ACS APPLIED MATERIALS & INTERFACES

Research Article

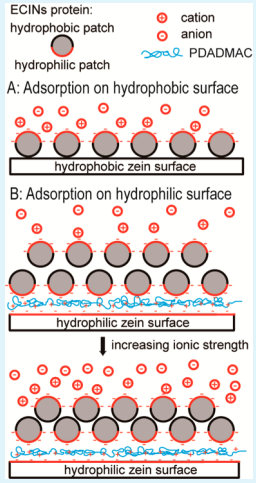
www.acsami.org

Improving Ice Nucleation Activity of Zein Film through Layer-by-Layer Deposition of Extracellular Ice Nucleators

Ke Shi, Hailong Yu, Tung-Ching Lee,* and Qingrong Huang*

Department of Food Science, Rutgers University, 65 Dudley, New Brunswick, New Jersey 08901, United States

ABSTRACT: Zein protein has been of scientific interest in the development of biodegradable functional food packaging. This study aimed at developing a novel zein-based biopolymer film with ice nucleation activity through layer-by-layer deposition of biogenic ice nucleators, that is, extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola*, onto zein film surface. The adsorption behaviors and mechanisms were investigated using quartz crystal microbalance with dissipation monitoring (QCM-D). On unmodified zein surface, the highest ECINs adsorption occurred at pH 5.0; on UV/ozone treated zein surface followed by deposition of poly(diallyldimethylammonium chloride) (PDADMAC) layer, the optimum condition for ECINs adsorption occurred at pH 7.0 and *I* 0.05 M, where the amount of ECINs adsorbed was also higher than that on unmodified zein surface. QCM-D analyses further revealed a two-step adsorption process on unmodified zein surfaces, compared to a one-step adsorption process on PDADMAC-modified zein surface. Also, significantly, in order to quantify the ice nucleation activity of ECINs-coated zein films, an empirical method was developed to correlate the number of ice nucleators with the ice nucleation temperature measured by differential scanning calorimetry. Calculated using this empirical method, the highest ice nucleation activity of ECINs on ECINs-modified zein film reached 64.1 units/mm^2, which was able to elevate the ice nucleation temperature of distilled water from −15.5 °C to −7.3 °C.



KEYWORDS: *extracellular ice nucleators (ECINs), zein, quartz crystal microbalance with dissipation monitoring (QCM-D), layer-by-layer deposition, biodegradable film*

INTRODUCTION

Biopolymers gain much attention on their potential as alternative biodegradable renewable packaging materials to reduce the usage of petroleum-based synthetic polymers. It is an emerging trends that using biopolymers in packaging materials, and it will become an evolutionary progress in food packaging industry.[1] One such example, zein is a major storage protein in corn and also the main coproduct of biofuel industry. Zein has been developed in many applications, such as coatings, fibers, inks, tissue engineering, and drug delivery.[2−5] But the most promising applications of zein are still biodegradable films, coatings, and plastics used for packaging. For example, utilizing the unique relatively hydrophobic nature of zein, paper coated by zein is suitable for the packaging of high-fat foods and sandwiches.[6] However, other than conventional applications, there are a limited number of studies on developing advanced zein-based functional films. The published research includes development of antimicrobial or antioxidant zein films by incorporating lysozyme, albumin proteins, disodium EDTA, and phenolic compounds.[7−9]

Extracellular ice nucleators (ECINs) isolated from *Erwinia herbicola* are biogenic ice nucleators that function in a freezing process, specifically on reducing the supercooling which is the stage when water cools below the freezing point without transforming into ice due to the lack of ice nucleators. Freezing is one of the best technologies for food preservation.[10,11] However, prominent supercooling makes the technology time- and energy-consuming and potentially harmful to the quality of frozen food.[12] ECINs provide heterogeneous ice nucleation sites for water molecules to bind and to form hydrogen bonds, so that the energy barrier during supercooling is significantly overcome and consequently water freezes at elevated subzero temperatures.[13] More importantly, by modifying the freezing process, ECINs were found to preserve the fish fillet, bakery yeast, and frozen bread dough from quality deterioration during the freeze−thaw process.[14,15] However, mixing ECINs into food is inconvenient and unfriendly to formulation and labeling. Thus, immobilization of ECINs has its practical significance on realizing the application of ECINs. Yet such technique is still lacking. It was reported that zein films are freeze/thaw stable, allowing their application in packing frozen foods.[16] Thus, zein could offer the carrier material for ECINs immobilization. In another study, zein-based functional films with ice nucleation activity were produced by immobilizing extracellular ice nucleators (ECINs) on film surface. It was demonstrated that the films preserved the baking quality of frozen bread dough.[17]

In the present study, the conditions for immobilizing ECINs through layer-by-layer deposition on zein film surface were investigated and the mechanisms of ECINs adsorption on two types of zein film surface were explored: untreated relatively hydrophobic surface and UV/Ozone (UVO) treated hydro-

Received: May 2, 2013
Accepted: October 9, 2013
Published: October 9, 2013

philic surface. In the second case, poly-(diallyldimethylammonium chloride) (PDADMAC) was adopted as the positively charged polyelectrolyte to help establish the multiple layers through electrostatic interactions, given that both ECINs and modified zein film surface are negatively charged at pH ≥ 5.[18] Quartz crystal microbalance with dissipation monitoring (QCM-D) technique was used to monitor the adsorption behavior of ECINs on zein films and to understand the deposition processes involved in zein films with different surface chemistry. Prior to this study, there was no method to quantify the ice nucleation activity on a solid surface. In this study, a new empirical quantitative method was developed and validated to assess the ice nucleation activity of zein-based films. The integration of extracellular ice nucleators (ECIN) into biopolymer thin films creates a novel biodegradable packaging materials which can be very advantageous in biomedical (e.g., storage of cells in frozen environments) and frozen foods applications with regard to energy saving and quality improvement.[15,17]

EXPERIMENTAL SECTION

Materials. The α-zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Poly-(diallyldimethylammonium chloride) (PDADMAC, also abbreviated as PDDA) solution, average M_w 200 000−350 000 (medium molecular weight), 20 wt % in H_2O, was purchased from Sigma Chemical Co. Milli-Q water was used throughout the experiment.

Preparation of Extracellular Ice Nucleators. The *Erwinia herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract medium under 18 °C. ECINs were isolated from the culture by sonication, filtration, and centrifugation as previously described.[15] Lyophilized ECINs were stored at −20 °C before use.

Ice Nucleation Activity Assay. INA defined as the activity units at −10 °C or higher was determined by the droplet-freezing assay[19] with some modifications. ECINs were dispersed into pH 7.0 Tris buffer to 10 mg/mL. Then 10-fold dilutions were prepared with distilled water. Twenty droplets, 10 μL each, were transferred onto the surface of polished aluminum pan which was then placed in −10.00 °C ethylene glycol/water bath, where the temperature was accurately controlled to ±0.01 °C by computerized heating/cooling devices. After 3 min, the number of frozen droplets was counted, and the value between 5 and 18 was considered to be statistically valid and the corresponding dilution was used for calculation using eq 1.

$$\mathrm{INA} = -\frac{\ln(1-f)}{V_d D} \tag{1}$$

Here f is the fraction of frozen droplets, calculated as $n_f/20$, where n_f is the number of frozen droplets; V_d is the volume of droplets (10 μL); and D is the dilution which gives a frozen droplet number in the range of 5−18, out of the 20 droplets.

