Polyanion/Gelatin Complexes as pH-Sensitive Gels for Controlled Protein Release

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ABSTRACT: Polyanion/gelatin complexes including poly(methacrylic acid) (PMAA)/gelatin, poly(acrylic acid) (PAA)/gelatin, and heparin/gelatin are investigated as pH-sensitive gels for controlled protein release. Polyanions can interact with gelatin and form amorphous precipitates within a certain pH range, which is affected by the polyanion nature. The entrapment efficiency of model proteins (myoglobin, cytochrome *c,* and pepsin) into the complexes is rather high (>80%). By using a modified colloid titration that mixes a solution of gelatin and model proteins titrated with polyanion solution, myoglobin and cytochrome *c* are found to interact with polyanions by electrostatic forces at low pH, while pepsin either interacts with the polyanion when the pH is below its isoelectric point (IEP) or complexes with gelatin at a pH above IEP_{pepsin}. At pH 7.4 all the complexes dissociate and proteins are rapidly released within a few hours. The complexes are stable and the proteins are retained within a certain pH range, which is related to the polyanion type (e.g., 5.0–2.0 for PMAA, 4.6–1.2 for PAA, and <4.3 for heparin). The three processes of complex formation, dissociation, and protein release have a good correlation. In addition, the protein release transition takes place within a rather narrow pH range (ca. 0.5 units) and the protein nature has little effect on the protein release profile. The high protein entrapment efficiency and good pH sensitivity of the protein release can be mainly attributed to the electrostatic attractive interactions between proteins and polyanion or gelatin. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 80: 1416–1425, 2001

Key words: pH-sensitive gels; polyelectrolyte complex; protein/polyelectrolyte complex; protein entrapment; pH-sensitive release

INTRODUCTION

The pH-sensitive hydrogels are of interest for various biomedical applications, such as artificial muscles and oral drug delivery. Recently, we designed a new laminated device composed of polyanhydrides and pH-sensitive gels for pulsatile protein release.[1] The unique advantage of this type of device is the synergistic effect between the degrading polyanhydrides and pH-sensitive gels, and the protein pulsed release pattern (i.e., the lag time prior to each pulsed release and pulse duration) can be finely modulated by selecting suitable polyanhydrides and pH-sensitive gels. A key point in the design of this device is to explore a pH-sensitive gel that can maintain a low swelling degree and retain the incorporated proteins until the external environment reaches a critical pH value (about 5.0). Many polyelectrolyte gels that normally possess pendant ionic groups in their polymeric networks exhibit a broad transition of equilibrium swelling degrees as a function

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of pH.[2] By introducing hydrophobic groups into the polyelectrolyte backbone, the gels show an abrupt pH-driven swelling transition.[3,4] However, it was found that protein loading into these gels was time consuming, protein was released from these gels via an ionic exchange driven release mechanism (i.e., the protein release mainly depends on salt concentration), and the pH transition of protein release was rather broad. The protein release rate from polyelectrolyte gels is also strongly affected by the protein nature, especially the isoelectric point (IEP).[5,6] In addition, these hydrogels cannot degrade or be eliminated *in vivo*. Hydrogen-bonding macromolecular complexes were also investigated as pH-sensitive gels.[7–9] Although the swelling transition of the complex gels occurs within a rather narrow pH range, the pH sensitivity of protein release was not satisfied. For example, we found that the cytochrome *c* (Cyt *c*) release transition from a poly(methacrylic acid) (PMAA)/polyethoxazoline (PEOx) complex takes place within a broad pH range and protein nature has a strong effect on its release behavior (unpublished results). Therefore, there are still no pH-sensitive gels that can meet our specific needs. In the last two decades, polyelectrolyte complexes were widely studied. Among them, protein/polyelectrolyte complexes attracted more attention because of their potential applications, such as protein separation, enzyme immobilization and stabilization, and so forth.[10,11] It was confirmed that the interactions between polyelectrolytes and proteins are principally ionic.[12–17] In addition, there were numerous convincing studies on the retention of protein bioactivity by complexation of proteins with polyelectrolytes under various harsh conditions, such as a strong acidic environment and organic solvents.[11,13,17,18–20] Various types of polyanion/gelatin complexes were used for microencapsulation of water-insoluble drugs by the complex coacervation method.[21,22] However, little attention was paid to the use of polyanion/gelatin complexes as a globular protein matrix and the deliver of these drugs in response to the pH. The formation of polyanion/gelatin complexes may strongly depend on the pH because of the polyampholyte nature of gelatin. Because globular proteins are polyampholytes, too, they may be able to interact with polyanions or gelatin at low pH and be efficiently entrapped into polyanion/gelatin complexes. Furthermore, protein release strictly follows complex dissociation and the transition of protein release may be within a rather narrow pH range. Here we investigate the entrapment of three model proteins [myoglobin (Mb), Cyt *c,* and pepsin] with different IEPs into three types of complexes [PMAA/gelatin, poly(acrylic acid) (PAA)/gelatin, and heparin/gelatin] and their characteristics of pH-sensitive release.

