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In Situ Fibril Formation of κ-Casein by External Stimuli within Multilayer Thin Films

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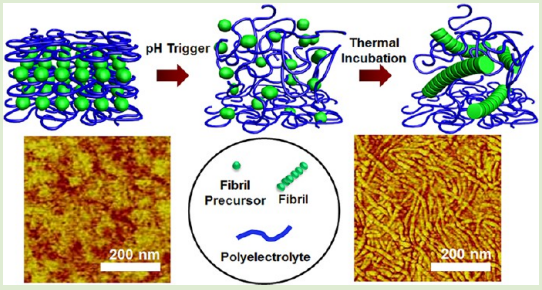
Supporting Information

ABSTRACT: We have developed the in situ fibrillation of κ-casein, employed as amyloid precursor, within multilayer films consisting of κ-casein and poly(acrylic acid) (PAA) prepared by the layer-by-layer (LbL) deposition. The fibrillation of κ-casein within the multilayered films is strongly dependent on the extent of intermolecular interactions between κ-casein and PAA. When films constructed initially at pH 3 were heat treated at the same pH, κ-casein did not transform into fibrils. However, when the films were subjected to heat treatment at pH 5, κ-casein was transformed into fibrils within multilayer films due to weakened intermolecular interactions between κ-casein and PAA. We also noted that the multilayer film was swollen at pH 5 by the charge imbalance within the film, which we believe gives enough mobility for κ-caseins to form fibrils with adjacent κ-caseins within the multilayer. The fibrils were found to be uniformly distributed across the entire film thickness, and the aspect ratio as well as the number density of fibrils increased as a function of incubation time. The present study reveals a strategy to realize in situ nanocomposites within LbL multilayer films simply by triggering the formation of protein fibrils by controlling the intermolecular interactions between amyloid precursors and polyelectrolytes (PEs).



Protein aggregates involving amyloid fibrils have recently received much attention in a variety of research areas such as medicine,[1−4] nanotechnology,[5,6] food,[7,8] and soft matter science.[9,10] One most widely known role of amyloid fibrils, induced by hydrogen bonds between parallel or antiparallel beta sheets, is their close connection to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.[11−13] However, not all amyloid fibrils are disease-related, and amyloid fibrils from nondisease-related proteins have great potentials for functional nanobiomaterials due to their highly ordered structure, robust mechanical properties, and elasticity.[14−16] Furthermore, those amyloid fibrils could be easily modified to generate specific anchoring sites for inorganic materials or biomolecules.[17,18] These merits make the amyloid fibrils very attractive and promising candidates for the development of advanced nanobiomaterials. Researchers have previously constructed protein fibril-based nanocomposites by adding fully matured protein fibrils into different bulk matrices such as silicon elastomer (poly(dimethylsiloxane)),[19] poly(L-lactic acid),[20] poly(ethylene glycol),[21] and epoxy resin.[22] These new types of nanocomposites, filled with optimal content of protein fibrils, possess tuned and balanced physical and mechanical properties such as stiffness and Young's modulus without sacrificing their thermal and elongation properties. More recently, nanocomposites containing protein fibrils and graphene sheets have been demonstrated to be biodegradable and highly conductive.[23]

The layer-by-layer (LbL) deposition has frequently been applied, due to its versatility, to develop functional multilayered thin films in various fields such as energy, environmental, and biomedical applications since its inception in the early 1990s.[24−26] The film thickness of the LbL films can easily be tuned with nanometer-scale resolution, and a wide variety of materials such as polymers, metals, ceramics, and biological molecules in different forms could be incorporated into the films at the desired location. Also, the LbL deposition is possible with all kinds of intermolecular interactions such as electrostatic interactions, hydrogen bonds, and hydrophobic interactions. The intensity of these intermolecular interactions between paring molecules could also be tuned by external stimuli such as pH or salts, resulting in the release or disintegration of the molecules as well as the swelling of the multilayer film.[27−29]

In the present study, we explored the in situ fibrillation of κ-casein within multilayer films consisting of κ-casein and PAA by the LbL deposition. The casein, which forms a unique calcium−phosphate transport complex, takes the largest portion of

