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Enzymatic desorption of layer-by-layer assembled multilayer films and effects on the release of encapsulated indomethacin microcrystals

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Abstract—Polyelectrolyte multilayer films were prepared through layer-by-layer (LbL) self-assembly of chitosan (CHI) and pyrene labeled poly(2-acrylamido-2-methylpropanesulfonic acid) (APy). After incubation in an enzyme pepsin solution, multilayer films were partially destroyed as detected by a decrease in fluorescence intensity due to enzymatic degradation of CHI and desorption of APy. The multilayer desorption rate was the highest at pH 4.0. Increasing temperature from 20 °C to 60 °C accelerated desorption. The enzymatic desorption was also observed from microcapsule walls made of CHI/alginate (ALG) multilayer films directly deposited on indomethacin (IDM) microcrystals by LbL self-assembly. After pepsin erosion, the IDM release from the microcapsule monitored by UV absorbance was obviously accelerated due to desorption. The influence of incubation time, pH, and temperature of the pepsin solution on the IDM release was investigated. The release rate was the fastest after incubation in the pepsin solution at pH 4.0 due to the highest activity of pepsin. Increasing incubation temperature from 20 °C to 60 °C, however, slowed down the release rate, which was considered to be due to the formation of more perfect and compact multilayer films through the chain rearrangement at higher temperatures. The CHI/ALG multilayer film was found to maintain its barrier function to the IDM diffusion even after 6-h incubation in the pepsin solution.

Keywords: Chitosan; Pepsin; Layer-by-layer; Enzymatic desorption; Controlled release

1. Introduction

Efficient micro-encapsulation of active ingredients, such as drugs, proteins, vitamins, flavors, gas bubbles, even living cells, is becoming increasingly important for a wide variety of applications from functional foods to drug delivery for biomedical applications.[1–5] In the last decade, layer-by-layer (LbL) self-assembly technique has been developed as a powerful method for the nano- and micro-encapsulation,[6,7] where polyelectrolyte multilayer films were elaborated on various particles through alternating deposition of oppositely charged polyelectrolytes.[8–14] Coverage with polyelectrolyte multilayer films on drug microcrystals can effectively control the diffusion rate of the drug from the interior to the outside. Thus, LbL multilayer microcapsules have been intensively used in drug controlled release. The multilayer encapsulation of ibuprofen (IBU),[15–17] furosemide,[18] vitamin K_3, insulin,[19,20] dexamethasone,[21,22] and indomethacin (IDM)[23] has been reported for prolonged release. Recently, we found that simply increasing the deposition temperature from 20 °C to 60 °C efficiently reduced the release rate of encapsulated IDM microcrystals owing to the increase in thickness and perfectness of chitosan/sodium alginate (CHI/ALG) multilayer films.[24] We proposed two new combination methods for sustained drug release of IBU. One was that IBU was adsorbed into porous $CaCO_3$ microparticles, and then the IBU-loaded microparticles were encapsulated with protamine sulfate/sodium

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poly(styrene sulfonate) (PRO/PSS) multilayer films through LbL self-assembly.[25] The other was that IBU-loaded poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) microparticles were prepared by conventional solvent evaporation method, and then the drug-loaded microparticles were encapsulated with CHI/ALG multilayer films.[26]

For the promising applications in the drug delivery field, biocompatible and non-toxic polyelectrolyte materials are the most fundamental conditions. Thus, the biopolymers are the most competitive candidates to use as absorption species for multilayer film microcapsules. But most of the biopolymers, such as chitosan and gelatin, are biodegradable in vivo, which is likely to cause damage to multilayer film microcapsules. Hence, it is necessary to investigate the stability of biopolymer multilayer film microcapsules in the body environments. The enzymatic degradation of planar multilayer films has been reported by some groups.[27–31] However, there are up to now few reports concerning the effect of enzymatic degradation of multilayer film microcapsules on their controlled release properties.[32,33]

In this work, based on our successful adjustment to the release rate by changing deposition temperature for the ALG/CHI multilayer film microcapsules directly on IDM microcrystals,[24] we turned to investigate the enzymatic decomposition of the multilayer films induced by incubation in pepsin solution. Fluorescence labeled polyelectrolytes were used to make the multilayer film desorption detectable. The effects of enzymatic desorption on the release rate of model drug IDM from the ALG/CHI multilayer film microcapsules were investigated.