EXPERIMENTAL

Materials

The PMAA and PAA were synthesized through free-radical polymerization of MAA and AA in our lab (MW = 38,000 for PMAA and 20,000 for PAA). The heparin (MW = 11,000, from porcine intestinal mucosa) was a gift from Jiuyuan Genetic Engineering Co. The gelatin (type B, IEP = 4.9) was purchased from Shanghai Chemical Co. The Mb (from horse heart), Cyt *c* (from bovine), and fluorescein isothiocyanate dextran (FITC-dextran, MW 71,200) were supplied by Sigma Chemical Co. The pepsin was obtained from Shanghai Chemical Co.

Turbidimetric Titration

Turbidity titrations were carried out at 37°C.[16,23] The turbidity was followed with a UV–vis spectrophotometer (Shimadzu 1201) at 420 nm and reported as 100-%turbidity, which was linearly proportional to the true turbidity for $T > 0.9$. Solutions were gently stirred, and the turbidity values were obtained after several minutes of stabilization in all cases. Two types of titration were involved in this study. In type I titration an 0.1N HCl solution was added to an initial mixed solution of polyanions and gelatin. The polyanion/gelatin weight ratio was fixed at the value where the maximum complex yield was obtained at pH 3.5 (i.e., PMAA/gelatin = 1.0, PAA/gelatin = 0.72, and heparin/gelatin = 0.25). A pH meter was used to monitor the pH change during titration. In colloid titration a mixed solution of gelatin and model proteins was titrated with an aqueous polyanion solution. The pH of the mixed solution was adjusted to the same value as that of the polyanion solution. The turbidity was recorded as a function of the titrant volume (V_t), and the end point of titration was indicated by the maximum turbidity.

IR Spectroscopy

The IR spectra[13] of native and complexed Mb were investigated to obtain information about the

formation of the linkages between the basic groups in the protein and the carboxy groups in PMAA. The mixed PMAA/Mb solution (pH 6.99, Mb/PMAA ratio = 0.05 by weight) was dried in a vacuum at room temperature. The uncomplexed Mb as a control sample was obtained by drying an aqueous solution adjusted to pH 6.99. The spectra measurements for both samples in a KBr disk were made with a Nicolet DX spectrometer.

Complex Preparation

Model proteins were dissolved in 100 mL of the 2.5 mg/mL mixed polyanion/gelatin solution (same polyanion/gelatin ratio as described above).[7] The complexes were formed by adjusting the pH of the solution to 3.5 with a 0.1*N* HCl solution under magnetic stirring; then they were centrifuged, washed with double-distilled water, and dried in a vacuum at room temperature.

Determination of Protein Entrapment in Complexes

The protein entrapment in the complexes was determined by the following procedures. After the pH of the mixed polyanion/gelatin solution was adjusted to 3.5, the sample was filtrated with a membrane filter (0.22-μm pore size). The protein concentration in the filtrate was measured with a UV–vis spectrophotometer at 420 nm for Mb and 410 nm for Cyt *c*. A calibration curve obtained by plotting the absorbency of the standard protein solution (pH 3.5) against the protein concentration was employed. The pepsin concentration was determined by high pressure liquid chromatography (HPLC, Toyo Soda Co., Tokyo). The protein entrapment in the complexes was calculated from the differences between the initial and the remaining protein in the filtrate.

Complex Dissociation and Protein Release

The complexes were ground with a mortar and pestle and sized with sieves; 20 mg of the complex granules (38–125 μm) were compressed into a disk (2.2-mm diameter, 2.0-mm thickness) by compression molding at 100 kg/cm^2 and 37°C. The blank or protein-loaded disks were immersed in 5 mL of 0.1*M* buffer solution with various pHs at 37°C. The dissolution fluid was removed and fresh solution was added periodically. The amount of gelatin released from the blank complexes was determined by a bicinchoninic acid (BCA) protein microassay. Model protein release was monitored at 420 nm for Mb and 410 nm for Cyt *c*. Pepsin release was detected by HPLC.

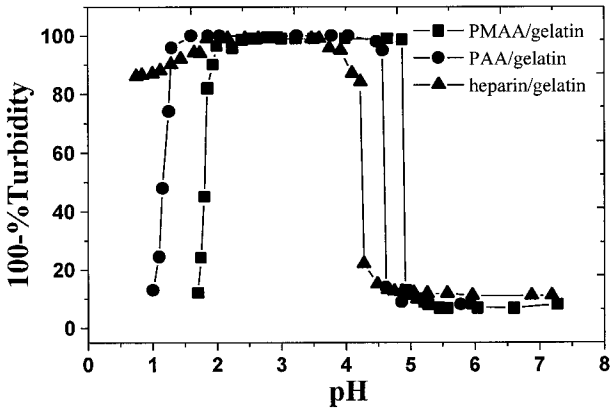

Figure 1 The turbidity changes of polyanion/gelatin systems versus the pH. The titration was with 0.1*M* HCl solution; [gelatin] = 8 mg/mL; [PMAA/gelatin] = 1.0; [PAA/gelatin] = 0.72; [heparin/gelatin] = 0.25 (n = 3).