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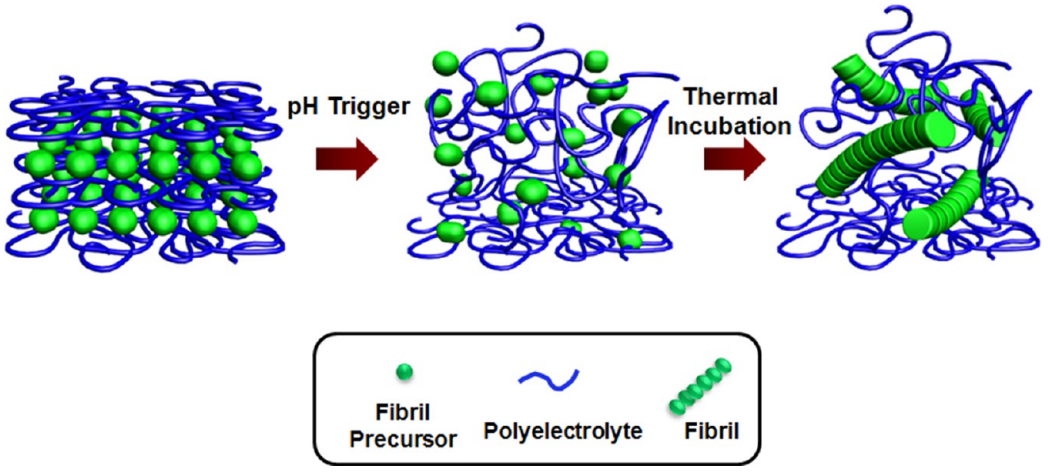

Figure 1. Schematic on the formation of in situ nanocomposite films by pH change and thermal incubation of multilayer films consisting of κ-casein and an oppositely charged PE.

bovine milk and consists of four different types of caseins: α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein. κ-Casein plays a role as a surface stabilizer of the whole casein colloidal particles at an interface between the hydrophobic caseins of the interior and the aqueous environment. The purified κ-casein from whole casein particles is in the oligomeric state, ranging from monomer to decamer, formed by three different types of intra- or interdisulfide bonds (Cys11−Cys11, Cys11−Cys88, and Cys88−Cys88).[30] However, the propensity toward extensive fibrillation is typically affected by the degree of dissociation of oligomeric κ-casein by the scission or reduction of disulfide bonds because the dissociated forms, only mono-, di-, and trimeric κ-casein, are the precursor to its amyloid fibril formation.[31] In addition, the thermal incubation accelerates the fibrillation kinetics. As a result, the fibrillation kinetics is accelerated, and the amount of fibrils is also increased by heat and the reducing agent for dissociation of κ-casein oligomer into monomer.

Figure 1 schematically depicts the process of developing in situ nanocomposite films through the transformation of κ-casein into amyloid fibrils within the LbL films which consist of κ-casein, as precursors of amyloid fibrils, and polyelectrolytes (PEs) with counter charges, constructed by the dip-based LbL deposition. Although thermal treatment and reduction of κ-casein oligomers are critical to transform the protein into amyloid fibrils in bulk solution, the additional important step for the κ-casein transformation within multilayered films is to control the strength of intermolecular interactions between κ-caseins and the pairing PEs to gain enough mobility for κ-casein. In other words, the interactions between κ-casein and PEs should be weak enough to form beta-sheets between κ-casein to realize amyloid fibrils within the film since the κ-casein do not transform into the fibrils when the intermolecular interactions between κ-casein and pairing PEs are too strong. At the same time, the pairing compounds within a multilayer flim should maintain certain intermolecular interactions to remain as a film without being delaminated.