To examine the environmental effect on ECINs' activity, solutions of different pH and ionic strength (IS) were prepared by using NaCl, HCl and/or NaOH.

Buffer Preparation. In the experiments that ECINs adsorbed on zein films, acetate buffers were prepared to pH 5.0 and 6.0. Tris buffers were prepared to pH 7.0, and 8.0. NaCl was used to adjust the IS in buffer.

UV/Ozone Treatment. Gold sensor crystals with or without coated zein films were exposed to ultraviolet emission from a low-pressure quartz-mercury vapor lamp at the distance of 2.54 cm. in the cabinet of a UV/ozone Cleaning System (model 42, Jelight Co. Inc., Irvine, CA).

Quartz Crystal Microbalance with Dissipation Monitoring. Gold sensor crystals (Q-Sense AB, Sweden) were precleaned by UVO treatment and wash liquid ($H_2O/H_2O_2/NH_3$, 5:1:1 v/v/v). Zein films were spin-coated on gold sensors as described in previous work.[20] QCM-D measurements were performed with a Q-SENSE D300 system (Q-Sense AB, Sweden) at 25 °C. Samples and buffers were conducted into the temperature-controlled chamber as gravitational flow. Depending on the test condition, acetate or Tris buffer was first introduced into the chamber to reach steady state with the film. Ten minutes after the baseline was established, samples (ECINs or PDADMAC) were introduced into the chamber to adsorb onto the surface of film. Minimal 5 min steady state was held until proceeding to the next step. The threshold to recognize steady state was that the change of frequency became slower than 1 Hz per 5 min. Each adsorption step was followed by a wash step using the buffer. During the steps, shifts in both frequency (ΔF) and energy dissipation (ΔD) were recorded simultaneously at the fundamental resonant frequency (5 MHz) along with the third (15 MHz), fifth (25 MHz), and seventh (35 MHz) overtones toward the end of each test.

Since biomolecules form viscoelastic layers which do not follow a rigid oscillation. the Voigt model was used to accurately estimate the mass of adsorption.[21] In this model, ΔF and ΔD are expressed by eqs 2 and 3.

$$\Delta F \approx -\frac{1}{2\pi\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3} + h_1\rho_1\omega - 2h_1\left(\frac{\eta_3}{\delta_3}\right)^2 \frac{\eta_1\omega^2}{\mu_1^2 + \omega^2\eta_1^2}\right\} \tag{2}$$

$$\Delta D \approx \frac{1}{\pi f\rho_0 h_0}\left\{\frac{\eta_3}{\delta_3} + 2h_1\left(\frac{\eta_3}{\delta_3}\right)^2 \frac{\eta_1\omega^2}{\mu_1^2 + \omega^2\eta_1^2}\right\} \tag{3}$$

where ρ_0 and h_0 are the density and thickness of crystal; η_3 is the viscosity of the bulk fluid (buffer); δ_3 [$= (2\eta_3/\rho_3\omega)^{1/2}$] is the viscous penetration depth of the shear wave in the bulk fluid; ρ_3 is the density of the bulk fluid, and ω is the angular frequency of the oscillation. The four parameters for the adsorbed layer are the thickness (h_1), the density (ρ_1), the viscosity (η_1), and the elastic shear modulus (μ_1). With ΔF and ΔD measured at the fundamental (1st) and the third, fifth, and seventh resonant frequency simultaneously, eight sets of data in one test could be modeled using eqs 2 and 3.

The density (ρ_3) and viscosity (η_3) of water at 25 °C are 0.997 g/mL and 0.890 cP, respectively. For ECINs, a common protein density of 1.22 g/cm^3[22] was adopted as ρ_1 for the software to calculate the thickness (h_1) of ECINs layer. The adsorbed mass was calculated by multiplying h_1 by ρ_1. The INA of ECINs used for adsorption on zein films is 1.61×10^6 units/mg.

Fourier Transform Infrared Spectroscopy (FT-IR). The Fourier transform infrared spectra were collected under ambient conditions, using a Thermal Nicolet Nexus 670 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a Smart MIRacle horizontal attenuated total reflectance Ge crystal accessory. Each spectrum was averaged by 256 scans with 4 cm^{-1} resolution.

Differential Scanning Calorimetry (DSC). DSC analyses were performed on a DSC 823E thermal analyzer (Mettler-Toledo Inc., Columbus, OH) supplied with liquid nitrogen and compressed nitrogen gas. Three batches of ECINs were individual prepared as described previously. Their INA was measured as 1.05×10^6, 8.56×10^5, and 3.57×10^6 units/mg, respectively. Each batch of ECINs was prepared into 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} mg/mL solutions. To measure the nucleation temperature of ECINs solutions, 10 μL ECINs solution was carefully transferred into 40 μL aluminum crucibles with lids. Temperature was decreased from 4 to −30 °C at a rate of −1 °C/min. An empty crucible was used as reference. To measure the nucleation temperature of water on films, the film was cut to exactly cover the bottom of crucible, and then 10 μL dH_2O was transferred carefully onto films and spread to cover the whole film surface. After the lid was closed, the crucible with sample was subjected to the same freezing condition as above. Crucible with films but without water was used as reference.

Statistical Analysis. One-way ANOVA and t test were performed to interpret the data at a confidence level of 0.95. The curve of nucleation temperature to number of ice nucleators was fitted by nonlinear regression, with the correlation coefficient calculated.

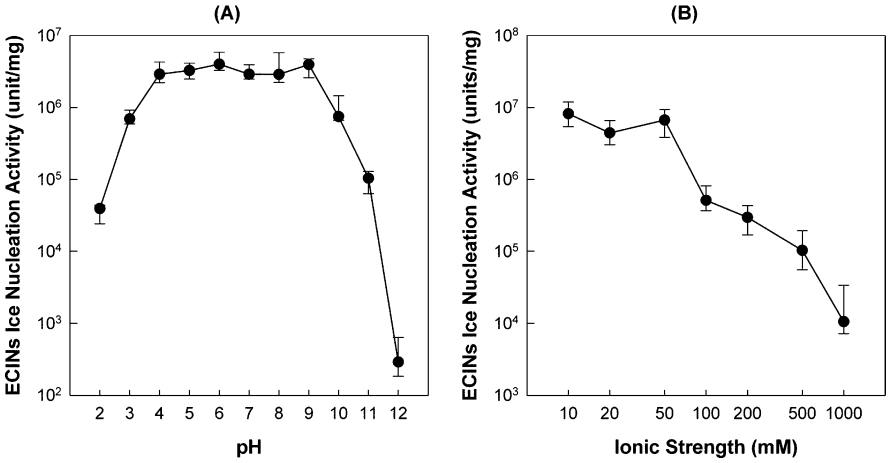


Figure 1. Effects of (A) pH and (B) ionic strength on INA of ECINs. Ionic strength for (A) was 0.02 M, and pH for (B) was 7.0.

RESULTS AND DISCUSSION

Effects of pH and Ionic Strength on the INA of ECINs. ECINs were protein-based ice nucleators containing lipid and carbohydrate moieties.[23] To investigate the optimum adsorption conditions on zein films, the effect of environmental physicochemical factors (pH and ionic strength, IS) on the INA of ECINs was first examined. As shown in Figure 1A, ECINs retained the activity in a wide pH range from pH 4.0 to 9.0. In extreme pH beyond this range, the ECINs activity dramatically diminished ($P < 0.05$).