RESULTS AND DISCUSSION

Polyanion/Gelatin Complex Formation

Figure 1 shows the type I turbidity titration curves of PMAA/gelatin, PAA/gelatin, and heparin/gelatin. The turbidity of the solutions is invariant with respect to the pH until the pH reaches a critical value (pH_Φ, 4.8, 4.5, and 4.2 for PMAA/gelatin, PAA/gelatin, and heparin/gelatin, respectively) at which abrupt changes are observed. As the pH progresses toward a lower critical value (designated pH'_Φ, 1.8 for PMAA/gelatin, 1.2 for PAA/gelatin), solutions of PMAA/gelatin and PAA/gelatin become optically clear while heparin/gelatin still remains turbid. The turbidity transition takes place within a rather narrow pH range (ca. 0.1 units). The above results indicate that the complex formation depends on the pH. Below its IEP (4.9), gelatin carries net positive charges, which can form salt bridges with negative charges on the polyanions, yielding water-insoluble complexes at pH_Φ. In addition, it can be seen that the pH_Φ is affected by the polyanion type. Park et al. studied the process of type I turbidity titration for various polyanion/protein pairs and found that the onset of association upon the addition of HCl to protein/polyanion solutions occurred at a well-defined pH (designated as pH_c, always above the IEP of the protein) for each

Table I Entrapment and Entrapment Efficiency of Model Proteins in Three Types of Polyanion/Gelatin Complexes

	Mb			Cyt *c*			Pepsin		
Protein Polyanion	PMAA	PAA	Heparin	PMAA	PAA	Heparin	PMAA	PAA	Heparin
Complex yield (%)	87.4	83.5	86.4	80.4	82.5	75.4	82.3	84.5	79.6
Entrapment (%)	19.2	19.6	18.8	19.4	19.7	19.7	18.3	18.8	18.2
Entrapment efficiency (%)	95.9	98.1	93.8	98.2	98.7	98.4	91.5	94.0	91.0

The protein/polyanion weight ratio was 0.4; the gelatin concentration was 0.25%; [PMAA/gelatin] = 1.0; [PAA/gelatin] = 0.72; [heparin/gelatin] = 0.25. The ultimate pH value was 3.5 (n = 3).

polymer/protein pair.[24] The pH_c corresponds to the formation of soluble polyanion/protein complexes and is followed, upon progressive pH decreases, by phase separation at pH_Φ (always below the IEP of the protein). At pH_Φ, water-soluble polyanion/protein complexes aggregate to form large moieties due to charge neutralization or hydrophobic interactions. Several factors affect the pH_Φ value, such as the linear charge density, molecular weight, polyanion structure, and protein/polyanion ratio, and a parameter correlating well with the pH_Φ has not been attainable so far. Because of the rather complicated phase separation process and the significant structure difference of the present studied polyanions, an attempt to correlate the titration process with the net charges presented by polyanions and gelatin was not the center of our investigation. The decrease in turbidity observed at the lower pH (<2.0) for PMAA/gelatin and PAA/gelatin revealed dissociation of the complexes. At lower pH, ionization of PMAA and PAA was depressed[25] and their charge density was too low to form salt bridges with gelatin. The pH'_Φ of PAA/gelatin was lower than that of PMAA/gelatin because of the stronger acidity of PAA than PMAA (pK_a of PAA and PMAA = 4.7 and 6.15,[25] respectively). For heparin/gelatin, this phenomenon was not observed because of the relatively strong acid nature of heparin. (The pK_a of heparin is ca. 1.94).[26] In addition, we noticed that the pH'_Φ for PMAA/gelatin and PAA/gelatin pairs was rather low, although PMAA (or PAA) can hardly dissociate below pH 3.0,[25] which can be attributed to the so-called induced effect for weak polyanions in the presence of polycations or polyampholytes.[27]

The turbidity of the mixed solution decreased with the increase in NaCl concentration and there was no phase separation occurring when 1*M* NaCl was added, indicating the main interaction between polyanions and gelatin was electrostatic.

The complex precipitates were isolated and dried in a vacuum. Large hard lumps were obtained, which could be ground to granules (<125 μm). Consequently, either implants or injectable granules of protein-loaded polyanion/gelatin complexes could be prepared to meet different needs. The gelatin content in the complexes was determined by BCA protein microassay method, and the composition of the complexes was calculated. The polyanion/gelatin ratio in the complexes nearly equaled the feed ratio, which corresponded to the maximum complex yields at the preparative conditions (the given feed ratio and ultimate pH value).