2. Materials and methods

2.1. Materials

Chitosan (CHI, molecular weight (MW) 15,000, Fluka), pepsin (Sigma) and polyethyleneimine (PEI, MW 25,000, Aldrich) were used as received. Alginate (ALG, Kimitsu Chemical Industries Co., Japan) was dialyzed and freeze-dried before use and its MW was 120,000 as determined by gel permeation chromatography (GPC) with a Waters apparatus using 0.1 mol/L of Na_2SO_4 aq soln as the eluant and narrowly distributed poly(ethylene oxide) as the standard. Poly(2-acrylamido-2-methylpropanesulfonic acid) labeled with 3 mol % of pyrene (APy) was synthesized in our laboratory as described in our previous paper.[34] Indomethacin (IDM, MW 357.8, Sigma) was gently ground into powder of micrometer with a pestle and mortar. Highly purified water was produced by deionization and filtration with a Millipore purification apparatus (resistivity >18.2 MΩ cm). Other chemicals were all analytical reagents and used as received.

Table 1. The viscosity change of chitosan solutions after the enzyme treatment

Enzyme	Control	Pepsin	Cellulase	Lipase	Lysozyme
$[\eta]$/mL/g	740.8	93.5	134.4	152.0	222.4
Decreasing percent/%	—	86.2	82.1	79.8	70.4

2.2. Enzyme treatment of chitosan

Four kinds of enzyme solns of pepsin, cellulase, lipase, and lysozyme (2 mL, pH 4.0, 5 g/L) were added to 20 mL 1 wt % chitosan solns, respectively, which was kept at 37 °C for 6 h. Then the viscosity of the chitosan solns were determined at 25 ± 0.05 °C, using a Ubbelohde viscometer (Table 1).

2.3. Fabrication of multilayer films

In order to detect the multilayer desorption induced by pepsin enzymatic degradation through monitoring fluorescence intensity, the multilayer film of CHI/APy containing fluorescent labels were prepared on a flat quartz slide as the substrate. Prior to use, the quartz slide was cleaned with the 'piranha' soln (7:3 98%H_2SO_4–30%H_2O_2) at 80 °C for 1 h and then dipped into 5:1:1 water–30%H_2O_2–29%NH_3 at 70 °C for 1 h accompanied with pure water rinse after each treatment.[34] The concentration of aqueous CHI and APy deposition solns was 1 g/L with 0.5 M NaCl and the CHI soln contained 0.3 vol % of AcOH for dissolution. Concentration of the PEI aq soln, used for the first layer, was 1 g/L without NaCl. Polyelectrolytes were alternately deposited on the quartz slide to form flat multilayer films PEI/Apy/$(CHI/Apy)_5$ by dipping the slide into a deposition soln for 20 min and rinsing with pure water (×3) for one layer.

Polysaccharides ALG and CHI were directly deposited on the IDM microcrystal of about 5 μm using layer-by-layer (LbL) self-assembly in aq solns as described previously.[24] The first layer was deposited by adding 1 mL of ALG soln (1 g/L, 0.5 M NaCl) into a dispersed soln of 100 mg IDM microcrystal at pH 4.0 adjusted by AcOH. The mixture was incubated for 15 min under gentle shaking. The excess ALG was removed by two refining circles of centrifugation (5000 rpm, 5 min)/washing/re-disperse in water. The following CHI layer was deposited in the same procedure with 1 mL of CHI soln (1 g/L, 0.5 M NaCl, 0.3 vol % AcOH). Alternate ALG and CHI layers were deposited successively in an identical way and microcapsule IDM-$(CHI/ALG)_5$ was achieved. To prevent the IDM from dissolving during the LbL process, the rinsing water used in this work was saturated with IDM as suggested by Qiu et al.[15] Microcapsules of IDM-$(CHI/ALG)_5$ were slightly rinsed with water before release experiments to eliminate the IDM residue adsorbed on the surface.

2.4. Enzymatic desorption of multilayer films

The multilayer film PEI/Apy/$(CHI/Apy)_5$ on the quartz slide was dipped in a quartz cuvette containing a pepsin soln of 0.5 g/L for a required time, and then air-dried prior to fluorescence intensity measurement. The fluorescence emission of the dipping soln in the quartz cuvette after removing the slide was also measured. For easy comparison, a blank soln without pepsin was used in the same procedure. The pH value of the pepsin soln was adjusted to 1.4 with the acetate/sodium acetate buffer. The incubation temperature was 37 °C.

