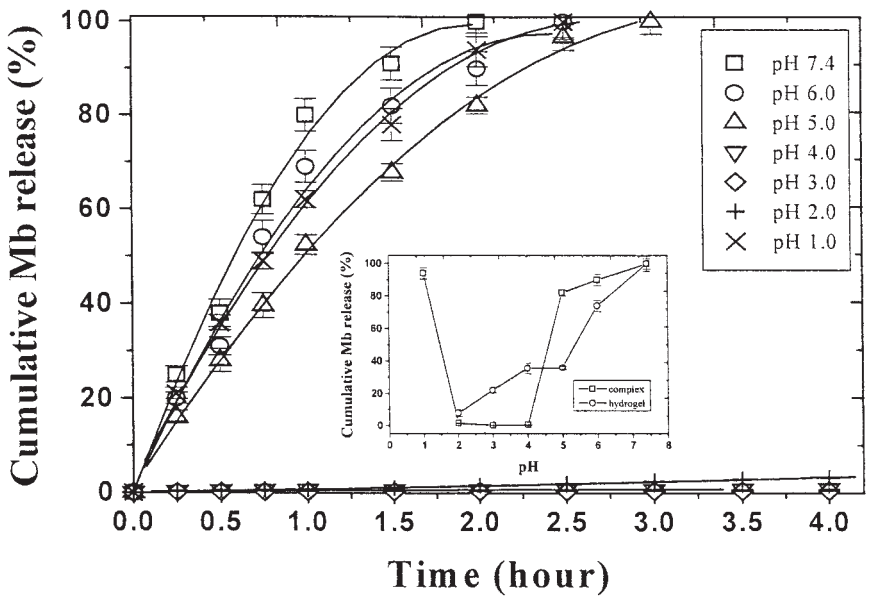


Figure 7 The effect of pH on Mb release from PASP/gelatin complex in 0.1*M* buffer solution. Mb loading percent was 10.93%. The inset graph shows the dependence of Mb release percent from PASP hydrogel (at 15 h) and PASP/gelatin complex (at 2 h) on pH (n = 3).

ergistic effect between polyanhydride degradation and erosion of the pH-sensitive complex, which has been discussed in detail previously.[11] When the device was soaked in pH 7.4 buffer solutions (Scheme 1), the outer PASP/gelatin complex layer dissociated and liberated the incorporated Mb, forming the first pulse of Mb release. When the complex eroded, the polyanhydride layer degraded and generated low pH environment (<4.0) within the device.[11] The low pH environment in the device stabilizes the inner pH-sensitive PASP/gelatin complexes and retains the incorporated proteins until the polyanhydride degrades completely. The erosion of the inner complex layer generated the second pulse of Mb release. The difference of Mb release duration from PASP/gelatin complex (2.0 h, Fig. 7) and the laminated device (12 h, Fig. 9) can be explained by the fact that the laminated device was