Protein Entrapment

The protein entrapment efficiency into matrices, either hydrophobic biodegradable polymers or hydrogels, was always low.[28,29] It was essential to explore a suitable matrix or method to circumvent this limitation. Table I shows the entrapment efficiency and entrapment of the three model proteins in three types of polyanion/gelatin complexes. Rather high entrapment efficiency was obtained for all model proteins in the three polyanion/gelatin complexes (>90%). There was no great discrepancy of the complex yield, protein entrapment efficiency for the three model proteins and three polyanion/gelatin complexes. It should be addressed here that the low complex yields observed were due to the operational loss; for example, the small complex particles could not be completely collected by centrifugation, while the protein entrapment efficiency was determined indirectly from the differences between the initial and the remaining protein in the filtrate, result-

Table II Dependence of Mb Entrapment on Mb/PMAA Ratio

Mb/PMAA	0.1	0.2	0.4	0.6
Complex yield (%)	89.3	88.6	89.2	79.5
Entrapment (%)	4.69	9.28	17.34	24.3
Entrapment efficiency (%)	93.79	92.85	86.67	80.12

PMAA/gelatin = 1.0; the gelatin concentration was 1.25%, and the ultimate pH was 3.5 (n = 3).

ing in the lower observed values for the complex yield than those for the protein entrapment efficiency. The dependence of the Mb entrapment and entrapment efficiency on the Mb/PMAA ratio is displayed in Table II. The lower the Mb/PMAA ratio, the higher the Mb entrapment efficiency and the lower the entrapment. Similar results were also reported by Calvo and colleagues who studied the entrapment of bovine serum albumin in chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles.[30] However, even with the large Mb/PMAA ratio employed, the high entrapment efficiency of Mb in PMAA/gelatin was still achieved (>80%) with high entrapment (ca. 24% by weight).

In contrast to the high entrapment efficiency of the model proteins in the polyanion/gelatin complexes, the entrapment efficiency of the nonionic polysaccharide (FITC-dextran) in the polyanion/gelatin complexes was rather low (<30%), which was possibly due to the lack of electrostatic attractive interactions with the polyanions or gelatin.

Interactions between Model Proteins with Polyanions or Gelatin

Wide attention has been paid to the electrostatic attractive interactions between polyanions and globular proteins at low pH and numerous polyanion/protein pairs have been found to form complexes that may exist in different forms such as soluble complexes, coacervates, or amorphous precipitates.[17] Type I turbidity titrations of model proteins/polyanions or model proteins/gelatin mixed solutions were conducted. The mixed solutions remained clear and no phase separation occurred over the studied pH range of 1.0–7.4; that is, the two model proteins could not form either coacervates or amorphous precipitates with polyanions and gelatin, respectively.

Colloid titration is widely used to study the stoichiometric complexation for water-insoluble polyelectrolyte/protein pairs. However, this method cannot be adopted for water-soluble complexes. In order to obtain information about the interactions of the model proteins with polyanion or gelatin, modified colloid titrations with mixed solutions of gelatin and protein titrated by PMAA solution were performed. The protein/gelatin ratio in the mixed solution was varied, maintaining the amount of gelatin constant, and the corresponding PMAA volume at the end point of titration was recorded. The results of the colloid titration for Mb and Cyt c at pH 4.0 are shown in Figure 2, which demonstrates that the PMAA volume at the end point of titration increases linearly with the increase in the two model proteins/gelatin ratio while the PMAA volume at the end point of titration did not change with the dextran/gelatin ratio because of the nonionic nature of dextran. The above results suggest that at the end point of titration the excess polyanions, except what was needed to neutralize the gelatin molecules, interacted with the model proteins by electrostatic force. At low pH, Mb and Cyt c carry positive charges (IEP_{Mb} was 6.99, 9.8 for Cyt c), which can form salt bridges with negative charges presented by PMAA molecules. The linear relationship between the polyanion volume at the end point of titration and the model protein/gelatin ratio indicated the stoichiometric complexation of the polyanion with the proteins. The δPMAA/protein ratio can be calculated to be 0.98 for Mb and 0.70 for Cyt c. (δPMAA is the subtraction of the PMAA amount

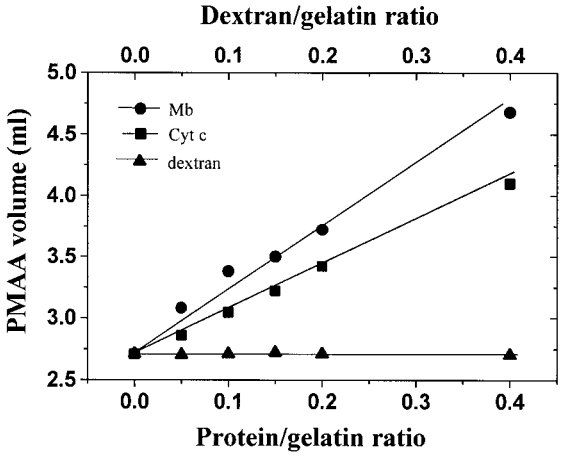

Figure 2 The dependence of the titrant volume of PMAA at the end point of colloid titration on the protein/gelatin ratio at pH 4.0; [PMAA] = 2 mg/mL; [gelatin] = 0.4 mg/mL, $V_{gelatin}$ = 25 mL (n = 3).

