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Development of a novel strategy for controlled release of lysozyme from whey protein isolate based active food packaging films

Bahar Basak Peksen Ozer [a, 1], Metin Uz [a, 1], Pelin Oymaci [b], Sacide Alsoy Altinkaya [a, *]

[a] Izmir Institute of Technology, Department of Chemical Engineering, Gulbahce Campus, 35430 Urla, Izmir, Turkey
[b] Izmir Institute of Technology, Department of Material Science and Engineering, Gulbahce Campus, 35430, Urla, Izmir, Turkey

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ABSTRACT

The purpose of this study is to develop a novel controlled release system based on pH-responsive polyacrylic acid (PAA)/lysozyme (LYS) complexes incorporated within a hydrophilic whey protein isolate (WPI) film matrix for active food packaging applications. Complex formation is simple under benign conditions that are suitable for preserving antimicrobial activity of the lysozyme. In addition, the pH-dependent charge density of complexes allowed a uniform distribution in the matrix. The properties of the complexes such as size, surface charge and hydrophilicity were varied by changing PAA/LYS ratio (0.1 and 0.3 w/w) and PAA molecular weight (2 kDa and 450 kDa). The effects of complex properties as well as mode of lysozyme incorporation into the films (100%-free, 50%-free+50%-PAA/LYS complex and 100%-PAA/LYS complex) on the LYS release rate, activity and antimicrobial efficacy of the films were investigated. The results have shown that ~100% LYS loading into the complexes is possible regardless of PAA molecular weight or PAA/LYS ratio. Incorporating lysozyme into the film in complexed form extended its release time from less than 24 h up to 500 h and reduced its diffusivity from ~10^{-9} to ~10^{-13} cm^2/s. The films including 50%-free-LYS+50%-PAA/LYS complex showed a 5.7 log reduction in bacterial population within 72 h whereas 100%-free-LYS containing film could not suppress *Listeria innocua* growth after 24 h. Overall, the results suggest that complexation of lysozyme with weak polyelectrolytes can be used as an effective strategy to achieve a long-lasting antimicrobial effect and that films prepared with such complexes have great potential as food packaging materials.

1. Introduction

Controlled release packaging is an emerging technology by which active compounds, such as antimicrobials and antioxidants, are released from packaging materials to the food surface at desirable rates to provide continuous replenishment of active compounds, inhibit bacterial growth and extend product shelf life (Mastromatteo, Mastromatteo, Conte, & Del Nobile, 2010). This technology overcomes the limitations of traditional approaches, in which active compounds are directly added into food formulation resulting in fast activity loss and the overloading of active compounds due to lack of surface targeting. Consequently, a greater level of food protection is achieved with controlled release packaging by using smaller amounts of active agents compared to the amounts used in traditional approaches (Appendini & Hotchkiss, 2002; Min & Krochta, 2005).

Lysozyme is one of the most commonly used natural proteins and has a great potential for antimicrobial packaging due to its stability over a wide range of temperature and pH values (James & McManus, 2012; Venkataramani, Truntzer, & Coleman, 2013). Different strategies have been applied in the literature to control the release rate of lysozyme. Most of these strategies focused on changing packaging material morphology by polymer concentration (Gemili, Yemenicioglu, & Altinkaya, 2009), plasticizer concentration (Min, Rumsey, & Krochta, 2008), additive concentration and type (Mastromatteo, Lecce, De Vietro, Favia, & Del Nobile, 2011), degree of crosslinking (Buonocore et al., 2003a; Buonocore, Del Nobile, Panizza, Corbo, & Nicolais, 2003b; Buonocore et al., 2004; Fajardo, Balaguer, Gomez-Estaca, Gavara, & Hernandez-Munoz, 2014; Ma, Tang, Yin, Yang, & Qi, 2013; Ozdemir & Floros, 2001) and number of layers (Buonocore et al., 2004; Conte, Buonocore, Nicolais, & Del Nobile, 2003). In another approach,

* Corresponding author. Izmir Institute of Technology, Department of Chemical Engineering, Gulbahce Campus, 35430, Urla, Izmir, Turkey.
E-mail address: sacidealsoy@iyte.edu.tr (S.A. Altinkaya).
[1] These authors contributed equally to this work.

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the pH of the release medium was changed to control release of lysozyme from the films (Benelhadj et al., 2016; Mendes de Souza, Fernández, López-Carballo, Gavara, & Hernández-Muñoz, 2010). In these studies, release times ranging from very short times (between 1 and 27 h) to times up to 5 d were reported (Benelhadj et al., 2016; Fajardo et al., 2014; Gemili et al., 2009). In addition to controlled release, the sustained antimicrobial efficacy of the films is required for application in food packaging. Fajardo et al. (2014) achieved the slow release of lysozyme from gliadin films cross-linked with cinnamaldehyde. However, antimicrobial tests conducted with these films showed that after 24 h the released amount of lysozyme was neither sufficient nor active enough to prevent the growth of bacteria, leading to an increase in colony formation unit (Fajardo et al., 2014).

Recently, potential uses of nanotechnology for developing food packaging with controlled release of active compounds have been identified. Antioxidants such as α-tocopherol, quercetin, Vitamin E, Vitamin B12, tea phenol and antimicrobial agents, *trans*-2-hexenal, cinnamon essential oil, nisin and thymol were incorporated into hydrophilic/hydrophobic films in encapsulated form (Bao, Xu, & Wang, 2009; Imran et al., 2012; Joo, Merkel, Auras, & Almenar, 2012; Koontz et al., 2010; Li, Yin, Yang, Tang, & Wei, 2012; Noronha, de Carvalho, Lino, & Barreto, 2014; Siró et al., 2006; Wu et al., 2015). Lysozyme was also encapsulated in zein microcapsules (Zhong, Jin, Davidson, & Zivanovic, 2009) and starch microgel (Li et al., 2012); however, these particles were not utilized for developing food packaging films. Although sustained release of lysozyme from zein capsules was observed, the use of these particles in hydrophilic food packaging materials seems to be highly challenging since homogeneous distribution of hydrophobic zein particles in hydrophilic film structures is a difficult task. In addition, antimicrobial activity of lysozyme might be significantly lost upon loading under high pressure conditions. Li, Kadam, et al. (2012) and Li, Yin, et al. (2012) prepared oxidized starch-based microgels and loaded them with lysozyme. The gels are then degraded by bacterial amylase, releasing lysozyme (Li et al., 2012). The main disadvantages of this encapsulation technique are the need for large amounts of oil and organic solvent, and the long time required for encapsulation. Furthermore, a prerequisite for the presence of amylase-positive, lysozyme-sensitive bacteria to induce lysozyme release restricts potential applications of these particles in food packaging formulations.