The charge density of the κ-casein (pI: 4.5−5.8)[32] is affected by pH of their surrounding environment due to the gain or loss of protons, and the pH dependence on the charge density of κ-caseins is demonstrated in Figure S1 (Supporting Information). Since the κ-casein is positively charged at pH 3 as confirmed by zeta potential measurements, two kinds of negatively charged PEs, poly(acrylic acid) (PAA) (M_w = 100 000) and poly(4-styrene sulfonic acid) (PSS) (M_w = 70 000), were employed as opposite polymers to construct multilayer films to verify the effect of controlled intermolecular interactions between κ-casein and oppositely charged PEs on the fibrillation of κ-casein within the multilayer films. Figure 2 shows the film thickness

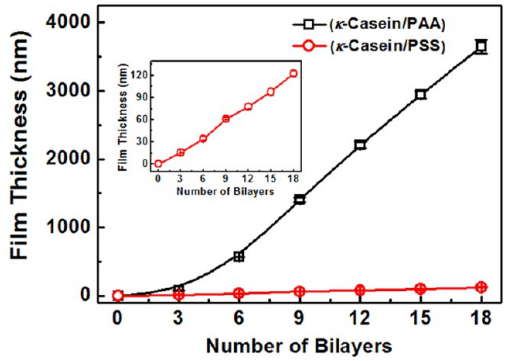

Figure 2. Film growth behavior of (κ-casein/PAA) and (κ-casein/PSS) multilayered films assembled at pH 3 on silicon substrates by the dip-based LbL deposition. An inset shows the magnified film thickness growth of (κ-casein/PSS) multilayers showing the linear growth.

growth curves of two different types of LbL films, indicating that the film growth behavior is significantly dependent on the type of polyanions used. The growth of (κ-casein/PAA) films exhibits an exponential-like growth behavior for the first few bilayers, followed by a linear growth from six bilayers showing the large increment in film thickness with each bilayer deposition. On the contrary, the growth of (κ-casein/PSS) bilayers is essentially linear with a very small thickness increment per each bilayer deposition. The average thickness per bilayer in linear growth regime is 261 ± 2 and 7 ± 0.5 nm for the (κ-casein/PAA) and (κ-casein/PSS) films, respectively. This tremendous difference is caused by the different building mechanisms of the LbL films. The degree of ionization of PSS, fully charged, is independent of the pH values, and the (κ-casein/PSS) films were constructed by electrostatic interactions at pH 3. On the other hand, the degree of ionization of PAA is strongly dependent on the pH values. The pK_a of PAA has been reported as a range of 5.5−6.5, and the degree of ionization is about 5% at pH 3 in bulk solution. However, the charge density

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of an absorbing PAA would increase substantially from its soluble-state value when it is incorporated into a multilayer film. The degree of ionization of PAA increases about 20−40% (pK_a value: 3.5−5.5) at pH 3 when PAA assembles with oppositely charged linear polymer, poly(allylamine hydrochloride) (PAH) and poly(diallydimethylammonium chloride) (PDAC).[33] Thus, we expect that the degree of ionization of PAA within the (κ-casein/PAA) multilayer film would increase when PAA is adsorbing on the positively charged κ-casein at pH 3. However, the degree of such increased ionization of PAA is insignificant when compared with PAA assembled with fully charged linear polymer PDAC and PAH since the zeta-potential of κ-casein (+13 mV) at pH 3 is low compared with the value of synthetic PEs.[34] Also, Cuisinier et al.[35] and Izumrudov et al.[34] have reported that protonated polycarboxylic acids of PAA and poly(methacrylic acid) and amide bonds of proteins are associated by hydrogen bonding. Thus, the amide bonds in κ-casein and protonated carboxylic acid should also form the hydrogen bonding. In addition, the amino acids such as Gln (Q), Asn (N), Asp (D), and Glu (E) in the κ-casein are also associated with protonated carboxylic groups in PAA at pH 3 by hydrogen bonding, and the number of the four different types of amino acids is 36 among 169 whole amino acids sequence in the κ-casein.

To ascertain the driving forces for the film construction, the (κ-casein/PAA) multilayer films were constructed in different deposition conditions: κ-casein (pH 3) with PAA at three different pH values (2, 3, and 4). The film growth curves (in Figure S2, Supporting Information) show the fastest growth of a multilayer film when assembled at pH 3/3 (denoting the pH of κ-casein/pH of PAA) when compared with other deposition conditions pH 3/4 and pH 3/2. The electrostatic interactions are believed to increase in the film assembled at pH 3/4, while the hydrogen bonding is more likely for the film assembled at pH 3/2. We believe that the deposition of a multilayer film with the highest bilayer thickness, assembled at pH 3/3, is mainly driven by the combined interactions of both hydrogen bonding and electrostatic interactions. The experimental data showing the different film growth behavior are given in Figure S2 (Supporting Information).