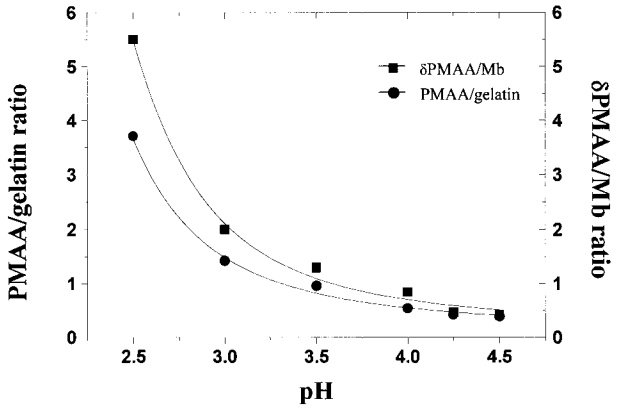

Figure 3 The effect of the pH on the PMAA/gelatin and δPMAA/Mb ratio at the end point of colloid titration in the absence and presence of Mb, respectively. The δPMAA was the subtraction of the PMAA amount at the end point of colloid titration in the presence and absence of Mb. [PMAA] = 2 mg/mL; [gelatin] = 0.4 mg/mL; V_{gelatin} = 25 mL (n = 3).

at the end point of titration in the presence and absence of model proteins, which can be regarded as the amount of PMAA interacting with the model proteins by electrostatic attractive force.) The Mb carries 2.03 mmol/g basic groups and Cyt *c* has 1.96 mmol/g, which may result in the fact that the δPMAA/Mb ratio is larger than δPMAA/Cyt *c* at pH 4.0. Quantitative calculation of the above δPMAA/protein ratio by the net charge carried by PMAA and proteins at pH 4.0 is complicated because of the weak acid nature of the studied polyanion, which has larger charge densities in the presence of a polycation or polyampholyte than the neat polymer because of the induced effect.[27]

Figure 3 shows the effect of pH on the PMAA/gelatin and δPMAA/Mb ratios at the end point of titration in the absence or presence of Mb. It can be seen that at pH 3.5 the PMAA/gelatin ratio is about 1.0, which correlates well with the maximum complex yield under the same conditions (see Experimental section). In addition, the PMAA/gelatin and δPMAA/Mb ratios both increase with the decrease in pH. Similar results were also reported for other polyanion/protein pairs.[12,13,16] It is well known that the charge densities of both PMAA and Mb depend on the pH because of their weak polyelectrolyte nature, which decreases as the pH progresses toward a low value for PMAA and increases for Mb. Therefore, when the pH value decreases, more PMAA is needed to neutralize Mb molecules, which convinces us of the ionic nature of the interactions between Mb and PMAA. It can also be seen that the PMAA/gelatin ratio is always smaller than the δPMAA/Mb ratio at the same pH, which may result from the higher content of basic groups for Mb. (The basic groups of gelatin are ca. 0.939 mmol/g.[31]) The trends of the colloid titration with Mb (or Cyt *c*)/gelatin mixed solution titrated with PAA or heparin are similar to that with PMAA. In addition, it was observed that no phase separation occurred for the PMAA/gelatin/Mb system following the addition of 1.0*M* NaCl into the mixed protein/gelatin solution, which was due to the charge-shielding effect of the microsalts and further substantiated the electrostatic interactions between PMAA and gelatin or Mb.

The electrostatic attractive interactions between PMAA and Mb can also be confirmed by IR analysis. An IR sample for the PMAA/Mb complex was prepared at IEP_{Mb} in order to remove the protons from the protonated basic groups in the Mb, which were free of salt linkages with PMAA anions. Figure 4 compares the spectra of the PMAA/Mb complex and native Mb. A new absorption (shoulder) exists at 2700–2400 cm^{-1} in the spectra of the PMAA/Mb complex, which can be assigned to amine salts (mainly $—\text{NH}_3^+$) and indicates that the complexation takes place through the formation of the salt bridges between the protonated basic groups in Mb and the carboxy ions in PMAA. Such results are similar to those of poly(vinyl alcohol sulfate)/trypsin pairs reported

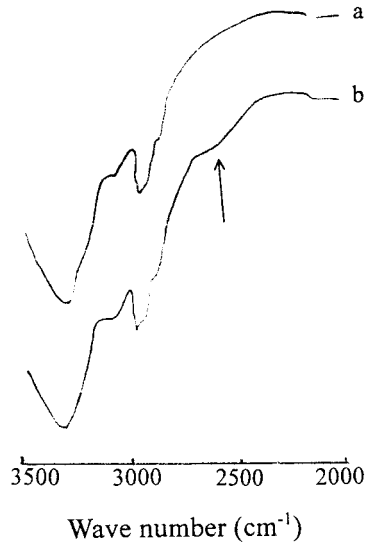

Figure 4 IR spectra of the native Mb (spectrum a) and the PMAA/Mb complex (spectrum b) in the KBr disk. The preparation methods of both samples are described in the text.