The review of literature studies has shown that achieving sustained release of lysozyme from hydrophilic food packaging materials while maintaining antimicrobial efficacy of the films over time is still a challenging issue. This is in large part because films made from hydrophilic polymers swell in the presence of water, increasing free volume between polymer chains, in turn accelerating lysozyme release. Considering the limitations of previous approaches, in this study we aimed to develop a new strategy for sustained lysozyme release that decouples the swelling of the film from the release rate. To achieve this, we first encapsulated lysozyme (LYS) into polyacrylic acid (PAA), a food-grade polyelectrolyte, to form LYS/PAA complexes. Subsequently these complexes were incorporated into whey protein isolate (WPI) films. Protein polyelectrolyte complex formation is mostly driven by the electrostatic interactions between oppositely charged groups in the protein and the polyelectrolyte (Chen, Walker, & John, 1992; Ghimire, Kasi, & Kumar, 2014; Kamiya & Klibanov, 2003; Kim, Hirasawa, & Kim, 2002). Under certain conditions, the complex is formed spontaneously by mixing solutions of proteins and polyelectrolytes. In addition to the simplicity of constructing ionic complexes, their responsiveness to pH and ionic strength offers a route to trigger the release of the encapsulated protein. We have selected PAA, a weak polyelectrolyte, because its charge density changes significantly with pH. This feature allows the control of LYS/PAA interactions and consequently, the control over the loading level and release of lysozyme. WPI has been chosen since it has excellent film forming and gas barrier properties. In addition, among many biopolymers, WPI is one of the abundant proteins isolated from milk as a by-product of the manufacture process of cheese or casein, which makes economic production of bio-based packaging materials possible. We have tuned the release rate of lysozyme from WPI films by varying the PAA/LYS ratio and PAA molecular weight. Three different types of WPI films have been prepared by incorporating 100% complexed lysozyme, 100% free lysozyme, or 50% free and 50% complexed lysozyme. The antimicrobial activity of the films was tested against *Listeria innocua*. To the best of our knowledge, this study is the first to report the release of lysozyme from complexes incorporated into a hydrophilic film and opens a new strategy in designing controlled release packaging using lysozyme-containing polyelectrolyte complexes.

2. Materials and methods

2.1. Materials

Lysozyme with an activity of ≥20,000 U/mg (MW: ~14,400 g/mole) was supplied from Applichem-BioChemica. *Micrococcus lysodeikticus* (ATCC # 4698) and polyacrylic acid (MW: 2 & 450 kDa) were purchased from Sigma-Aldrich. Whey protein isolate powder was provided by Davisco Foods International Inc. (Le Sueur, Minnesota, USA). Glycerol and acetic acid were purchased from Panreac. Trifluoroacetic acid and acetonitrile were supplied by Roth and Merck, respectively. *Listeria innocua* (ATCC® 33090™) obtained from ATCC was used in antibacterial tests. Peptone used for cultivation of bacteria and Muller Hinton agar and broth used as bacterial growth medium were provided by Merck. All the buffers and solutions used in the experiments were prepared with ultrapure water following standard laboratory procedures.

2.2. Preparation and characterization of PAA/LYS complexes

PAA/LYS complexes were prepared by the precipitation of lysozyme with polyacrylic acid through charge neutralization and polymer bridging mechanisms (Chen et al., 1992; Ghimire et al., 2014; Kim et al., 2002). Four different complexes were produced through changing the molecular weight of PAA (2 and 450 kDa) and PAA/LYS ratio (PAA/LYS: 0.1 and 0.3 w/w). For the complex preparation, PAA solution in 10 mM phosphate buffer at pH 7.0 was mixed with LYS solution in the same buffer to get the final PAA/LYS ratio of 0.1 and 0.3 w/w. Following 1 min mixing at 300 rpm, the suspension was left at room temperature for 2 h to complete the precipitation of particles. The complexes were collected by centrifugation (at 5000 rpm for 5 min) and supernatants were analyzed by using High Pressure Liquid Chromatography (HPLC) (Agilent Model 1100) equipped with a C18 reverse phase column (Fortis, United Kingdom) to determine the lysozyme loading (Min et al., 2008; Pellegrino & Tirelli, 2000). For the analysis, 20 μL of supernatant containing lysozyme was injected to the column at 30 °C and eluted at a flow rate of 1 mL/min with water-acetonitrile gradients in aqueous 0.1% trifluoroacetic acid. The amount of lysozyme in the supernatant was detected and loading capacity was calculated using a calibration curve (Fig. S.1.) as described in Supplementary Information.

Following the phase separation and centrifugation, the complexes prepared were diluted with the sodium acetate (0.05 M, pH 4.5) and their size and zeta potential values were measured by dynamic light scattering (DLS) (Malvern Zetasizer NanoZS). The contact angle values were also assessed to determine the surface

wettability behavior (hydrophobicity/hydrophilicity) of the complexes by using Attention Theta optical tensiometer. The pellets of complexes were prepared by pressing 60 mg of complex powder at 7 bar. The change in the contact angle of water droplet 6 μL in volume was recorded for 10 s (1 frame per sec). The values were analyzed by averaging the three independent replicates on three different spots conducted for each sample.

2.3. Preparation and characterization of LYS incorporated WPI films

Three different types of WPI films were prepared by incorporating a) 100% free lysozyme b) 100% complexed lysozyme and c) 50% free and 50% complexed lysozyme. In all modes of incorporation (free, complexed with PAA and combination of free and complexed LYS), the total LYS amount (80 mg) in WPI films was kept constant. First, a solution composed of 5% (w/w) WPI in deionized water was denatured at 90 °C for 30 min and then, cooled to room temperature. Lysozyme, either in free or complexed form, was dissolved and dispersed in water and then, it was added dropwise to the WPI solution. The pH of the WPI solution was adjusted to 3.0 with 1 N HCl prior to free lysozyme addition. Glycerol was used as a plasticizer in each film formulation and the WPI/glycerol ratio was kept constant at 1. The film forming solution was stirred for 30 min, cast over a polypropylene substrate and finally dried overnight at 25 °C and 40% relative humidity.