The microcapsules IDM-$(CHI/ALG)_5$ were immersed in the pepsin soln of 0.5 g/L for a specified time at a given pH and temperature, and rinsed following two repeat refining circles of centrifugation (5000 rpm, 5 min)/washing/re-dispersal with water. The pepsin soln for treatment was saturated with IDM to prevent drug leakage from the microcapsules.

2.5. In vitro release

Microcapsules IDM-$(CHI/ALG)_5$ after the pepsin treatment were allowed to release at pH 7.4 and 37 °C. The released amount of IDM from the microcapsule was in situ monitored by UV absorbance at 320 nm.[24] The bare IDM microcrystal and IDM microcapsules were, respectively, immersed in pH 7.4 phosphates buffer in a 3 mL quartz cuvette. UV absorbance was measured as a function of dipping time and converted to the IDM concentration in the cuvette with a calibration curve.

2.6. Characterization

Fluorescence intensity and UV absorbance were measured with Hitachi F-4500 and Hitachi UV-3010 spectrometers, respectively. The multilayer film morphology was observed with a Seiko SII atomic force microscope (AFM) in the tapping mode at room temperature.

3. Results and discussion

3.1. Enzymatic desorption of planar multilayer films

Chitosan (CHI) has been found to be enzymatically degradable.[35–39] For the LbL multilayer film, Serizawa et al. found that the CHI/dextran multilayer was desorbed in the presence of chitosanase,[27] which is a special enzyme for CHI and can be isolated from a commercial pepsin preparation.[40] Kumar et al. directly used pepsin to degrade CHI and prepare low molecular weight CHI.[38,39] Considering the fact that pepsin is abundant in the gastric juice, we have chosen to investigate the enzymatic desorption of multilayer films containing CHI.

The multilayer film PEI/APy/$(CHI/APy)_5$ was deposited on the quartz slide through LbL assembly to demonstrate the enzymatic desorption induced by the pepsin erosion. The electrostatic attraction was the dominant driving force for the oppositely charged APy and CHI adsorbed alternately on the surface of the quartz slide and the first PEI layer was adopted to ensure the following adsorption. Pyrene labeled APy was used to make the multilayer film desorption detectable with fluorescence spectra. Fluorescent intensity is proportional to the APy amount deposited on the slide. The linear increase in the fluorescence intensity with the layer number shown in Figure 1 confirmed the formation of the polyelectrolyte multilayer film.[34]

With the degradation of multilayer film PEI/APy/$(CHI/APy)_5$, APy was partially dissolved into the pepsin solution, leading to a decrease in fluorescence intensity of the film. The fluorescence intensity change of the multilayer film PEI/APy/$(CHI/APy)_5$ against the incubation time t is displayed in Figure 2. The film maintains its emission intensity during 7-h incubation in water of pH 1.4 without pepsin at 37 °C. In contrast, the fluorescence intensity of the film decreases over the period of 7-h incubation in pepsin solution of pH 1.4 at 37 °C with a corresponding increase in the fluorescence intensity of the incubation solution. The desorption of the CHI/APy multilayer film is slow within the first 2 h, and then accelerated, finally to reach a half of its original intensity value after 7 h incubation with pepsin. The results from Figure 2 obviously indicate the gradual desorption of the CHI/APy multilayer film due to the chitosan degradation by pepsin.

AFM images of the surface morphology of multilayer film PEI/APy/$(CHI/APy)_5$ before and after pepsin treatment at pH 1.4 and 37 °C for 4 and 6 h are depicted in Figure 3. The original CHI/APy multilayer film appears