In most cases, pH dependency of protein activity was determined by the pK values of certain amino acids involved directly in catalytic reactions or in maintaining the protein conformation.[24] As for ECINs, the INA depends on the overall structure of the lipoglycoprotein complexes,[25] which may account for the relative insensitivity to pH variation.

In addition to pH, the effect of IS was also examined. The maximum INA was detected at low IS, from 0.01 to 0.05 M (Figure 1B). Under higher ionic strength (IS), ECIN activity was inhibited ($P < 0.05$). This finding was consistent with the previous report.[26] Based on these findings, the effects pH (from 5.0 to 8.0) and IS (from 0.02 to 0.10 M) on the adsorption of ECINs on zein film surfaces were investigated in the following sections.

Adsorption of ECINs on Untreated Surface of Zein Films. Original untreated zein films have a relatively hydrophobic surface. After UV-ozone (UVO) treatment, the surface is hydrophilic, bearing carboxylic acid groups that are negatively charged at pH ≥ 5.[20] On the other hand, it was suggested that ECINs have both hydrophobic and hydrophilic patches on their surface.[25,27] Therefore, it was hypothesized that ECINs could adsorb onto untreated zein surface via hydrophobic interaction or onto UVO-treated negatively charged zein surface via electrostatic interaction. The changes of surface hydrophobilicity during UV/ozone treatment and protein adsorption were monitored by measuring the static water contact angles. Coated with zein, the gold crystal had a static surface water contact angle at about 80°. UVO-treatment (8 min) decreased the surface contact angle to about 20°. Coated with PDADMAC, the contact angle was slightly increased to about 35°. The detailed changes of surface elemental composition and contact angle before and after UV/ozone treatment have been reported in our pervious paper.[20] In order to find the optimum adsorption conditions to generate highest INA activity on zein films, the adsorption of ECINs on both untreated and UVO-treated zein film surface were investigated.

First, the adsorption of ECINs on untreated zein films was monitored in real time by QCM-D as ΔF and ΔD curves. Since the ΔF and ΔD at first resonant frequency were not used by the software for data fitting, the curves of $\Delta F3$, $\Delta F5$, $\Delta F7$, $\Delta D3$, $\Delta D5$, and $\Delta D7$ obtained from a representative test are plotted in Figure 2. The curves revealed a real-time adsorption

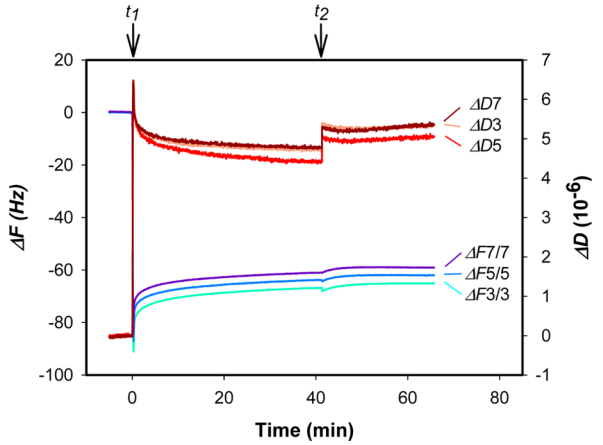


Figure 2. Frequency shift (ΔF) and energy dissipation shift (ΔD) induced by the adsorption of 0.1% ECINs solution at pH = 5.0 and I = 0.05 M on the zein-coated gold crystal surface. ΔF and ΔD were measured simultaneously at three overtones (n = 3, 5, 7) and normalized by their overtone numbers. The arrows indicate the time for the injection of sample (t_1) and rinsing with buffer (t_2).

of ECINs: at t_1, ECINs solution flowed in and the adsorption caused a rapid decrease of frequency and increase of energy dissipation, which gradually slowed down until steady state. After 40 min, both ΔF and ΔD became stable, suggesting the adsorption and desorption reached equilibrium. Subsequently, the buffer flowed in at t_2 for washing step, which mostly removed the reversibly adsorbed ECINs, shown as a slight increase of frequency. Meanwhile, the energy dissipation, ΔD, further increased after the washing step, suggesting after washing, the ECINs layer became even more loosely bound.

The frequency shifts at the third overtones for adsorption under various pH were plotted in Figure 3A. While increasing pH from 5.0 to 8.0, the frequency shifts decreased. Moreover,