The morphology of the films was examined using a scanning electron microscope (SEM) (FEI Quanta) with an accelerating voltage of 5 kV. The films were freeze-fractured under liquid nitrogen and coated with a gold layer prior to observation. The existence of the lysozyme and PAA/LYS complexes in the cross section of the films was observed through a transmission electron microscopy (TEM-FEI, Tecnai G^2 Spirit Bio(TWIN)). For the TEM analysis, the films were placed inside epoxy blocks with dimensions of 8 mm × 4 mm x 10 mm and about 100 nm ultrathin TEM specimens were cut at room temperature by using an ultra microtome equipped with a diamond knife. The samples were visualized at an acceleration voltage of 80 kV.

2.4. Determination of release kinetics of lysozyme

The films with an area of 16 cm^2 were placed in tightly sealed flasks containing 40 mL of release medium (0.05 M Na-Acetate buffer at pH 4.5). Then, the flasks were incubated at 10 °C under continuous shaking at 100 rpm. Lysozyme released from the films into buffer solution was quantified by HPLC analysis (Min et al., 2008; Pellegrino & Tirelli, 2000). The volume of the release medium was kept constant by replacing the collected sample volume with the same amount of buffer each time.

2.5. Determination of diffusion coefficient of lysozyme

The diffusion coefficient of lysozyme in the films was determined by combining the experimental release kinetics measurements with the mathematical model as described in our previous studies (Gemili et al., 2009; Uz & Altınkaya, 2011). The model is based on Fick's second law (Crank, 1975). The total amount of lysozyme desorbed from the film at any time t due to Fickian diffusion ($M_{Fickian}$) is given by the following expression:

$$M_{Fickian} = M_\infty \left[1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n} \exp\left(-Dq_n^2 t / L^2\right)\right] \quad (1)$$

where $\alpha = \frac{a}{L}$ and the q_n's are the non-zero positive roots of

$$\tan q_n = -\alpha q_n \quad (2)$$

and a is given by

$$a = \frac{V_{sol}}{KA_m} \quad (3)$$

In Equations 1 through 3, M_∞, V_{sol}, A_m and K represent the amount of lysozyme desorbed at equilibrium, volume of the release medium, area of the film and the partition coefficient indicating the ratio of lysozyme concentration in the film to that in the solution at equilibrium, respectively.

2.6. Determination of released lysozyme activity

Lysozyme activity was calculated by measuring the decrease in absorbance of *Micrococcus lysodeikticus* suspension prepared in 0.05 M Na-phosphate buffer at pH 7.0 with a concentration of 0.26 mg/mL. Next, 1.15 mL of this suspension was mixed with 0.1 mL of collected lysozyme sample and incubated at 30 °C for 5 min. The decrease in absorbance of *Micrococcus lysodeikticus* was monitored at constant temperature (30 °C) for 120 s at 660 nm wavelength by using a UV/VIS spectrophotometer (PerkinElmer, Lambda 45, USA) equipped with a constant temperature cell holder. Activity was calculated from the slope of the initial linear portion of absorbance versus time curve and expressed as Unit/film area. A representative calculation is shown in Supplementary Information.

2.7. Determination of time dependent antibacterial activity of films

Time dependent antibacterial activities of the selected films were measured by the colony counting method. Films with area of 4 cm^2 were placed in Muller Hinton broth medium (10 mL) and incubated for 24, 48 and 72 h at 4 °C under continuous shaking (100 rpm) for the release of lysozyme to the broth medium. At the same time, *Listeria innocua* was seeded on Muller Hinton agar and placed in incubator for the growth during 24 h at 37 °C. At the end of 24 h, the grown bacteria were collected from the agar and dispersed in 0.1% peptone water and its McFarland value was adjusted to 0.5, which corresponds to the bacterial concentration of 10^8 cfu/mL. After serial dilutions, the bacteria concentration in peptone water was adjusted to 10^4 cfu/mL. At the end of each incubation period, the films were removed from the broth medium and 0.1 mL of the bacteria solution in peptone water was added to the 10 mL film free broth medium; therefore, the initial bacteria concentration in the broth medium was 100 cfu/mL. Following this, the flasks containing the bacteria and released lysozyme were incubated at 37 °C under continuous shaking during 24 h. Finally, samples were taken from the flasks, spread on Muller Hinton agar plates and further incubated at 37 °C during 24 h. The colonies formed were counted to determine the activity of the released lysozyme from the films.

2.8. Statistical analysis

Throughout this study, significant differences between groups were evaluated using ANOVA analysis by Tukey's method with 95% confidence interval. The results are presented as average ± standard deviation calculated from at least three independent experiments. In each of these experiments, three replicates were performed for all nine film samples.

3. Results and discussion

3.1. Characterization of complexes

PAA-LYS complex formation was dominated by charge neutralization through a pH-dependent protonation and deprotonation binding mechanism (Ghimire et al., 2014), followed by a shear-induced collision based polymer bridging mechanism (Chen et al., 1992; Kim et al., 2002). For all complex formulations, almost ~100% LYS loading into complexes was achieved regardless of the PAA molecular weight or PAA/LYS ratio (Table 2). This is attributed to strong electrostatic interactions between the negatively charged PAA (isoelectric point (IEP): pH 4.7) and positively charged LYS (IEP: pH 11.2) at the loading pH (pH = 7) (Kamiya & Klibanov, 2003). In previous studies, lysozyme was encapsulated into chitosan nanoparticles (Deng, Zhou, & Luo, 2006), poly(lactic-co-glycolic acid) (PLGA) based solid lipid nanoparticles (Xie et al., 2008) and PLGA and poly(L-lactide) (PLLA) based double walled microspheres (Kokai et al., 2010) for pharmaceutical and biomedical applications; in the first two studies the loading capacities were reported as 35% and 65%, respectively. For food applications, lysozyme alone (Li et al., 2012) or lysozyme and calcein together (Were, Bruce, Davidson, & Weiss, 2003) were encapsulated into starch microgels and phosphatidylcholine nanoparticles at the loading capacity of 1.2 mg protein/mg dry gel and 61%, respectively. Our loading capacities for lysozyme are higher than the values reported in previous studies. In addition, our loading protocol is simpler than previous methods since complexation allows loading and particle formation simultaneously in one step while in other studies nanoparticles or microspheres are formed first prior to lysozyme addition. The results in Table 2 show that both the size and zeta potential of the complexes changed with the PAA molecular weight and PAA/LYS ratio. The molecular weight of PAA was shown to have a significant effect on the size of the complexes. An increase in the molecular weight or PAA/LYS ratio resulted in the formation of larger sized complexes, since not every negatively charged carboxylic acid group of PAA can be paired with positively charged amine groups in lysozyme (Chieng & Chen, 2010; Chollakup, Beck, Dirnberger, Tirrell, & Eisenbach, 2013; Schmitt & Turgeon, 2011). The complexes formed from high molecular weight PAA had a higher charge both in deionized water and in Na acetate buffer due to the presence of more charged groups in longer polymer chains. All of the complexes carry net negative charge in deionized water under typical loading conditions (pH 7), which proved encapsulation of positively charged lysozyme by negatively charged PAA at pH 7 (Ghimire et al., 2014). The presence of PAA on the outer surface of the complex is critical to protect bioactivity of lysozyme during film formation.