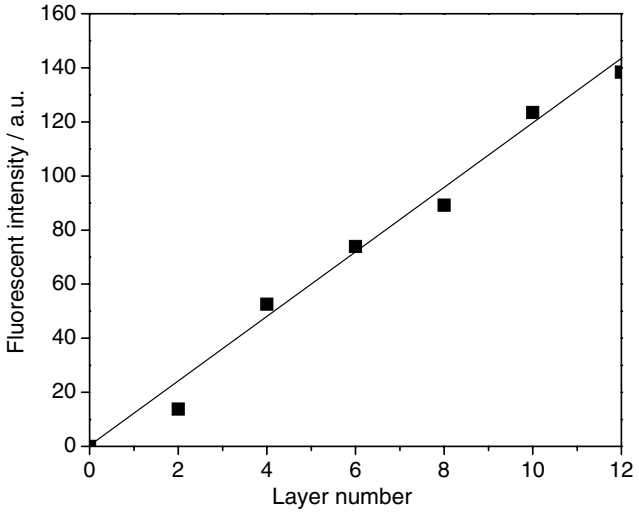

Figure 1. Fluorescence intensity of film PEI/APy/$(CHI/Apy)_5$ on the quartz slide as a function of the layer number.

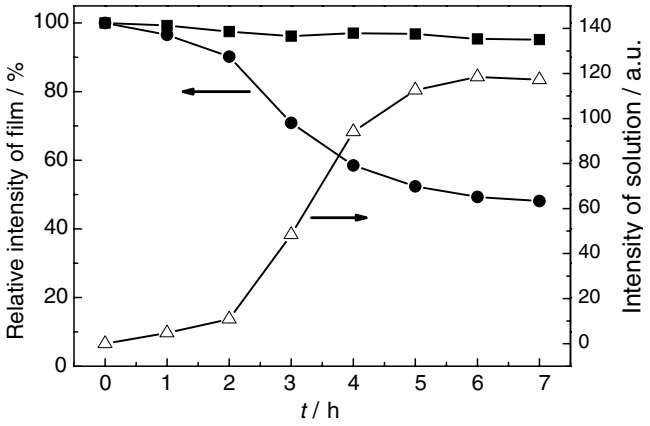

Figure 2. Fluorescence intensity relative to incubation time $t = 0$ as a function of t for film PEI/APy/(CHI/Apy)$_5$ on the quartz slide treated with (solid circle) or without (solid square) pepsin at pH 1.4 and 37 °C, and fluorescence intensity of the pepsin treatment solution (open up triangle).

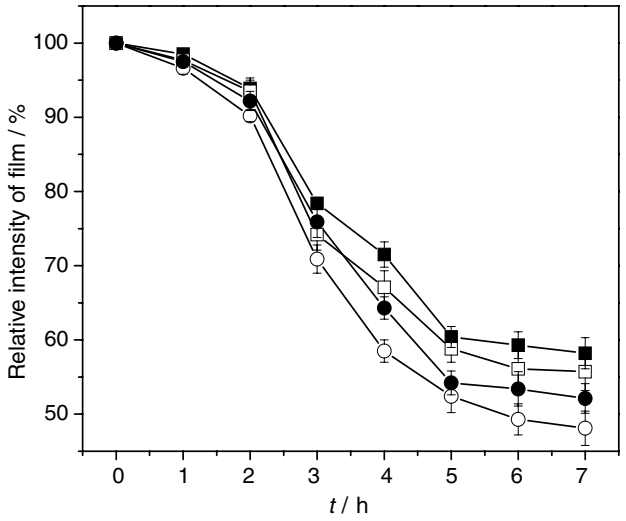

Figure 4. Fluorescence intensity of film PEI/APy/(CHI/Apy)$_5$ on the quartz slide as a function of incubation time t after incubation in the pepsin solution at pH 1.4 (solid square), 2.5 (open square), 4.0 (open circle), 5.5 (solid circle) and 37 °C.

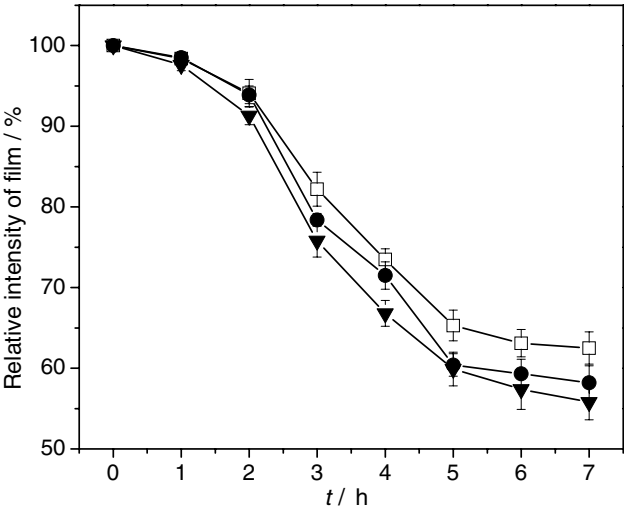

Figure 5. Fluorescence intensity of film PEI/APy/(CHI/Apy)$_5$ on the quartz slide as a function of incubation time t after incubation in the pepsin solution at pH 1.4 and 20 °C (open square), 37 °C (solid circle), 60 °C (solid down triangle).