dx.doi.org/10.1021/am4016457 | ACS Appl. Mater. Interfaces 2013, 5, 10456−10464

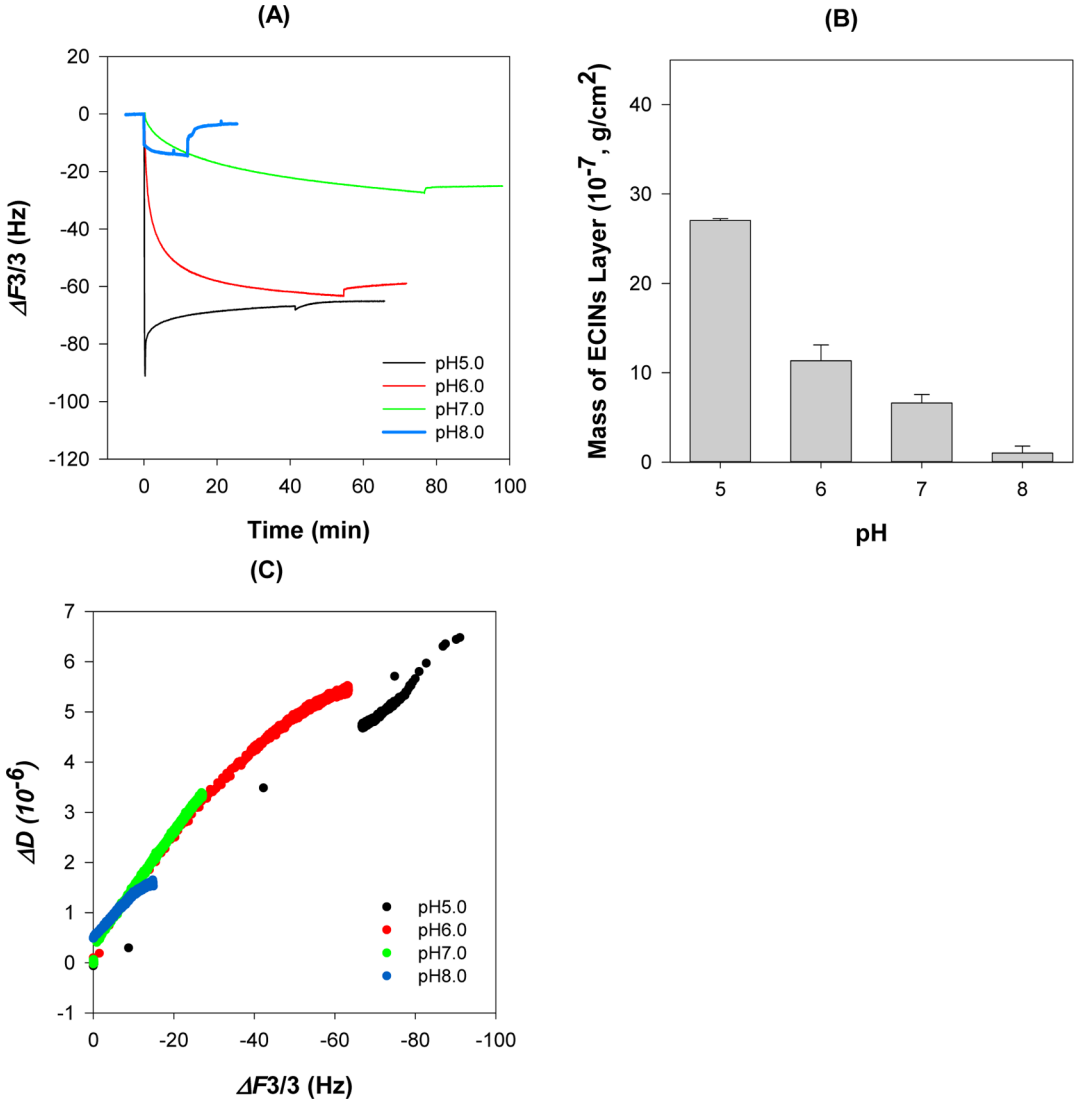


Figure 3. Effect of pH on (A) the change of frequency ($\Delta F_3/3$), (B) the mass of adsorbed ECINs layer on untreated zein surface, and (C) $\Delta D-\Delta F$ plots. The ECINs solutions were prepared to the concentration of 0.1% w/v in 0.02 M buffers at various pH values. The total ionic strength of the solutions was fixed at 0.05 M.

the pattern of adsorptions also changed. At pH 5.0, 6.0 and 7.0, there was only small frequency increase after washing step, indicating that most of the adsorption at these conditions was irreversible. In contrast, at pH 8.0, the adsorbed ECINs were rarely retained after washing step, indicating a mainly reversible adsorption at this pH. Voigt model was used to estimate the adsorbed mass (Figure 3B). The highest adsorption of ECINs occurred at pH 5.0 and increasing pH reduced the adsorption.

In order to investigate the adsorption behavior of ECINs on untreated zein surface, ΔD was plotted against ΔF in Figure 3C. The plots for pH 6.0 to pH 8.0 shared similar initial slope values (about 0.12) which are clearly larger than the initial slope at pH 5.0 (about 0.07). Because a smaller slope of the plot corresponds to a more compact and rigid layer and vice versa,[28] this indicated that, at pH 5.0, ECINs adsorbed onto zein films in a more compact molecular arrangement and formed a more rigid layer than the adsorption at higher pH. Also, plots for pH 6.0–8.0 showed a bending as the adsorption proceeded, suggesting a two-step adsorption at these conditions, where the first step revealed larger frequency change with a deeper slope compared to the second step. These multiple adsorption stages were commonly observed in adsorptions of biological materials, where the initial adsorption usually involved a quick addition of biomolecules which formed a loose layer, resulting in a quick change of F and larger slope of ΔD to ΔF; in the second stage, the adsorption slowed down and the adsorbed biomolecules reorganized to form a more compact layer.[29]

Adsorption of ECINs on UVO-Treated Surface of Zein Films. It was previously demonstrated that the surface of zein film could be modified by UVO treatment to form carboxyl groups.[20] In this study, similar UVO treatment was applied to zein film surface to make it negatively charged, and structural changes of zein film surface were monitored by ATR- FTIR as shown in Figure 4. UVO treatments resulted in the formation of a broad shoulder peak in the wavenumber range between 1710 and 1760 cm^{-1}, which corresponded to the formation of negatively charged carboxyl group.[20] When the UVO treatment reached 12 min, the integrity of zein film was damaged, as evidenced by the decrease of overall spectral intensity.

Considering that modified zein surface with carboxyl groups and ECINs both bear negative charges at pH 5–8,[20] a positively

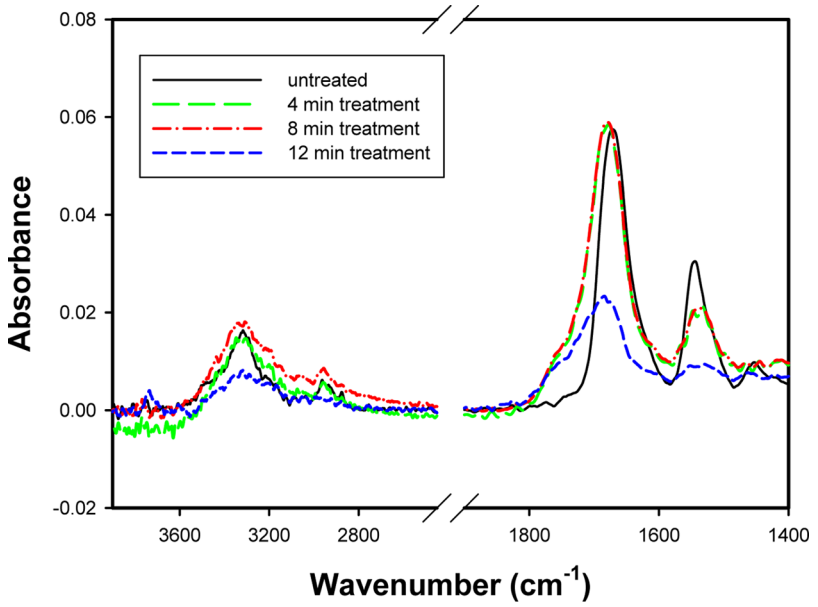


Figure 4. FT-IR spectra of zein surface before and after 4, 8, and 12 min UVO exposure.

charged layer was needed to establish electrostatic interactions. PDADMAC is a strongly dissociated quaternary ammonium polycation and low-molecular-weight polymer that was demonstrated to form compact, rigid and flat layer when adsorbed onto fiber web.[30] It was previously demonstrated to successfully mediate the adsorption of negatively charged bacterial cells onto the negatively charged surface of hemicelluloses and wood extractives.[30] As shown in Figure 5, after 4, 8, and 12 min UVO treatment of the zein surface, the adsorption of PDADMA was compared on $\Delta F3/3$. Three repeats for each condition were plotted individually to reveal the difference in repeatability. Adsorption on films with 8 min UVO treatment was most reproducible and was chosen for the following experiments.

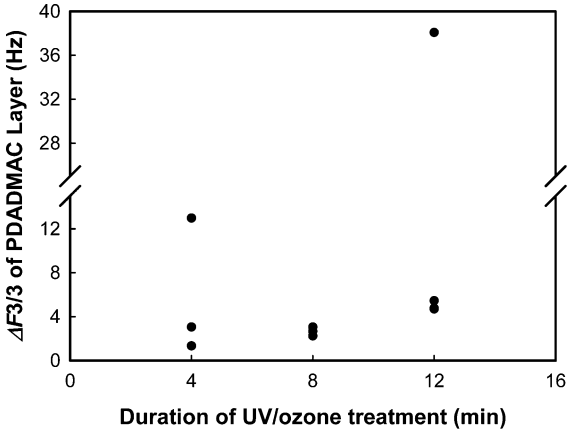


Figure 5. Adsorption of PDADMAC on UVO-treated zein surface monitored by QCM-D. The PDADMAC solution was prepared to the concentration of 0.1% in 0.02 M Tris buffers at pH 7.0 and I 0.05M.