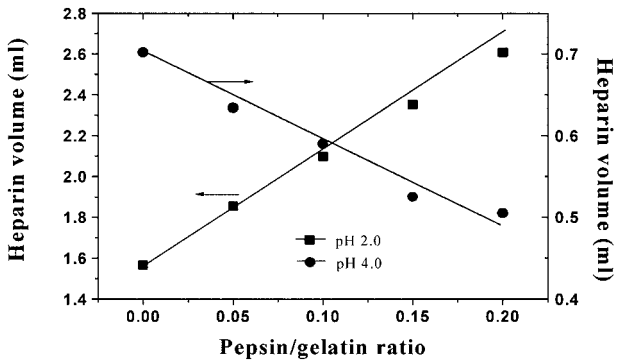

Figure 5 The dependence of the titrant volume of heparin at the end point of colloid titration on the pepsin/gelatin ratio at pH 2.0 and 4.0; [heparin] = 4 mg/mL; [gelatin] = 0.4 mg/mL; $V_{gelatin}$ = 25 mL (n = 3).

by Kokufuta and Takahashi.[13] The results of the characterization of water-soluble polyanion/model protein complexes by dynamic light scattering will be reported elsewhere.

Figure 5 shows the results of colloid titration with a pepsin/gelatin mixed solution titrated with heparin at pH 2.0 and 4.0. It can be seen that the heparin volume at the end point of colloid titration increases with the increase in the pepsin/gelatin ratio at pH 2.0, while the reverse trend is observed at pH 4.0. At pH 2.0 pepsin carries positive charges (IEP_{pepsin} = ~2.5, according to Tsuboi et al.[16]), similar to the situation for Mb/gelatin or Cyt *c*/gelatin solution titrated by PMAA at pH 4.0, which can neutralize negative charges carried by heparin, resulting in more heparin being needed at the end point of titration as the pepsin/gelatin ratio increases. At pH 4.0 pepsin presents negative charges, which can form salt bridges, and part of the positive charges are presented by the gelatin molecules. Thus, less heparin is needed to neutralize gelatin. Similar results were obtained for the pepsin/PAA/gelatin system. Tabata et al. also found that basic fibroblast growth factor could ionically interact with negatively charged, acidic gelatin.[32]

Complex Dissociation and Protein Release

The dissociation behavior of the blank complexes is shown in Figure 6, which demonstrates that dissociation of the complexes is greatly affected by the pH. The complexes remain stable at low pH (<5.0) while they dissociate rapidly within 4 h at pH 7.4. Dissociation also takes place at lower pH (1.0) for PMAA/gelatin and PAA/gelatin. The Mb release behavior in various pH buffer solutions is depicted in Figure 7. Similarly, the protein delivery strongly depends on the pH. At pH 7.4 Mb releases rapidly within a few hours, while the release rate is rather low below pH 5.0 (<10%