Hydrophilicity of the complexes, which is critical to achieve uniform distribution in the hydrophilic WPI matrix, was determined through contact angle measurements. The results in Table 3 indicate that all of the complexes have a hydrophilic character with

Table 1
Codes, properties and diffusion coefficient of lysozyme in the films.

Code of the film	Incorporation mode of LYS	Free LYS %	LYS% in complex form	Code of PAA/LYS complex	PAA MW (kDa)	PAA/LYS ratio	Diffusion coefficient (cm^2/s)
WPI-100% free LYS	Free	100%	–	–	–	–	1.54×10^{-09} m
WPI-50% free LYS+50% 2S/LYS Complex	Free + Complex	50%	50%	2S/LYS	2	0.1	1.26×10^{-10} a,e,i,m
WPI-50% free LYS+50% 2B/LYS Complex	Free + Complex	50%	50%	2B/LYS	2	0.3	3.25×10^{-11} a,f,j,m
WPI-50% free LYS+50% 450S/LYS Complex	Free + Complex	50%	50%	450S/LYS	450	0.1	4.75×10^{-11} b,e,k,m
WPI-50% free LYS+50% 450B/LYS Complex	Free + Complex	50%	50%	450B/LYS	450	0.3	1.67×10^{-11} b,f,l,m
WPI-100% 2S/LYS Complex	Complex	–	100%	2S/LYS	2	0.1	1.42×10^{-11} c,g,i,m
WPI-100% 2B/LYS Complex	Complex	–	100%	2B/LYS	2	0.3	1.08×10^{-11} c,h,j,m
WPI-100% 450S/LYS Complex	Complex	–	100%	450S/LYS	450	0.1	1.58×10^{-11} d,g,k,m
WPI-100% 450B/LYS Complex	Complex	–	100%	450B/LYS	450	0.3	4.00×10^{-13} d,h,l,m

(a-d): Complexation of lysozyme with PAA at the PAA/LYS ratio of 0.1 and 0.3 caused significantly different lysozyme diffusivities in the films including 50% or 100 complexed lysozyme ($p < 0.05$).
(e-h):Complexation of lysozyme with 2 kDa and 450 kDa PAA caused significantly different lysozyme diffusivities in the films including 50% or 100% complexed lysozyme ($p < 0.05$).
(i-l): The diffusion coefficients of lysozyme in the films incorporated with 50% complexed lysozyme are significantly different than those in the films including 100% complexed lysozyme ($p < 0.05$).
(m):The diffusion coefficients of lysozyme in the films incorporated with 50% or 100% complexed lysozyme are significantly different compared to the diffusion coefficient in the film including only 100% free lysozyme.

Table 2
Codes and properties of PAA/LYS complexes.

				Zeta potential (mV)		Size (nm)
Complex code	PAA MW (kDa)	PAA/LYS ratio (Wt/wt)	Loading capacity (%)	In DI water (pH 7)	In NaAcetate (pH 4.5)	In DI water
2S/LYS	2	0.1	97.4 ± 1.4	$-7.0 \pm 0.7^{a,b,g}$	$10.2 \pm 0.9^{d,g}$	$31.9 \pm 7.53^{i,k}$
2B/LYS	2	0.3	99.5 ± 1	$-8.7 \pm 0.3^{a,c,h}$	$10.7 \pm 0.2^{f,h}$	$361.9 \pm 40.2^{i,l}$
450S/LYS	450	0.1	99.9 ± 1.2	-11.1 ± 1.2^{b}	$12.0 \pm 0.3^{d,e}$	$427.5 \pm 51.2^{j,k}$
450B/LYS	450	0.3	99.1 ± 0.9	-13.6 ± 1.1^{c}	$14.9 \pm 1.1^{e,f}$	$665 \pm 17.5^{j,l}$

(a,i): Zeta potentials and sizes of the complexes prepared at the PAA (2 kDa)/LYS ratio of 0.1 and 0.3 are significantly different ($p < 0.05$).
(b,d):Zeta potentials of the complexes (PAA/LYS:0.1) prepared with 2 kDa and 450 kDa PAA are significantly different ($p < 0.05$).
(c,f):Zeta potentials of the complexes (PAA/LYS:0.3) prepared with 2 kDa and 450 kDa PAA are significantly different ($p < 0.05$).
(e,j):Zeta potentials and sizes of the complexes prepared at the PAA (450 kDa)/LYS ratio of 0.1 and 0.3 are significantly different ($p < 0.05$).
(g,h):Zeta potentials of the complexes (PAA(2 kDa)/LYS:0.1) in water and in NaAcetate are significantly different ($p < 0.05$).
(k): Size of the complexes (PAA/LYS:0.1) prepared with 2 kDa and 450 kDa PAA are significantly different ($p < 0.05$).

Table 3
Contact angle measurement results.