The PDADMAC-mediated adsorption of ECINs on UVO-treated zein film surface was monitored by QCM-D (Figure 6), which involved injection of PDADMAC at time t_1 and a following washing step at t_2, in addition of injecting ECINs at time t_3 and the second washing step at t_4.

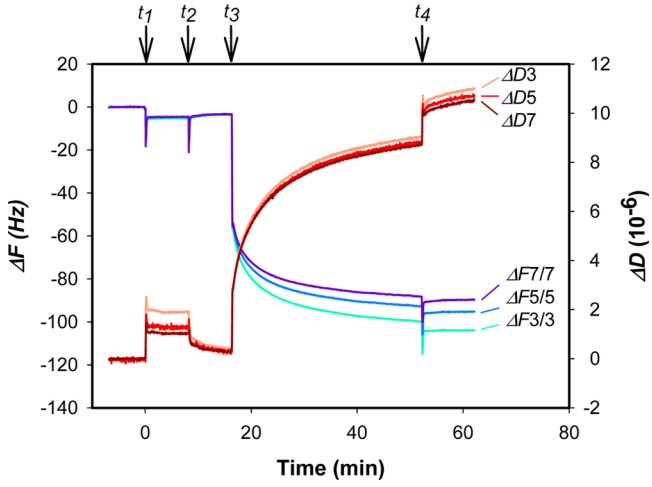


Figure 6. Frequency shift (ΔF) and energy dissipation shift (ΔD) induced by the adsorption of 0.1% PDADMAC and then 0.1% ECINs solution at pH = 7.0 and I 0.05 M on the 8 min UVO-treated zein-coated gold crystal surface. ΔF and ΔD are measured simultaneously at three overtones (n = 3, 5, 7) and normalized by their overtone number. The arrows indicate the time for the injection of PDADMAC (t_1), rinsing with buffer (t_2), injection of ECINs (t_3), and rinsing with buffer (t_4).

The effect of pH on adsorption of ECINs was first examined (Figure 7A). Among the tested pH conditions, the adsorption of PDADMAC and ECINs at pH 5.0 behaved differently compared to at higher pH. Less PDADMAC was adsorbed at pH 5. From pH 6 to 8, the same amount of PDADMAC was adsorbed on UVO-treated zein surface. The ECINs layers formed at pH 5.0 were more compact than those formed at higher pH, suggested by smaller slope of ΔD to ΔF in Figure 7C. However, in contrast to the maximum ECINs adsorption on untreated zein surface occurring at pH 5, in the case of UVO-treated hydrophilic surface, neutral pH was preferred under which the highest mass of adsorbed ECINs reached about 4 $\mu g/cm^2$ (Figure 7B).

dx.doi.org/10.1021/am4016457 | ACS Appl. Mater. Interfaces 2013, 5, 10456−10464

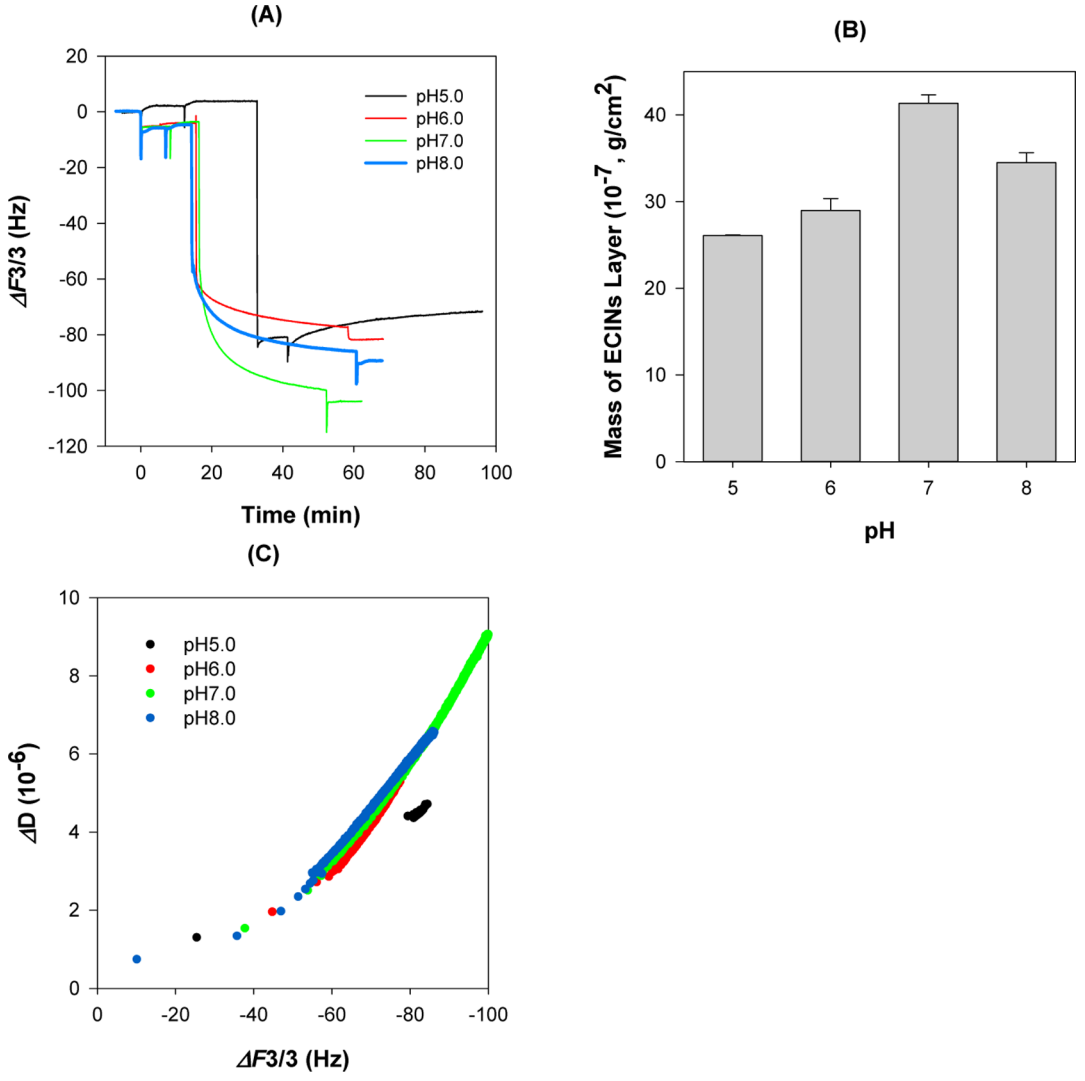


Figure 7. Effect of pH on the (A) change of frequency ($\Delta F3/3$) and (B) mass of adsorbed ECINs layer on the surface of UVO-treated-zein/PDADMAC bilayer and (C) $\Delta D-\Delta F$ plots. The ECINs and PDADMAC solutions were prepared to the concentration of 0.1% in 0.02 M buffers at various pH, and I 0.05M.

Changes of frequency and mass of ECINs adsorption on the surface of UVO-treated-zein/PDADMAC bilayer at various IS are plotted in Figure 8A and B. While IS ranged from 0.02 to 0.10 M, adsorption of PDADMAC preferred higher IS, but a higher adsorption of ECINs occurred at an intermediate level of IS at 0.05 M. On the other hand, a decrease in the slope of the $\Delta D-\Delta F$ curve with increased IS suggested that higher IS drove the ECINs protein to form a more compact and rigid layer (Figure 8C).