smooth with the average roughness about 8.9 nm (the root-mean-squared surface roughness calculated over the scan area using the SII software). After incubation in the pepsin solution, the film surface became rougher with an average roughness of 23.7 and 44.5 nm for the films incubated for 4 and 6 h, respectively, and some obvious pits and/or holes appear at the surface. The present change in surface morphology reflects the gradual corrosion of the multilayer film due to the enzymatic degradation of CHI. Fredin et al. also found that the poly(β-aminoester)/PSS multilayer film roughness increased gradually during the desorption but not by the enzymatic erosion.[41]

Because the enzyme activity is sensitive to the environmental condition, such as pH and temperature, we have tested the influence of desorption conditions on the multilayer film PEI/APy/(CHI/APy)$_5$. The decrease in fluorescence intensity with incubation time t of the multilayer film PEI/APy/(CHI/APy)$_5$ incubated in the pepsin solution at 37 °C and different pHs is shown in Figure 4. The decrease in fluorescence intensity with incubation time t of the multilayer film PEI/APy/(CHI/APy)$_5$ incubated in the pepsin solution at pH 1.4 and different temperatures is shown in Figure 5. This intensity decrease is caused by the dissolution of APy in the multilayer film due to the degradation of its counterpart CHI. The film desorption became the fastest at pH 4.0,

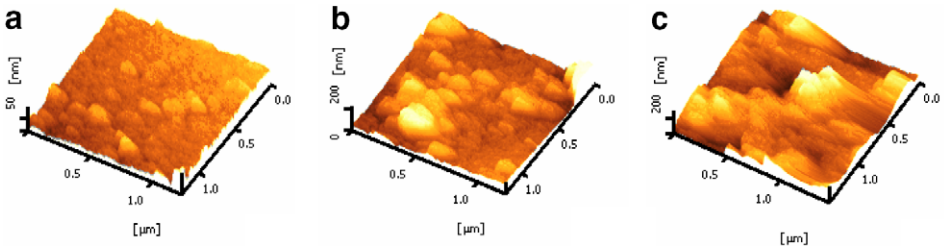

Figure 3. AFM image (1.2 μm × 1.2 μm) of film PEI/APy/(CHI/Apy)$_5$ on the quartz slide before (a) and after incubation in the pepsin solution at pH 1.4 and 37 °C for 4 h (b) and 6 h (c).

while at pH 1.4 the desorption was the slowest within the tested pH range of 1.4–5.5 (Fig. 4). This phenomenon can be attributed to the relatively higher activity of pepsin at pH 4.0 than at other pH,[31] consequently causing the fastest desorption of the CHI/APy multilayer film. Besides pH, the incubation temperature also affects the pepsin activity. As the temperature increases from 20 to 60 °C, the multilayer desorption is somewhat speeded up (Fig. 5). This temperature dependence can be understood on two factors: one is the existence of an optimum temperature for the pepsin enzymatic reaction, another is the acceleration at higher temperature of the molecular mobility to facilitate the multilayer desorption.

3.2. Release of encapsulated IDM affected by pepsin erosion

To study the effect of pepsin erosion to multilayer films on the release of encapsulated drugs, the IDM microcrystal of about 5 μm was directly encapsulated with polysaccharides CHI and ALG through LbL self-assembly. The pyrene labeled polyelectrolyte APy was not used in the encapsulation. IDM was used as a model drug only due to its low solubility in aqueous solution during the LbL assembly of CHI and ALG alternately deposited directly on its microcrystals. These polysaccharides were chosen for their biocompatibility and suitable for medical applications.