Complex code	Left-angle	Right-angle
2S/LYS	$34.62 \pm 3.43^{a,e,g}$	$32.74 \pm 6.11^{c,h,j}$
2B/LYS	$30.86 \pm 2.71^{b,e,g}$	$30.42 \pm 2.89^{d,h,j}$
450S/LYS	$61.82 \pm 0.84^{a,e,f}$	$63.13 \pm 0.77^{c,h,i}$
450B/LYS	$62.26 \pm 1.59^{b,e,f}$	$61.34 \pm 2.89^{d,h,i}$
Lysozyme	46.07 ± 1.45^{e}	46.54 ± 3.60^{h}
PAA (450 kDa)	71.52 ± 2.86^{f}	71.27 ± 2.72^{i}
PAA (2 kDa)	23.13 ± 0.10^{g}	21.35 ± 0.36^{j}

(a-d): Left and right-contact angles of the complexes prepared with 2 kDa and 450 kDa PAA are significantly different ($p < 0.05$).
(e,h): Left and right-contact angles of the complexes are significantly different ($p < 0.05$) from those of free lysozyme.
(f,i): Left and right-contact angles of the complexes are significantly different ($p < 0.05$) from those of free PAA (450 kDa).
(g,j): Left and right-contact angles of the complexes are significantly different ($p < 0.05$) from those of free PAA (2 kDa).

contact angle values less than 90°. The changes in the contact angles of free PAA (Contact angle ~22° or ~71°) and LYS (Contact angle ~46°) upon complexation were considered as an indication of successful complex formation. Complexes prepared from high molecular weight PAA (450 kDa) were found to be more hydrophobic and their contact angle values (~62°) are closer to the contact angle of free PAA (~71°). This result suggests that lysozyme is encapsulated inside the PAA layer. On the other hand, complexation of lysozyme with low molecular weight PAA resulted in a contact angle value between those of free LYS and PAA. This may possibly be due to presence of lysozyme molecules not only inside the complex but also on the surface as well.

3.2. Characterization of WPI films incorporated with PAA/LYS complexes

The effect of free LYS or PAA/LYS complex on the structure of the WPI films was observed with SEM cross section images of the selected films (Fig. 1). Both additives resulted in denser film structure which can be attributed to the formation of a stabilized network by possible electrostatic interaction or hydrogen bonding between whey protein isolate, PAA and LYS (Howell & Li-Chan, 1996; Sheinerman, Norel, & Honig, 2000). This network promotes the uniform distribution of complexes and free lysozyme in the WPI film as confirmed by SEM images.

The shape, size and distribution of the free LYS and complexes in the same films are shown in TEM images (Fig. 2). Although most of the complexes seemed to be distributed homogenously, some agglomerates were also observed which might be due to the effect of drying during the film formation. The sizes of the complexes in the film structure were measured around 20 nm which is close to their sizes measured by DLS as shown previously in Table 2. (~31 nm).

3.3. Release kinetics of lysozyme

The PAA/LYS complexes have a pH-dependent surface charge-reversal features, as their zeta potentials varied from negative to positive with a change in pH value of media from 7 to 4.5 (Table 2). At acidic pH values, stability of the complexes is lost as a result of repulsion between lysozyme and PAA which are both positively charged and the release of lysozyme is induced. The release profiles obtained with the films including 100% free lysozyme, 100% complexed lysozyme or 50% free and 50% complexed lysozyme are shown in Figs. 3 and 4. The code of the films is listed in Table 1. The results indicate that the release of lysozyme is greatly influenced by the mode of lysozyme incorporation and complexation conditions (molecular weight of PAA and PAA/LYS ratio). A significant difference in the release rate of lysozyme was found between two formulations of WPI film (100% free lysozyme and 100% complexed lysozyme). The highest release rate was observed for the film prepared with 100% free lysozyme and the lowest release rate was measured from the film including lysozyme complexed with 450 kDa PAA. Migration of free lysozyme into the release medium was completed in less than 24 h. On the other hand, at the end of 500 h only 20% of complexed lysozyme released from the WPI-100% 450B/LYS Complex film. To prevent rapid consumption of lysozyme or insufficient growth inhibition for the bacteria due to very fast or slow release, we incorporated lysozyme both in free (50%) and in complexed form (50%). As expected, the release rate of lysozyme from these films was found to be between 100% free and 100% complexed lysozyme incorporated films. In order to control the release rate, we have changed not only mode of incorporation of lysozyme but also molecular weight of PAA and PAA/LYS ratio during complexation. We observed that lysozyme is released more quickly from the films integrated with the 2 kDa PAA lysozyme complexes than from those with the 450 kDa counterpart (Fig. 3A and B). This observation can be explained by the larger sizes of 450 kDa PAA/LYS complexes and the location of lysozyme in the complex. Lysozyme is completely wrapped by the 450 kDa PAA since fully extended length of this PAA is much longer than the circumference of the lysozyme molecule (Chieng & Chen, 2010). This is confirmed by the contact angles of 450 kDa complexes closer in magnitude to the contact angle of free PAA (450 kDa) rather than that of free LYS. Although ~100% lysozyme loading was achieved for complexes with 2 kDa PAA, the chain lengths of low MW PAA are not sufficiently long to wrap around all the lysozyme to completely embed it in the complex. Consequently, some fraction of lysozyme is located on the complex surface which easily detaches from the surface and diffuses through the film. Slower lysozyme release from the films integrated with 450 kDa complexes can also be attributed

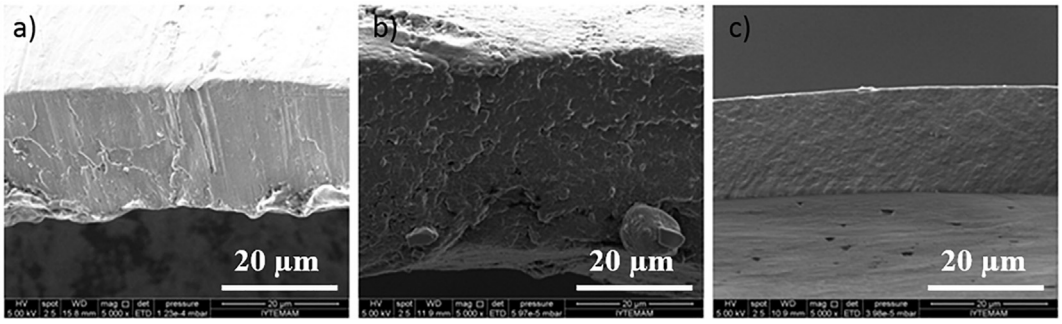

Fig. 1. SEM cross section images of selected films a) WPI film, b) Free LYS incorporated film and c) PAA/LYS complex (2S/LYS: PAA MW of 2 kDa and PAA/LYS ratio of 0.1) incorporated WPI film. Magnification 5000×.