Comparing the adsorption of ECINs onto an untreated zein film surface and a PDADMAC mediated UVO-treated hydrophilic zein surface, the maximum ECINs adsorption was achieved on the UVO-treated hydrophilic zein surface at pH 7.0 and IS 0.05 M.

Discussion on Mechanisms of Adsorption. The possible mechanisms involved in ECINs adsorption on the two types of zein films were illustrated in Figure 9. ECINs were suggested to contain large hydrophobic and hydrophilic patches on the surface to orient the water molecules so that the hydrogen bonds preferentially formed.[25,27] Thus, ECINs have potential affinities with either hydrophobic surface or hydrophilic surface. Comparing the two situations, adsorption by hydrophobic force dominated on a relatively hydrophobic surface of zein film (static water contact angle of 80°[20]), while mainly electrostatic forces were involved in the adsorption of ECINs on the UVO-treated-zein/PDADMAC bilayer. Similar as observation with other amphiphilic biomolecules, when adsorbed on a more hydrophobic surface, monolayer coverage by ECINs was expected with their hydrophobic patches facing toward the surface and the hydrophilic patches toward the buffer.[28] When the surface was hydrophilic, the ECINs molecules turned around having the hydrophilic patches interact electrostatically with the surface. In this case, more ECINs were needed to shield each other's hydrophobic patches from buffer.[28] This contributed to the phenomenon that more ECINs adsorbed to a hydrophilic surface than a more hydrophobic surface (Figures 3B and 7B).

The adsorption of ECINs was pH-dependent probably due to the change of surface net charge of ECINs at different pH. Using pH-dependent turbidimetric titration, we previously observed that the isoelectric point (pI) of ECINs was closed to 4.2.[18] At pH 5.0, the net charge on the surface of ECINs proteins was close to zero, making the surface of ECINs more likely to build hydrophobic interactions with the nonpolar

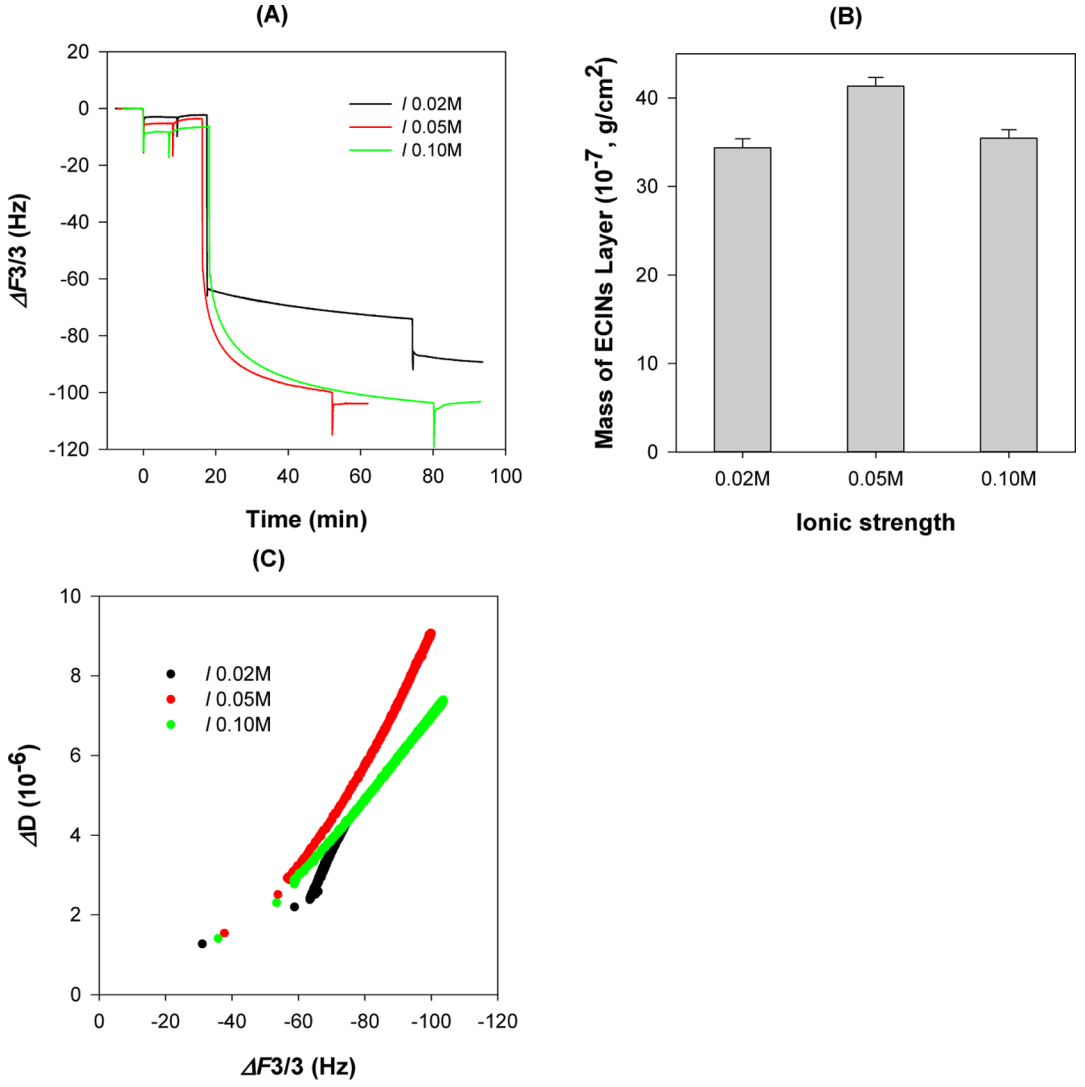


Figure 8. Effect of IS on the (A) change of frequency ($\Delta F3/3$) and (B) mass of adsorbed ECINs layer on the surface of UVO-treated-zein/PDADMAC bilayer and (C) $\Delta D-\Delta F$ plots. The ECINs and PDADMAC solutions were prepared to the concentration of 0.1% in 0.02 M pH 7.0 Tris buffers adjusted to various ionic strengths.

groups of untreated zein. This is the reason that on the untreated zein surface, pH 5.0 was the favorable condition for ECINs adsorption (Figure 3B). At higher pH, under which the surface of ECINs became more negatively charged, the adsorption behaviors of ECINs changed, evidenced by different $\Delta D-\Delta F$ curves between pH 6–8 and pH 5 (Figures 3C and 7C). At pH 8, the surface negative charge on ECINs molecules was largely increased which hindered the establishment of hydrophobic interactions, resulting in the nonspecific reversible adsorption of ECINs at this condition.

On the hydrophilic zein surface, the maximum adsorption of ECINs occurred at neutral pH rather than at higher pH under which ECINs were expected to bear more negative charge and formed stronger electrostatic forces with the PDADMAC polycation layer. The possible reason is that, even after UVO treatment, zein film surface still contained some nonpolar amino acids that formed relatively hydrophobic local regions. These regions were not covered by PDADMAC and attracted ECINs directly via hydrophobic interactions. Similar to what is observed in Figure 3A, when pH increased from 7 to 8, adsorption via hydrophobic interactions became reversible and some of the ECINs dissociated after rinsing with buffer. At pH 5, less PDADMAC was adsorbed on UVO-treated zein film than that at pH 6–8 (Figure 7A), which leads to lower adsorption of ECINs on hydrophilic surface at pH 5, in addition to the factor from less surface charges on ECINs molecules.