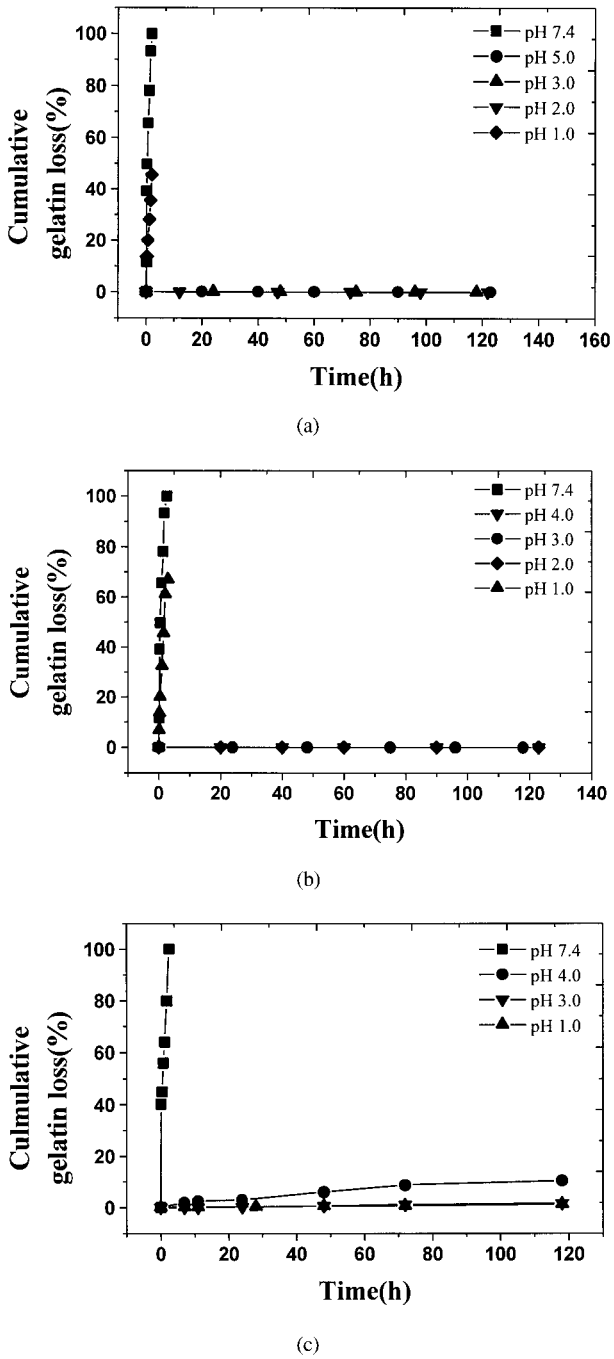

Figure 6 Gelatin loss curves from the polyanion/gelatin complex: (a) PMAA/gelatin, (b) PAA/gelatin, (c) heparin/gelatin. There was 20 mg of complexes in 0.1*M* buffer at 37°C (n = 3).

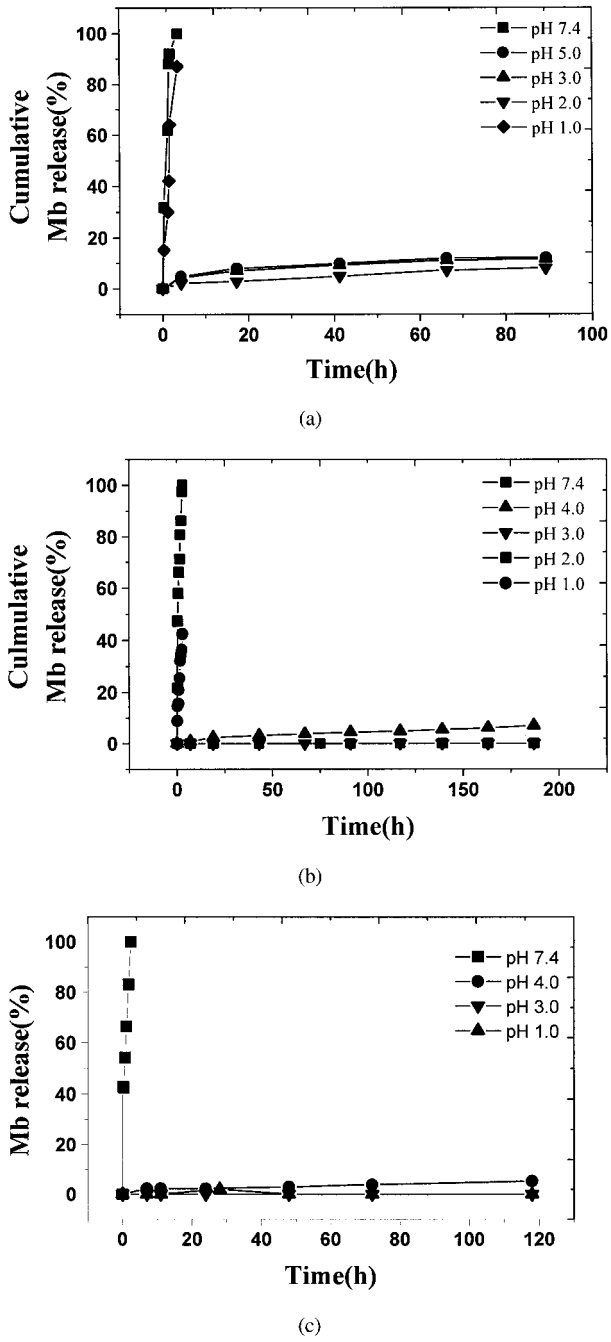

Figure 7 Mb release curves from polyanion/gelatin complexes with about 19.0% Mb entrapment: (a) PMAA/gelatin, (b) PAA/gelatin, (c) heparin/gelatin. There was 20 mg of Mb-loaded complexes in 0.1*M* buffer at 37°C ($n = 3$).