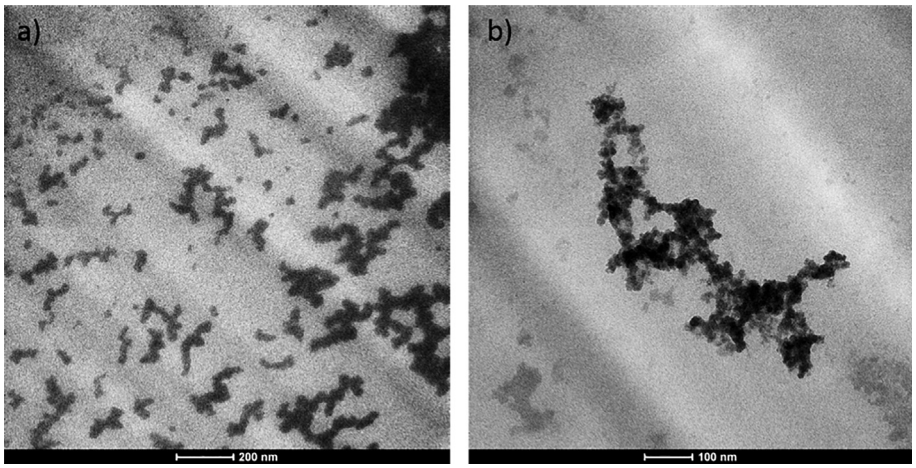

Fig. 2. TEM cross section images of a) free LYS (Scale bar: 200 nm) and b) PAA/LYS complex (2S/LYS: PAA MW of 2 kDa and PAA/LYS ratio of 0.1) incorporated WPI films (Scale bar: 100 nm).

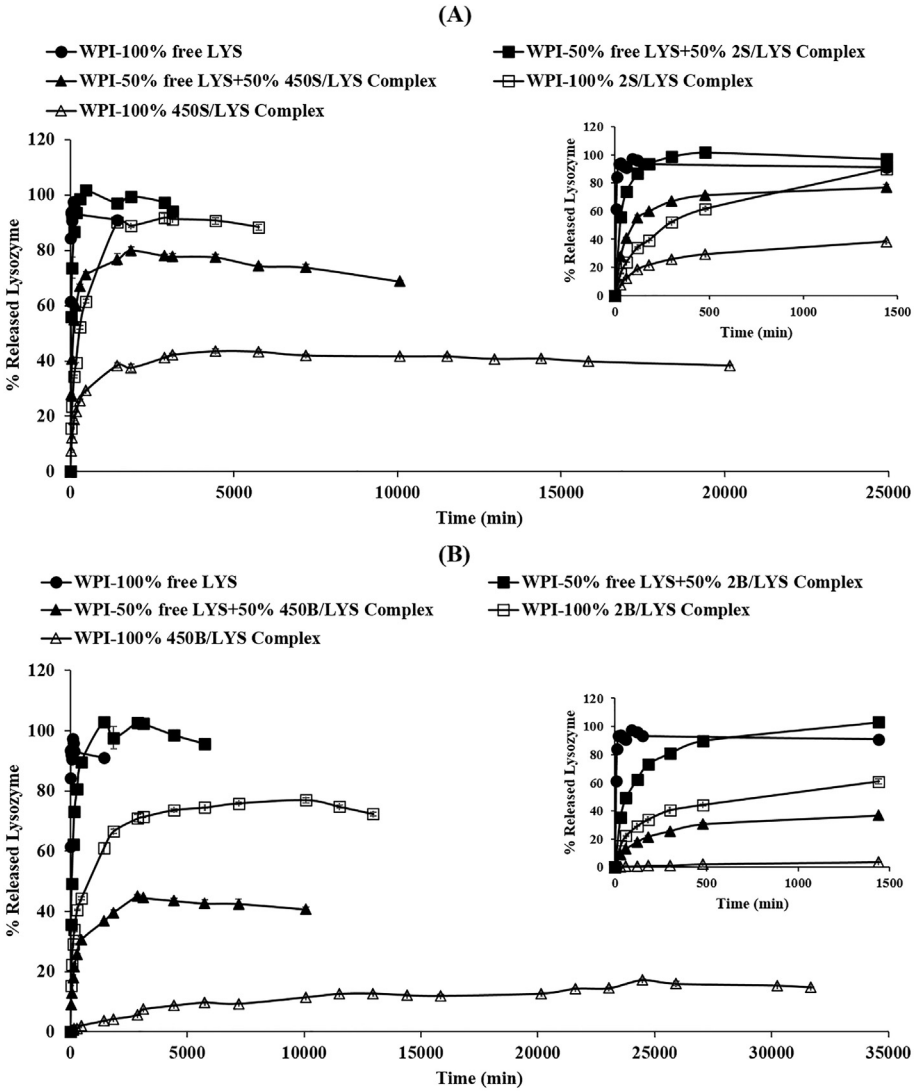

Fig. 3. Effect of PAA molecular weight on the release kinetics of LYS A) PAA/LYS ratio:0.1, B) PAA/LYS ratio:0.3.

to relatively more hydrophobic nature of these complexes and stronger binding of lysozyme to PAA compared to 2 kDa counterparts (Table 3). In addition to PAA molecular weight, the PAA/LYS ratio was also found to be effective in controlling the release rate (Fig. 4A and B). Increasing this ratio from 0.1 to 0.3 resulted in larger complexes (Table 2) and a correspondingly slower release rate from the films integrated with these complexes. Previous investigators attempted to control the release rate of lysozyme by changing film morphology with polymer concentration (Gemili et al., 2009), plasticizer concentration (Min et al., 2008), degree of cross linking (Buonocore et al., 2003a, b; Buonocore et al., 2004; Fajardo et al., 2014; Ma et al., 2013; Ozdemir & Floros, 2001) and pH of the release medium (Benelhadj et al., 2016; Mendes de Souza et al., 2010). Some of these strategies resulted in short release times ranging between 1 and 27 h (Bayarri, Oulahal, Degraeve, & Gharsallaoui, 2014; Benelhadj et al., 2016; Gemili et al., 2009), while slow release of lysozyme up to 5 d was also reported (Fajardo et al., 2014). In comparison, the results in Figs. 3 and 4 demonstrated that it was possible to extend the release time from less than 24 h up to 500 h through incorporating complexed lysozyme instead of free lysozyme in the films. The pH-dependent release of lysozyme from the complexes provides release-on-demand functionality to the films by preventing undesirable release during loading or film preparation, allowing release only at the pH of food in the package. The release profiles of all the films were fit to Equation (1) to estimate the diffusion coefficient of lysozyme in the films. The results listed in Table 1 show that complexation of lysozyme with 450 kDa PAA at the PAA/LYS ratio of 0.3 dramatically reduced its diffusivity in the film from ~10^{-9} to ~10^{-13} cm^2/s. Calculated diffusivities are apparent values since the model only takes into account mass transfer by diffusion and does not consider dissociation kinetics of the complex (Crank, 1975; Koontz et al., 2010). We note however that the fit of model to the experimental data for the films including complexed lysozyme was as good as the fit to the release data of free lysozyme containing films (Fig. S.2 Supplementary Information). This indicates that the rate of dissociation of complex is fast compared to the rate of diffusion of lysozyme in the film; consequently, the release of lysozyme from the films is controlled by the diffusion of lysozyme.