In addition to the pH effect, higher IS enhanced the hydrophobic force among ECINs molecules, and thus drove the ECINs to form a close-packed layer as suggested by a smaller slope of ΔD to ΔF in Figure 8C.[28]

Quantification of Ice Nucleation Activity on Film. The Vali method is a relatively accurate method to quantify the activity of heterogeneous ice nuclei.[19] Its procedure involves series dilution and intensive sampling as described in the method section, which is time-consuming and burdensome. Moreover, the method is limited to test liquid which could be diluted. Because the ice nucleators on films are unable to be diluted, a novel method is required to determine the activity of ice nucleators on a surface.

Relationship between nucleation temperature and number of ice nuclei in a defined volume has been observed in some previous studies.[19,32] Recently, Zhang and his colleagues described this relationship using eq 4.

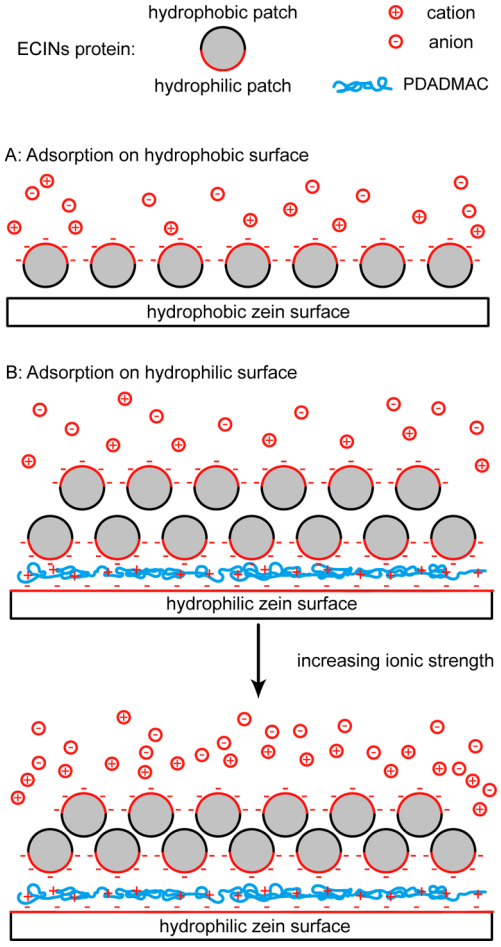


Figure 9. Schematic of the adsorption of ECINs on the untreated zein surface (A) and UVO-treated hydrophilic zein surface (B, top: low ionic strength; bottom: high ionic strength).

$$T_s = A + Bx\ e^{Kx} \quad (4)$$

where T_s was the degree of supercooling (difference of nucleation temperatures and freezing point) and x was the number of ice nucleators.[33] For deionized water whose freezing point is close to 0 °C, the degree of supercooling equals to the absolute value of ice nucleation temperature. However, when we plotted the ice nucleation temperature versus the number of ice nuclei (see Figure 10), we found a modification of eq 4 to eq 5 better fitted the data obtained from three independent batches of ECINs with varied ice nucleation activity. The fitting correlation coefficient (R^2) by using eq 5 was 0.979, versus 0.572 by using eq 4.

$$T_s = A + B \log \mathrm{IN}\ e^{K \log \mathrm{IN}} \quad (5)$$

where T_s is nucleation temperature measured by DSC; *IN* is the number of ice nucleators. By nonlinear regression, *A*, *B*, and *K* were calculated as −9.69, 0.44, and 0.17, respectively. The difference between eqs 5 obtained in this study and eq 4 is possibly due to two reasons. First, this study examined a five-magnitude range in the number of ice nucleators from 1 to 10^5 units, whereas eq 4 was based on data from one-magnitude range from 1×10^4 to 17×10^4 units/mL. Second, this study used DSC to detect the nucleation temperature of 10 μL of liquid, whereas the other study used freezing curves to determine the nucleation temperature in 6 mL of liquid.

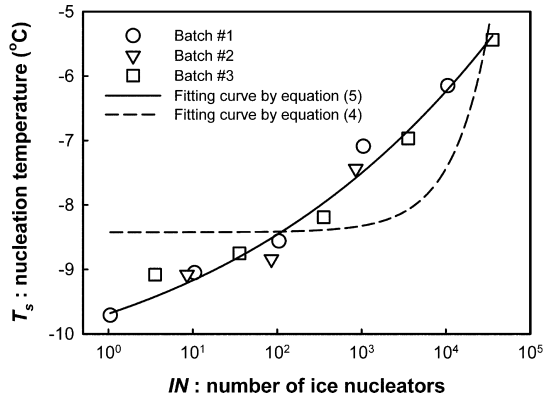


Figure 10. Modeling to the nucleation temperature of water containing ice nucleators. ECINs isolated from three batches of bacteria were used for test as described in the Experimental Section, shown as data in three different symbols. The curve represents modeling result.

Because heterogeneous ice nucleation is a stochastic process, the accurate measurement on nucleation temperature becomes more difficult in a larger volume.[19,34,35] Due to the same reason, many studies on heterogeneous ice nucleators preferably tested several magnitudes of concentration of ice nucleators.

QCM-D method is well-established to measure the mass of adsorption of substances on surfaces.[36] This method was used to verify our developed empirical equation and its applicability in quantifying INA on surfaces. Films were prepared using the optimum adsorption condition with ECINs solution at two concentrations, 1 and 0.1 mg/mL, and the activity of ECINs on the film was determined by our developed DSC method and by QCM-D method (Table 1). In one method, the INA of films was calculated from the nucleation temperature of water on films using eq 5. On the other hand, the mass of adsorption was measured by QCM-D and the activities of the films were then calculated by multiplying the mass of adsorption with the INA of ECINs (1.61×10^6 units/mg). With both loading concentrations of ECINs, the two methods gave consistent values for the INA on zein films, suggesting that the developed method based on nucleation temperature was applicable in quantifying the INA of films.

CONCLUSIONS

To develop zein-based ice nucleation films, the effect of different conditions on ECINs adsorption was examined. First, it was found that ECINs' activity was better retained at pH 4.0−9.0 and IS 0.01−0.05 M. Using QCM-D analysis, the adsorption behavior of ECINs were investigated on a untreated zein film and a PDADMAC-mediated UVO-treated zein film. At pH 5.0, which was close to the pI of ECINs, the adsorption of ECINs on untreated zein films reached maximum compared to adsorption at higher pH, possibly because hydrophobic interaction favored this pH. On the other hand, neutral pH and IS 0.05 M was preferred for immobilization of ECINs on the surface of UVO-treated-zein/PDADMAC bilayer. Comparing the adsorption of ECINs at all conditions, the maximum ECINs adsorption was achieved on the hydrophilic zein surface at pH 7.0 and IS 0.05 M.