To elucidate the effects of controlled intermolecular interactions between κ-casein and the PEs on the fibrillation of κ-casein within the LbL films, both the films, initially assembled at pH 3, were thermally treated at 80 °C with pH 3, 5, and 8 in the presence of DTT (20 mM). Prior to observation of the fibrillation within the multilayered films, only κ-casein was thermally treated at 80 °C with pH 3, 4, 5, and 8 in the presence of DTT in bulk solution (Figure S3, Supporting Information), to verify the fibril formation as a function of pH. While the fibril morphologies incubated at pH 3, 4, and 8 are similar to well-dispersed fibrils, the κ-casein incubated at pH 5 shows the coexisting phase of random aggregates and fibrils, presumably due to lack of electro-repulsive force between κ-caseins close to pI. Figure 3 is the AFM phase images of two different types of LbL films: the $(\kappa\text{-casein/PAA})_{6.5}$ films on the left column and the $(\kappa\text{-casein/PSS})_{6.5}$ films on the right column. The images of (a) and (b) are the surface morphologies of each film assembled at room temperature and pH 3. The images of (c, d), (e, f), and (g, h) are the surface morphologies of the films thermally treated for 6 h at pH 3, 5, and 8, respectively. The films thermally treated at pH 3, the same pH value in which the multilayer films were initially deposited, do not show the formation of amyloid fibrils because