within 120 h). The differences in Mb release from PMAA/gelatin, PAA/gelatin, and heparin/gelatin complexes at pH 1.0 are also observable with the rapid release from PMAA/gelatin or PAA/gelatin and no release from heparin/gelatin. The results of Cyt *c* and Pepsin release from three types of polyanion/gelatin complexes are similar to that of the Mb release (results not shown). As discussed above, at low pH Mb and Cyt *c* interact with polyanions by electrostatic attractive force; pepsin complexes either with polyanions or gelatin and polyanion/gelatin complexes remain stable, resulting in a rather low release rate of proteins from the complexes. At pH 7.4 all polyanion/gelatin complexes become dissociable, rendering the protein liberated. Moreover, depression of ionization of the weak polyanion (PMAA and PAA) at lower pH (1.0) leads to the release of gelatin and the model proteins from the complexes. In contrast to the pH-sensitive protein release from the polyanion/gelatin complexes, the pH of the dissolution fluid has little effect on FITC-dextran release (>60% within 6 h for $1.0 < \text{pH} < 7.4$), further supporting the above conclusions.

The dependence of PMAA/gelatin complex formation, dissociation, and Mb release on pH is shown in Figure 8. It can be seen that the transition of protein release takes place within a rather narrow pH range. For instance, only 5% Mb releases from the PMAA/gelatin complex at pH 5.0 within 6 h, while 68% Mb is liberated at pH 5.5, which correlates well with the dissociation profile of the complex. In addition, the pH range of the two above transitions agrees with that of the turbidity variation. Similar results were also found for the other two types of complexes. For example, heparin/gelatin complex forms when the pH is below 4.2, dissociates rapidly above pH 4.3, and the model proteins release from the complex only at a pH of > 4.3. As for the

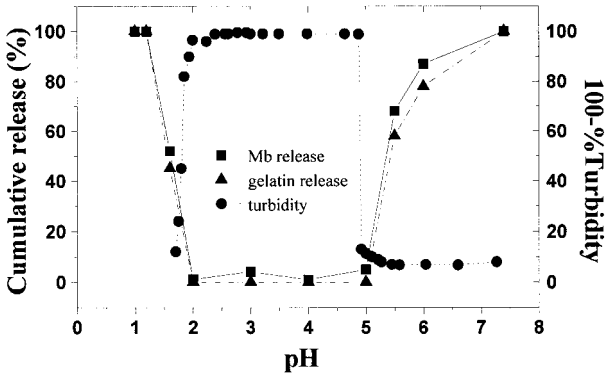

Figure 8 The dependence of PMAA/gelatin complex formation, dissociation, and Mb release on the pH. The complex dissociation was determined by the gelatin release at 6 h, and the Mb release percent was also obtained at 6 h.

PAA/gelatin complex, the pH range of complex formation is 4.5–1.2, and 4.6–1.2 for maintaining both a stable complex and protein retention. It is obvious that the complex dissociation is the reverse process of complex formation and the protein release is concomitant with the complex dissociation attributable to the electrostatic attractive interactions of proteins with polyanions or gelatin, which results in the good correlation between the three different processes. Such a correlation makes it possible to obtain a specific pH range of protein release just by investigating the profile of type I turbidity titration of a suitable polyanion/gelatin system. We observed that the protein release profiles from the complexes were nearly superimposable for 0.1 and 0.2*M* ionic strength (results not shown). Because the gastrointestinal ionic strength is about 0.15*M* and the pH is about 2.0 in the stomach and 7.4 in the intestine,[33] a PAA/gelatin and heparin/gelatin complex may be used for intestine protein delivery.

The maxima of the Soret bands (407 nm) of Mb released from the polyanion/gelatin complexes, as well as the β and α bands due to the $\pi \rightarrow \pi^*$ electronic transitions in the heme (505 and 637 nm, respectively), corresponded to the native state of oxidized horse Mb.[34] In addition, the enzymatic activity of the pepsin released from the complexes was measured to be the same as that of the original enzyme. Such results indicated that the proteins released from the polyanion/gelatin complexes remained unchanged.

CONCLUSION

Three types of polyanion/gelatin complexes were investigated to incorporate proteins and deliver protein in response to pH. Three model proteins could be efficiently loaded into complexes with high entrapment. By using the modified colloid titration method, we found that the model proteins could complex with polyanions or gelatin by electrostatic attractive interactions. At pH 7.4 all the complexes dissociated and proteins were rapidly released. The heparin/gelatin complex remained stable and retained the entrapped protein below pH 4.3, the range of which was 5.0–2.0 for PMAA/gelatin and 4.6–1.2 for PAA/gelatin. The complex formation, dissociation, and protein release had a good correlation. The transition of protein release took place within a rather narrow pH range. In addition, the protein nature had little effect on the protein release. The high protein entrapment efficiency and good pH-sensitive release can be attributed to the complexation between proteins with polyanions or gelatin. Such characteristics of polyanion/gelatin complexes as easy preparation, high entrapment efficiency, good pH sensitivity, and erodibility make them useful in the design of a new laminated device for pulsatile protein release, as well as in oral protein delivery.

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