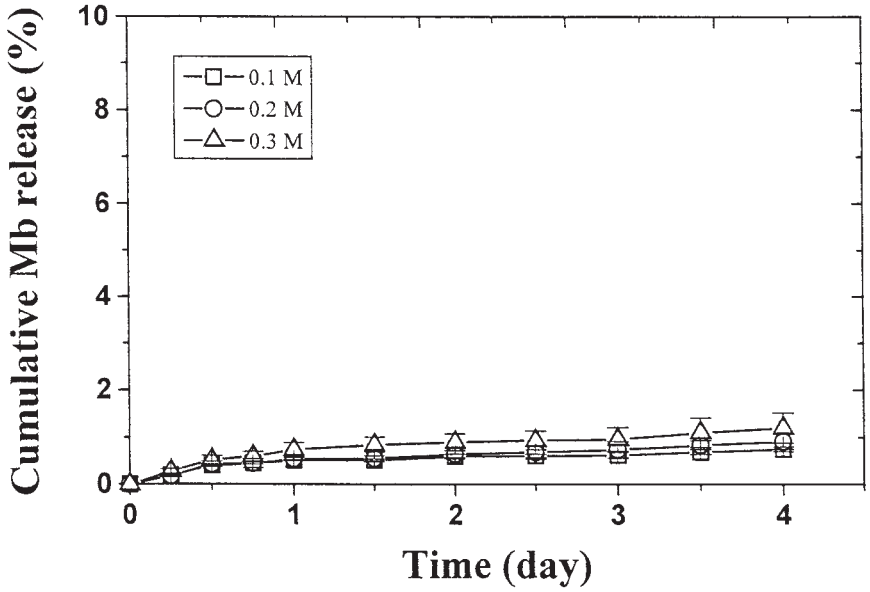


Figure 8 The effect of NaCl concentration on Mb release from PASP/gelatin complex at pH 4.0. Mb loading percent was 10.93% (n = 3).

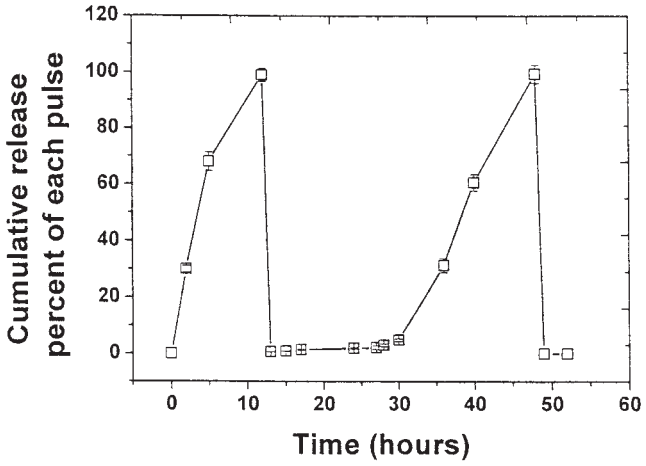


Figure 9 Mb release from the laminated device composed of P(TMA-gly-*co*-SA)-*b*-PEG and PASP/gelatin complex. Each complex layer was compressed with 20 mg of complex powder and 20 mg of polyanhydride was used for compression of a isolating layer ($n = 3$).

coated with hydrophobic membrane and the erosion of the complex layer can only occur through the side faced toward the aqueous media. The above results indicate the feasibility of combining biodegradable pH-sensitive PASP/gelatin complex with hydrophobic polymers for finely modulating protein release.

Mb assay

The maxima of the soret bands (407 nm) of Mb released from the laminated device, as well as β and α bands due to the $\pi \rightarrow \pi^*$ electronic transitions in heme (505 and 637 nm, respectively), correspond to the native state of oxidized horse Mb.[27] In addition, both the retention time and absorbency peak of Mb released are identical to those of native Mb as noted by capillary electrophoresis analysis, which indicates that there is no aggregation or degradation occurring during Mb entrapment into the complex and release from the laminated device (results not shown). On the contrary, Mb aggregation was observed when it was directly incorporated into P(TMA-gly-*co*-SA)-*b*-PEG matrices in previous study.[11] It was reported that the protein stability under various harsh conditions, such as acidic environment, organic solvents, etc., could be enhanced by complexation of proteins with polyelectrolytes.[28–31] It was also proposed that protein degradation and denaturation in the inner acidic environment of PLGA during degradation could be prevented by maintaining the protein as a native insoluble aggregate or excipient complex.[32] The above results also indicate that Mb complexation within PASP/gelatin complex can improve its stability under acidic/hydrophobic environment.

CONCLUSIONS

Both swelling of PASP hydrogel and complexation of PASP and gelatin depend on pH of buffer solution. Absorption of Mb into PASP hydrogel is affected by both pH and NaCl concentration and entrapment is incomplete even at the optimized conditions. In contrast, Mb can be incorporated into PASP/gelatin complex with both high loading percent and entrapment efficiency. Mb is liberated from PASP hydrogel gradually via ion exchange-driven mechanism. However, Mb can hardly be liberated from PASP/gelatin complex within pH 2.0–4.0. Pulsatile Mb release can be achieved from a laminated device composed of polyanhydride and PASP/gelatin complex. Biodegradable PASP/gelatin complex can be a promising pH-sensitive matrix for controlled protein release by combining biodegradable hydrophobic polymers.

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