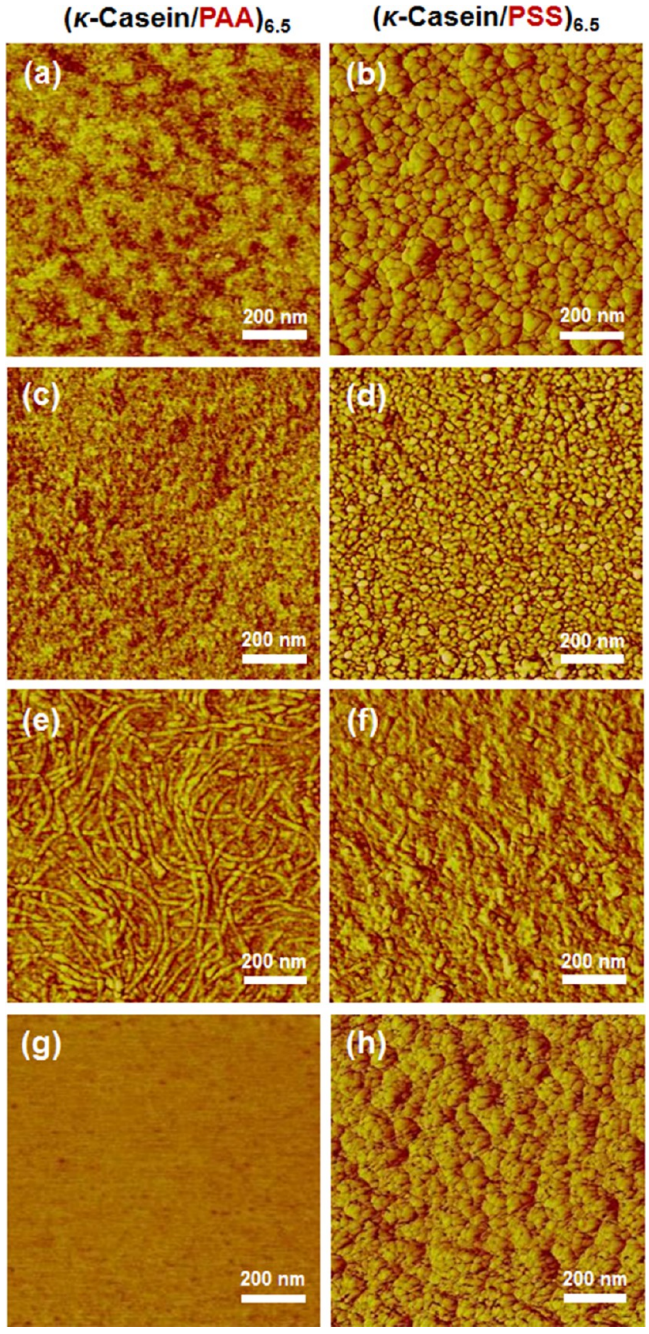

Figure 3. AFM phase images of the surface morphologies of $(\kappa\text{-casein/PAA})_{6.5}$ (left column) and $(\kappa\text{-casein/PSS})_{6.5}$ (right column) multilayer films. (a) and (b): the surface morphologies of films assembled at room temperature and pH 3; (c) and (d), (e) and (f), and (g) and (h): the surface morphologies of films thermally incubated at pH 3, 5, and 8 in DTT solution (20 mM).

of strong intermolecular interactions between κ-casein and pairing PEs (PAA) maintained, hindering the mobility of κ-casein to form fibrils. When these films were thermally incubated at pH 5, the κ-casein was successfully fibrillated within the (κ-casein/PAA) multilayer films but not within the (κ-casein/PSS) multilayer films. We believe that the decrease in the number of hydrogen bonds between κ-casein and PAA by the deprotonation of carboxylic acid groups in PAA and the reduced electrostatic attractions due to the change in zeta potential of κ-caseins from positive to neutral values when pH

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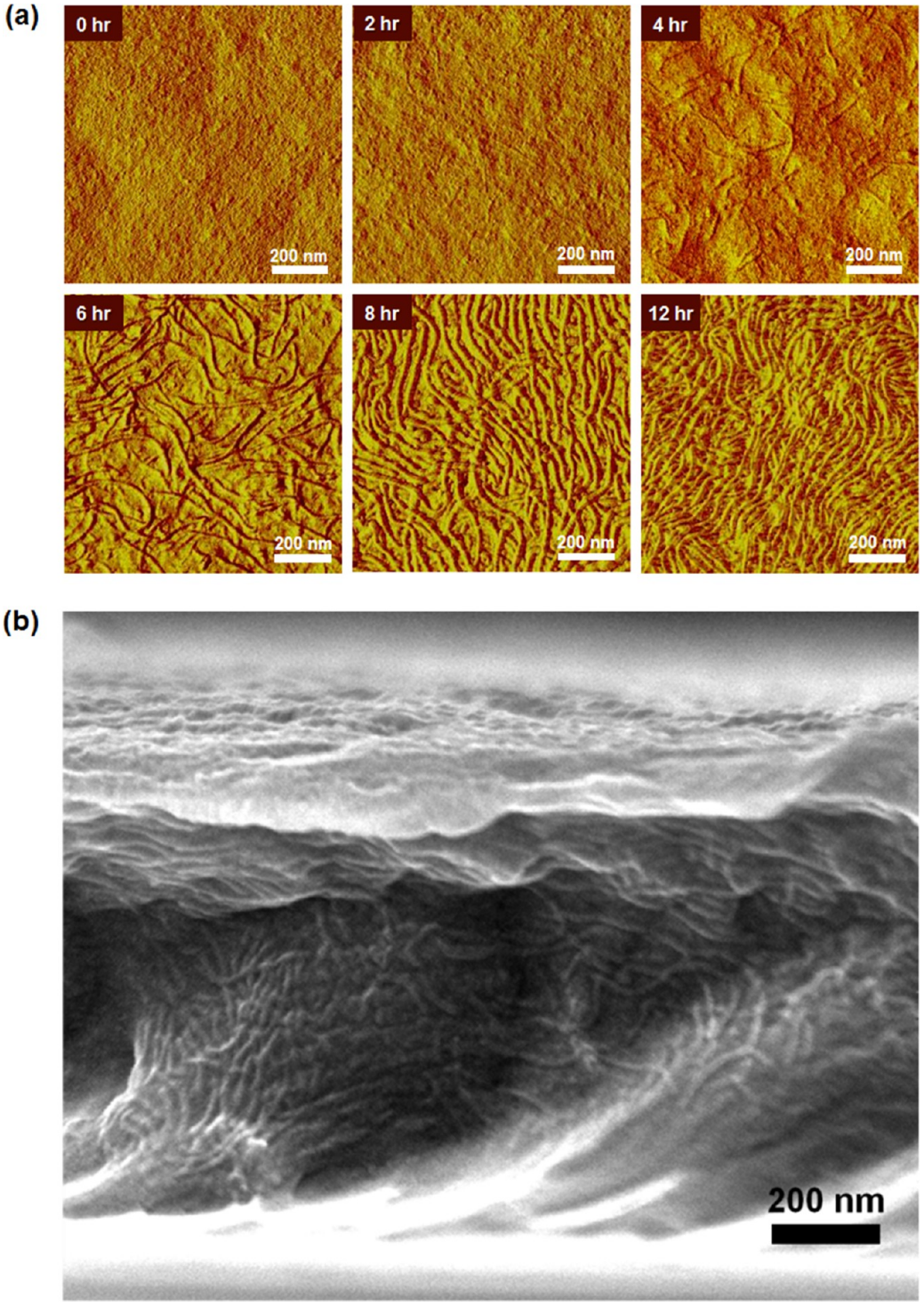