Min et al. (2008) reported the change in the diffusion coefficients of lysozyme in edible WPI coatings from ~0.7×10^{-9} to 3×10^{-12} cm^2/s by increasing WPI:Glycerol ratio from 1:1 to 3:1. Although changing WPI:Glycerol ratio seems to be an effective strategy in controlling the release rate of lysozyme through edible coatings, it was shown that increasing glycerol content in WPI films decreases tensile strength of the films (McHugh & Krochta, 1994). For this reason, this strategy cannot be used to achieve sustained release of lysozyme from self-standing WPI films. In our study,

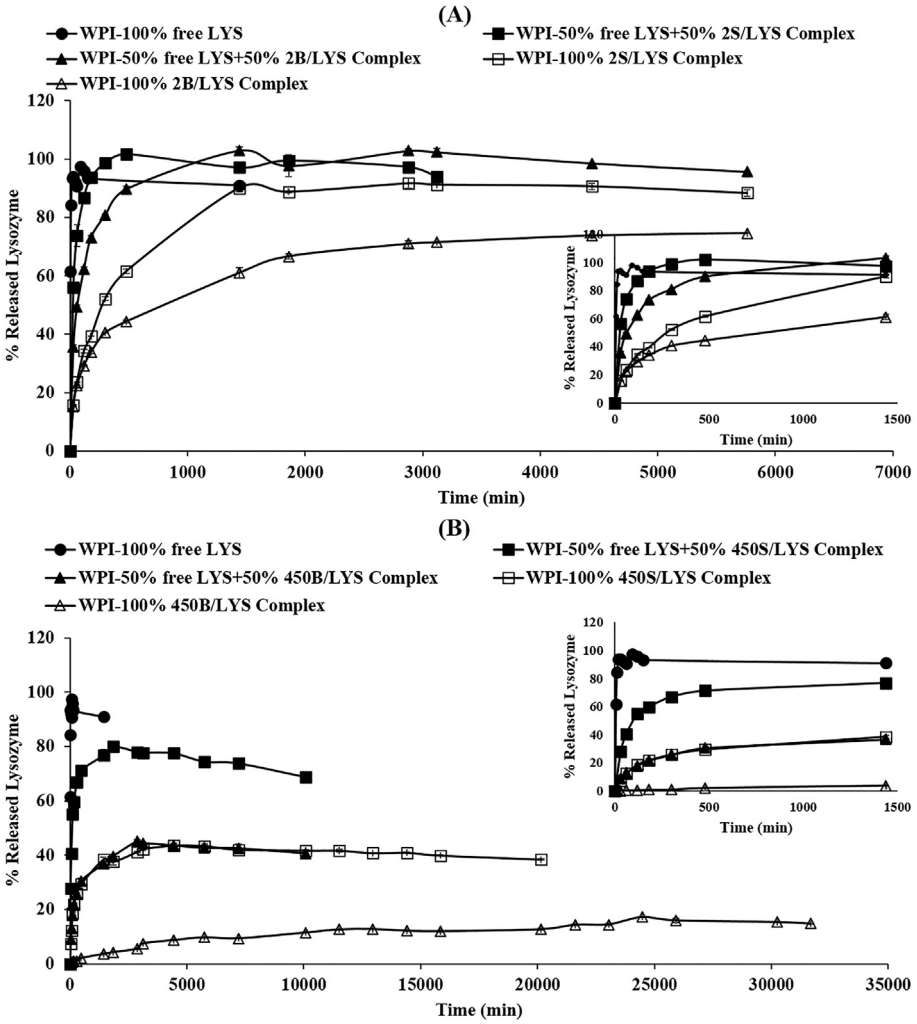

Fig. 4. Effect of PAA/LYS ratio on the release kinetics of LYS A) PAA MW:2 kDa B) PAA MW:450 kDa.

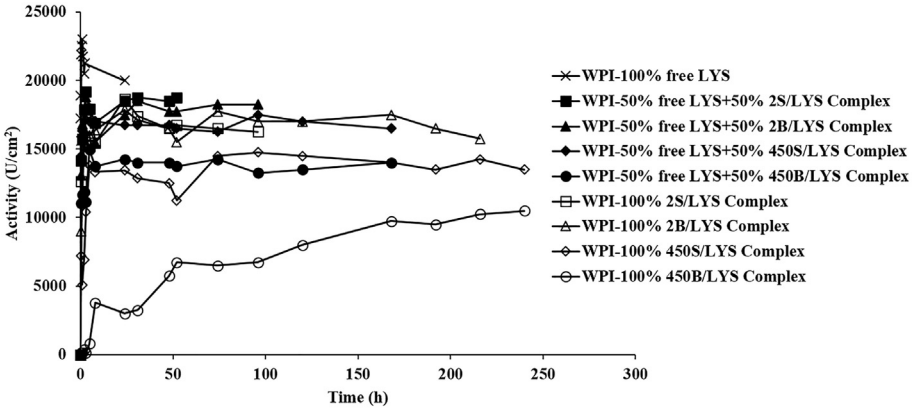

Fig. 5. Time dependent released LYS activity of the prepared films.