In order to quantify the ice nucleation activity of films, a new empirical method was developed to quantitatively correlate the number of ice nucleators with the nucleation temperature measured by DSC. The INA of the ECINs-immobilized zein

dx.doi.org/10.1021/am4016457 | ACS Appl. Mater. Interfaces 2013, 5, 10456−10464

Table 1. Comparison to the INA of Zein Films Measured by DSC versus Those Measured by QCM-D

ECINs concentration in film preparation	DSC method			QCM-D method	
	nucleation temperature (°C)	no. of ice nucleators (units)[a]	INA (units/mm^2)[b]	mass of adsorption (× 10^{-7} g/cm^2)	INA (units/mm^2)[c]
1 mg/mL	−7.31 ± 0.11	1508.2 ± 337.9	64.1 ± 14.4[a]	41.3 ± 1.0	66.5 ± 1.6[a]
0.1 mg/mL	−8.40 ± 0.11	118.7 ± 34.1	5.0 ± 1.4[b]	2.8 ± 0.8	4.5 ± 1.3[b]

[a]Calculated from nucleation temperature using eq 5. [b]Area of films for DSC test was 23.54 mm^2. [c]Calculated from mass of adsorption using INA of ECINs as 1.61 × 10^6 units/mg.

films was assessed using the method developed and verified by comparing to the values obtained from QCM-D method. The INA values obtained by our novel developed method were statistically same as the values from QCM-D method which has been well established in quantifying mass of adsorbed substances on surface.

AUTHOR INFORMATION

Corresponding Authors

*(Q.H.) Tel: 848-932-5514. E-mail: qhuang@aesop.rutgers.edu.

*(T.-C.L.) Tel: 848-932-5536. E-mail: lee@aesop.rutgers.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the United States Department of Agriculture National Research Initiative (#2009-35603-05075).

ABBREVIATIONS

DSC = differential scanning calorimetry
ECINs = extracellular ice nucleators
FT-IR = Fourier transform infrared spectroscopy
INA = ice nucleation activity
IS = ionic strength
PDADMAC = poly(diallyldimethylammonium chloride)
PDDA = poly(diallyldimethylammonium chloride)
QCM-D = quartz crystal microbalance with dissipation monitoring
UVO = UV/ozone

REFERENCES

(1) Rhim, J. W.; Ng, P. K. W. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 411−433.
(2) Wang, H. J.; Gong, S. J.; Lin, Z. X.; Fu, J. X.; Xue, S. T.; Huang, J. C.; Wang, J. Y. *Biomaterials* **2007**, *28*, 3952−3964.
(3) Miyoshi, T.; Toyohara, K.; Minematsu, H. *Polym. Int.* **2005**, *54*, 1187−1190.
(4) Luo, Y. C.; Zhang, B. C.; Whent, M.; Yu, L. L.; Wang, Q. *Colloids Surf., B* **2011**, *85*, 145−152.
(5) Luecha, J.; Hsiao, A.; Brodsky, S.; Liu, G. L.; Kokini, J. L. *Lab Chip* **2011**, *11*, 3419−3425.
(6) Andersson, C. *Packag. Technol. Sci.* **2008**, *21*, 339−373.
(7) Gucbilmez, C. M.; Yemenicioglu, A.; Arslanoglu, A. *Food Res. Int.* **2007**, *40*, 80−91.
(8) Mecitoglu, C.; Yemenicioglu, A.; Arslanoglu, A.; Elmaci, Z. S.; Korel, F.; Cetin, A. E. *Food Res. Int.* **2006**, *39*, 12−21.
(9) Arcan, I.; Yemenicioglu, A. *Food Res. Int.* **2011**, *44*, 550−556.
(10) Archer, D. L. *Int. J. Food Microbiol.* **2004**, *90*, 127−138.
(11) Roos, Y. H.; Karel, M.; Kokini, J. L. *Food Technol.* **1996**, *50*, 95−108.
(12) Zaritzky, N. E. In *Managing Frozen Foods*; Kennedy, C. J., Ed.; CRC Press: Boca Raton, FL, 2000; pp 111−136.
(13) Zasypkin, D. V.; Lee, T.-C. *J. Food Sci.* **1999**, *64*, 473−478.
(14) Zhu, X.; Lee, T.-C. *Int. J. Food Sci. Technol.* **2007**, *42*, 768−772.
(15) Shi, K.; Yu, H.; Lee, T.-C. *J. Cereal Sci.* **2013**, *57*, 237−243.
(16) Padua, G. W.; Rakoronirainy, A.; Wang, Q. In *The Food Biopack Conference Proceedings*; Weber, C., Ed.; Royal Veterinary and Agricultural University: Copenhagen, Denmark, 2000; pp 84−88.
(17) Shi, K.; Yu, H.; Jin, J.; Lee, T.-C. *J. Cereal Sci.* **2013**, *57*, 430−436.
(18) Shi, K. Ph.D. Thesis, Rutgers University, New Brunswick, NJ, 2012.
(19) Vali, G. *J. Atmos. Sci.* **1971**, *28*, 402−409.
(20) Shi, K.; Kokini, J. L.; Huang, Q. R. *J. Agric. Food Chem.* **2009**, *57*, 2186−2192.
(21) Voinova, M. V.; Rodahl, M.; Jonson, M.; Kasemo, B. *Phys. Scr.* **1999**, *59*, 391−396.
(22) Andersson, K. M.; Hovmöller, S. *Z. Kristallogr.* **1998**, *213*, 369−373.
(23) Li, J.; Lee, T.-C. *Trends Food Sci. Technol.* **1995**, *6*, 259−265.
(24) Nielsen, J. E.; Borchert, T. V.; Vriend, G. *Protein Eng.* **2001**, *14*, 505−512.
(25) Hew, C. L.; Yang, D. S. C. *Eur. J. Biochem.* **1992**, *203*, 33−42.
(26) Phelps, P.; Giddings, T. H.; Prochoda, M.; Fall, R. *J. Bacteriol.* **1986**, *167*, 496−502.
(27) Mizuno, H. *Proteins: Struct., Funct., Genet.* **1989**, *5*, 47−65.
(28) Rodahl, M.; Hook, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B. *Faraday Discuss.* **1997**, *107*, 229−246.
(29) Zhou, C.; Friedt, J. M.; Angelova, A.; Choi, K. H.; Laureyn, W.; Frederix, F.; Francis, L. A.; Campitelli, A.; Engelborghs, Y.; Borghs, G. *Langmuir* **2004**, *20*, 5870−5878.
(30) Leino, T.; Raulio, M.; Salkinoja-Salonen, M.; Stenius, P.; Laine, J. *Colloids Surf., B* **2011**, *86*, 131−139.
(31) Shi, K.; Yu, H.; Lakshmana Rao, S.; Lee, T.-C. *J. Agric. Food Chem.* **2012**, *60*, 5988−5993.
(32) Bigg, E. K. *Proc. Phys. Soc. B* **1953**, *66*, 688−694.
(33) Zhang, S. Z.; Wang, H. Y.; Chen, G. M. *J. Food Eng.* **2009**, *94*, 248−253.
(34) Vali, G. *J. Atmos. Sci.* **1994**, *51*, 1843−1856.
(35) Wilson, P. W.; Heneghan, A. F.; Haymet, A. D. J. *Cryobiology* **2003**, *46*, 88−98.
(36) Jonsson, M. P.; Jonsson, P.; Hook, F. *Anal. Chem.* **2008**, *80*, 7988−7995.