Figure 4. (a) Surface morphologies of the (κ-casein/PAA)$_{6.5}$ multilayer films thermally treated at pH 5 in DTT solution (20 mM) as a function of incubation time. (b) A SEM cross-sectional image of a (κ-casein/PAA)$_{6.5}$ multilayer film thermally treated for 12 h in DTT solution (pH 5, 20 mM), for which the surface morphology is shown in (a).

changes from 3 to 5 (Figure S1, Supporting Information) all contributed to a gain in the mobility of κ-casein within the film to form fibrils. We also note that the fibril morphology within the film is quite different from the fibrils formed with the treatment at pH 5 in the bulk solution (Figure S3, Supporting Information): well-distributed fibrils within the film without massive κ-casein aggregates. This is due to the fact that well-dispersed κ-casein was uniformly deposited when the initial deposition condition was pH 3 and was transformed into fibrils when thermally treated in pH 5 of DTT solution without massive aggregation of κ-casein owing to intermolecular interactions between κ-casein and pairing PEs within the multilayered films.

Although the degree of charge density of κ-casein varies by changing pH so much like weak PEs, the fibril formation was not achieved when the κ-casein was initially strongly bound to

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PSS, presumably due to the negligible change in the intermolecular interactions between κ-casein and PSS, because the degree of ionization of a weak PE is strongly dependent on the type of oppositely charged PEs within the LbL films.[36] We also note that when the multilayer films were thermally incubated at pH 8 (κ-casein/PAA) film was completely dissociated, while the (κ-casein/PSS) film maintained over 90% of its original film thickness. In addition, the $(\kappa\text{-casein/PAA})_{6.5}$ film was thermally incubated at pH 4, a midway between pH 3 and 5, in the presence of DTT to clarify how sensitively the fibrillation of κ-caseins within LbL films is affected by pH stimuli. The fibrils formed at pH 4 show the morphology similar to the fibrils formed at pH 5 except the fact that the number density of fibrils within the film is lower than the number density of fibrils formed at pH 5, as shown in the Supporting Information (Figure S4). These results demonstrate that the sensitive control of intermolecular interactions between κ-caseins and pairing PEs within multilayered films is crucial in transforming κ-caseins into fibrils within the multilayer films.