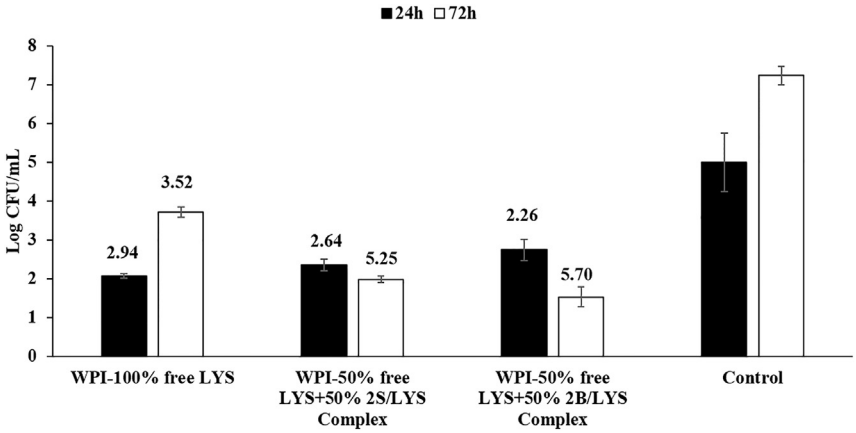

Fig. 6. The time dependent antibacterial activity of selected films. ($p < 0.05$).

lysozyme release time is controlled in a broad range by changing the mode of lysozyme addition and adjusting the complex properties such as size, charge and hydrophilicity/hydrophobicity without changing film casting conditions.

3.4. The released LYS activity

The activity of LYS released from the WPI films with respect to time appears in Fig. 5. The highest and the lowest activities were detected for the films containing 100% free (WPI-100% free LYS) and 100% complexed lysozyme with 450 kDa PAA (WPI-100% 450B/LYS Complex), respectively. For practical applications of antimicrobial food packaging films, it is important to maintain sufficient activity of antimicrobial agent for a long time so that bacteria growth is inhibited. As seen from Fig. 5, the activity of lysozyme released from the films including only uncomplexed free lysozyme showed a maximum at the end of 1 h and then started to decline. Adding lysozyme into the films in complexed form significantly reduced its release rate. Consequently, lysozyme activity from these films was lower compared to free lysozyme containing film; however, the released activity remained at a constant level after an initial sharp increase. To determine change in the activity of lysozyme upon complexation, the activity of unused fresh lysozyme with an equivalent amount to the maximum released amount was measured. It was found that no significant activity loss was observed due to complexation, especially with 2 kDa PAA (Supplementary Information, Table S.1.). At the end of 72 h, the highest lysozyme activities were observed in films containing 50% free and 50% complexed lysozyme. Free lysozyme in the films released very quickly to the medium, increasing the activity. This was followed by the sustained release from the complexes keeping LYS amount and lysozyme activity in the release medium constant with time. The results in Fig. 5 clearly showed that it is possible to achieve a sustained lysozyme activity through the mode of LYS incorporation and PAA/LYS complex properties.

3.5. Time dependent antimicrobial activity of the films

The antimicrobial effectiveness of three selected films (WPI-100% free LYS, WPI-50% free LYS+50% 2S/LYS Complex and WPI-50% free LYS+50% 2B/LYS Complex) with respect to time was determined against *L. innocua*. The films incorporated with only complexed lysozyme were not chosen due to low lysozyme released from these films. On the other hand, the film containing only free lysozyme which showed the burst release was tested as a control while the films including 50% free and 50% complexed lysozyme were chosen to illustrate the advantage of sustained release.

According to the results in Fig. 6, although the WPI film including only free lysozyme (WPI-100% free LYS) showed the highest inhibition for bacteria growth (~2.9 log reduction with respect to control) at the end of 24 h, this growth suppression was unsustainable; consequently, bacteria concentration increased from ~2 to ~3.7 log cfu/mL by 72 h. In contrast, *L. innocua*

population decreased continuously over 3 d when incubated with the films including both free and complexed lysozyme. The decrease in the antimicrobial efficacy of the film with only free lysozyme can be explained by the burst release within 1 h (Figs. 3 and 4) resulting in a first sharp increase, followed by the loss of lysozyme activity (Fig. 5). As Fig. 6 shows, the suppression of bacterial growth with the films including both free and complexed lysozyme increased from ~2.6 to 5.2 log reduction for the WPI-50% free LYS+50% 2S/LYS Complex film and from ~2.3 to 5.7 log reduction for the WPI-50% free LYS+50% 2B/LYS Complex film at the end of 72 h as a result of sustained lysozyme release from these films. Compared to the other studies in which the antimicrobial activity of the lysozyme containing films against *L. innocua* was tested, the films prepared in this work showed higher antimicrobial activity. For instance, Barbiroli et al. (2012) reported 0.5 log reduction in *L. innocua* population compared to control case at the end of 24 h incubation. In another study by Fajardo et al. (2014), the highest log reduction for *L. innocua* was determined as 2.13 at the end of 24 h. However, after this time the quantity and activity of released lysozyme was insufficient to prevent bacterial growth, leading to an increase in colony formation unit from ~7.5 to 8.5 log cfu/mL by 32 h (*i.e.*, log reduction decreased from 2.13 to 1.43 by increasing incubation time from 24 to 32 h) (Fajardo et al., 2014). In conclusion, the results in Fig. 6 confirmed the hypothesis that the sustained release of an active agent from food packaging films is required for maintaining long-term antimicrobial effect.

4. Conclusion

We have developed new whey protein isolate based antimicrobial food packaging films by incorporating lysozyme into the films in free and complexed form. Complexation of lysozyme with polyacrylic acid was found to be an effective tool for controlling its release from the films. The properties of the complexes, such as size, surface charge and hydrophilicity, were easily controlled by changing PAA molecular weight or PAA/LYS ratio. The pH-dependent charge density of PAA was used to maximize lysozyme encapsulation, maintain uniform dispersion of the complexes in the WPI film and control complex stability. The tunability of these properties enabled optimization of the lysozyme release rate. Our results suggest that the disadvantage associated with the burst release of lysozyme from the hydrophilic matrixes can be eliminated by adding lysozyme into the film in complexed form. Complexation significantly reduced the release rate of lysozyme, allowing sustained release for extended periods. The WPI film prepared by adding only free lysozyme could not maintain its antimicrobial activity after 24 h. However, the WPI films incorporating both complexed and free lysozyme sustained antimicrobial activity over time by supplementing the initial burst release from the free lysozyme with a slower sustained release from the complexed lysozyme. The strategy used in our study for achieving sustained lysozyme release can be applied to other antimicrobial proteins/peptides by complexing with a suitable polyelectrolyte and incorporating into WPI or other hydrophilic films. The prerequisite for film formation in the presence of complexes is to adjust pH so that the complexes and the matrix polymer have opposite charges. The films prepared in this study may have a great potential for food preservation and shelf-life extension. Further work is needed to evaluate the effectiveness of these films in the preservation of real food products.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2016.07.001.

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