Based on understanding enzyme erosion of multilayer film PEI/Apy/(CHI/Apy)$_5$ on the quartz slide, we further studied the IDM release from multilayer microcapsule IDM-(CHI/ALG)$_5$ after pepsin treatment. Firstly, we examined the incubation time effect on the release of IDM from microcapsule IDM-(CHI/ALG)$_5$ after pepsin treatment at pH 1.4 and 37 °C. The IDM release profiles from microcapsule IDM-(CHI/ALG)$_5$ after pepsin corrosion compared with that from the same microcapsule but only incubated in an acetic acid solution of pH 1.4 at 37 °C for 6 h without pepsin are shown in Figure 6. The release rate from microcapsule IDM-(CHI/ALG)$_5$ only treated with acetic solution without pepsin is similar with that of the same microcapsule without any treatments. This means that the accelerated IDM release from the pepsin treated microcapsules is induced by enzymatic erosion of the multilayer walls. With the Fick's first law of diffusion, the IDM release from the ALG/CHI multilayer microcapsule has been quantitatively described.[21] By fitting the release data in Figure 6, we evaluated the diffusion coefficient D for IDM passing through the ALG/CHI multilayer film of the microcapsule accordingly. As the incubation time increased, the release rate increased gradually and the diffusion coefficient D raised from 3.5×10^{-9} to 13.3×10^{-9} cm^2/s. After pepsin erosion, the release of encapsulated IDM microcrystals was accelerated due to the increase in permeability of the ALG/CHI multilayer film covering on the drug. However, even after incubation in the simulating gastric juice for 6 h, the ALG/CHI multilayer film still maintained the barrier capability to diffusion of the encapsulated IDM when compared with the release of bare IDM microcrystals.

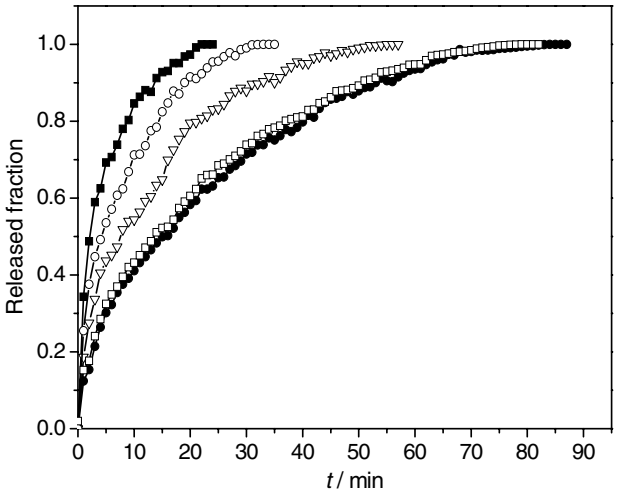

Figure 6. Release profiles from IDM (solid square) and microcapsule IDM-(CHI/ALG)$_5$ after pepsin treatment for 0 h (solid circle), 4 h (open circle), 6 h (open down triangle) compared with that from microcapsule IDM-(CHI/ALG)$_5$ after immersion in an acidic solution of pH 1.4 at 37 °C for 6 h without pepsin (open square).

The release profile of IDM from microcapsule IDM-(CHI/ALG)$_5$ after incubation for 4 h in pepsin solution of given pH at 37 °C are displayed in Figure 7. The release rate increases when the pH of the pepsin solution increases from 1.4 to 4.0, and then decreases when pH decreases from 4.0 to 5.0. This change is due to the higher activity of pepsin at pH 4.0 than that at other pH as found from pepsin desorption of the CHI/APy multilayer film on quartz slide (Fig. 4). As the incubation pH increases from 1.4 to 4.0, D increases from 10.4×10^{-9} to 15.3×10^{-9} cm^2/s, and then is reduced to

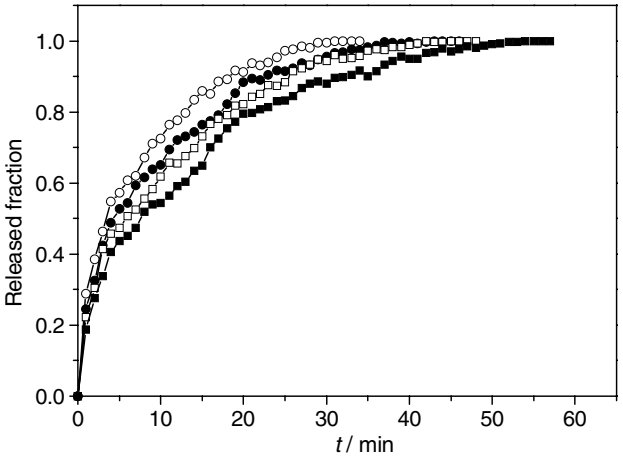

Figure 7. Release profiles from microcapsule IDM-(CHI/ALG)$_5$ after incubation in the pepsin solutions at pH 1.4 (solid square), 2.5 (open square), 4.0 (open circle), 5.5 (solid circle) and 37 °C for 4 h.