Furthermore, to confirm the pH sensitivity of intermolecular interactions between κ-casein and charged polyelectrolytes for the formation of fibrils in bulk solutions, κ-casein solutions were mixed with two types of polyelectrolytes (i.e., PAA and PSS) at pH 3 and thermally incubated for 12 h at pH 3, 5, and 8 in the presence of DTT, which are the same conditions for the film experiment. At pH 3, both mixtures showed big aggregates of clusters consisting of κ-casein and PAA or PSS. However, in the case of κ-casein/PAA mixtures, the massive aggregates, initially formed by the complexation at pH 3, were gradually dissociated by the increase in pH with κ-casein fibrils formed at pH 5 and 8 by thermal incubation. In contrast, the κ-casein/PSS mixtures were found to be less influenced by pH adjustment without forming fibrils, although the size of clusters somewhat decreased at pH 8 (Figure S5, Supporting Information). Although there have been several reports on the kinetics of fibril formation of proteins affected by charged macromolecules,[37,38] the type of pairing PEs, such as weak and strong PEs, mainly determines the fibril formation of κ-casein within LbL films.

Also, the swelling behavior of two different types of the films, (κ-casein/PAA) and (κ-casein/PSS) films, is monitored with quartz crystal microbalance with dissipation monitoring (QCM-D), which were both subject to post-treatment at pH 5 of water. We set the bilayer number at 3.5 for both film systems such that the film swelling could be monitored by QCM-D. We noted the significant decrease in the frequency ($\Delta f_3/3 = -110$ (Hz)) when the $(\kappa\text{-casein/PAA})_{3.5}$ film was post-treated at pH 5, indicating that the film was gradually swollen up to 12 h by the pH change. On the other hand, there is no such changes in the frequency for the $(\kappa\text{-casein/PSS})_{3.5}$ film. We believe that the swelling of weak PE-based $(\kappa\text{-casein/PAA})_{3.5}$ film is induced by the charge imbalance (i.e., deprotonation of carboxylic acids in PAA) within the film, originating from the pH change. This film swelling is believed to assist the increase in the mobility of κ-caseins within the multilayer film to form fibrils (Figure S6, Supporting Information). On the basis of the results given in Figure 3 and Figures S4, S5, and S6 (Supporting Information), we conclude that reduced intermolecular interactions between κ-casein and PAA, leading to the film swelling, would give enough mobility for κ-casein to form beta-sheet stacks with adjacent κ-casein within the multilayer. In other words, κ-casein is successfully fibrillated within the LbL films when the hydrogen bonding among κ-casein beta-sheets is stronger than the intermolecular interactions between κ-casein and PAA, thus indicating that the fibrillation process within the film is competitive.

Figure 4(a) shows the AFM images of the surface morphologies of the $(\kappa\text{-casein/PAA})_{6.5}$ multilayer films thermally incubated at 80 °C in a DTT solution of pH 5, as a function of incubation time. We have noticed that the length (or the aspect ratio) as well as the packing density of fibrils increase with the incubation time. For the first 2 h of thermal incubation, there was no transformation of κ-casein into fibrils observed. A small fraction of fibrils started to appear from 4 h of thermal incubation, and the fibrillation persisted and became more pronounced up to 12 h of incubation. The SEM image shown in Figure 4(b) is the cross-sectional image of a $(\kappa\text{-casein/PAA})_{6.5}$ film thermally treated for 12 h at 80 °C in the presence of DTT at pH 5. It is interesting to note that the κ-caseins were not only fibrillated on the surface of the (κ-casein/PAA) LbL film but also fibrillated throughout the film, and the fibrils were also fully entangled together. The mechanical characterization of such nanocomposite films containing amyloid fibrils is now under way with surface probe microscopy with nanomechanics characterization.

In summary, we have explored the formation of in situ nanocomposite films consisting of weak PEs (PAA) and κ-casein, employed as biocompatible amyloid fibril precursors, constructed by the dip-based LbL deposition. The κ-casein was transformed into amyloid fibrils without film delamination by combined external stimuli such as pH, reductant, and heat. The aspect ratio as well as the fibril density of such amyloid fibrils within the multilayer films were controlled by incubation time. The formation of in situ nanocomposite films, triggered by external stimuli, could open up new types of nanocomposite platforms suitable for a wide range of advanced materials and biomedical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedure, zeta-potential measurements and turbidity measurements of κ-caseins, QCM-D results, film growth curves of (κ-casein/PAA) films (when the pH of κ-casein is 3, PAA is 2, 3, and 4), and additional AFM and TEM images are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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