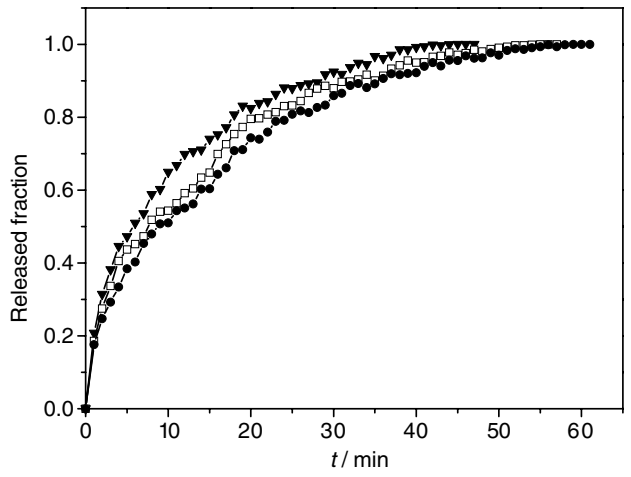

Figure 8. Release profiles from microcapsule IDM-(CHI/ALG)$_5$ after incubation in the pepsin solution at pH 1.4 and 20 °C (open square), 37 °C (solid circle), 60 °C (solid down triangle) for 4 h.

13.8×10^{-9} cm^2/s when pH becomes 5.5. As expected, the enzymatic erosion of the multilayer film causes a faster release for the encapsulated drug.

Besides pH, increasing temperature also speeds up the enzymatic desorption of the flat CHI/APy multilayer (Fig. 5). Thus, we would expect a faster release of IDM from microcapsule IDM-(CHI/ALG)$_5$ after incubation in pepsin solution at higher temperature. However, the experimental results illustrated in Figure 8 indicate a decrease in release rate when incubated at higher temperature. Increasing incubation temperature has two simultaneous effects on the CHI/ALG multilayer film: one is to speed up the enzymatic desorption as found from the flat CHI/APy multilayer film (Fig. 5) and another is to accelerate the chain rearrangement through the segment movement, which leads to a more perfect multilayer structure. The second effect seems to be dominant in the pepsin treated CHI/ALG multilayer film, so that the diffusion coefficient D decreases from 15.0×10^{-9} to 8.42×10^{-9} cm^2/s as the incubation temperature increases from 20 °C to 60 °C. Annealing of the CHI/ALG multilayer microcapsule at 60 or 80 °C after formation has been found to contribute little to decrease the release rate.[24] The possible reason seems to be that when the multilayer stacking is loose during the deposition and/or pepsin treatment, increasing temperature can make the multilayer thicker and more perfect through the segment rearrangement. But if the multilayer is in the condensed state already, the subsequent annealing at moderate temperature cannot change its structure significantly.

4. Conclusions

By following the fluorescence emission of pyrene labeled polyelectrolyte, the desorption of multilayer films containing the polysaccharide CHI prepared by LbL self-assembly was observed to be induced by enzymatic degradation of pepsin to CHI in the films. The multilayer desorption rate is the highest when in the pepsin solution of pH 4.0 and increasing the temperature from 20 to 60 °C also accelerated the desorption. Indomethacin microcrystals were directly encapsulated with CHI/ALG multilayer films through LbL self-assembly to investigate the effect of this enzymatic desorption on the release. The fastest release rate was realized when incubated in the pepsin solution at pH 4.0 due to the higher activity of pepsin. But increasing incubation temperature slowed down the release rate owing to the enhanced rearrangement of the polyelectrolyte chains in the capsule wall. The CHI/ALG multilayer film was found to maintain its barrier function to the IDM diffusion even after 6-h incubation in the pepsin solution.

Acknowledgments

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