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Nanoscale properties of biopolymer multilayers

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ABSTRACT

Layer by Layer (LbL) deposition is a simple and inexpensive method of multilayer self-assembly, that relies on the electrostatic interactions of oppositely charged polyelectrolytes. Functional biopolymers used to build such nanostructured multilayers can keep serving their inherent functions in food systems. A good example is chitosan, a positively charged biopolymer which has antimicrobial properties and forms stable complexes with negatively charged polymers. This study investigates the potential of commercially available food grade biopolymers to substitute synthetic polyelectrolytes when building nanostructured multilayers. Chitosan molecular weight, carrageenan charge density and pectin degree of esterification and amidation was varied to investigate their respective effects on the nanoscale morphology and wettability of these surfaces. Extracellular Ice Nucleators (ECINs), functional lipoglycoproteins of negative charge known to increase ice nucleation temperatures in food systems, were used to confirm the suitability of biopolymer multilayers for fabricating functional food grade nano-thin films. High molecular weight chitosan and iota-carrageenan created the most appropriate biopolymer pair to serve this purpose, as demonstrated by the nanoscale evaluation of these ultra-thin layers. ECINs successfully adsorbed onto the multilayer system, and ice nucleation activity of the nano-thin film was confirmed via the cooling bath experiments.

1. Introduction

Layer by Layer (LbL) deposition technique is an alternative way to fabricate thin films on surfaces to the traditional Langmuir-Blodgett method which requires special instruments and presents limitations to the substrate size and shape, as well as film stability and quality (Decher, 1997). LbL deposition relies mainly on the electrostatic interactions of oppositely charged polyelectrolytes for multilayer self-assembly (Zhang, Chen, & Zhang, 2007). This allows the building of multilayer systems more stable than those obtained by physical adsorption, due to the stronger electrostatic attraction among the layers and with the substrate (Cai, Rechtenbach, Hao, Bossert, & Jandt, 2005). There are only two basic requirements for LbL deposition; one is that the polyelectrolytes of interest are soluble in water and the other that they carry opposite electrostatic charges. The LbL technique has several advantages such as (1) it is very easy to perform and no expensive or complicated instruments are required, (2) different building blocks can be incorporated into the multilayer system to obtain the desired properties and (3) it can be applied to any charged substrate, of any shape or size (Zhang et al., 2007).

Nano-thin multilayers nanostructured from oppositely charged proteins or polysaccharides provide perfect foundations for delivering functional substance making up the top layer of the multilayer system, such as negatively charged Extracellular Ice Nucleators (ECINs). ECINs are substances that can initiate nucleation by organizing water molecules into an ice-like pattern at relatively high temperatures (-2 °C) (Duman, 2001). They are used in food systems to increase freeze-thaw resistance, modify food texture and deliver energy savings in freeze drying and concentration applications (Li-Jung, Chen, Tzeng, Chiou, & Jiang, 2005; Zhu & Lee, 2007) There are no reports regarding the activity of ice nucleators when applied as a nano-thin layers in food products.

ECIN nano-thin films have been fabricated using non-food grade synthetic oppositely charged cationic poly (diallyldimethyl ammonium chloride) and anionic poly (styrenesulfonate) (Gezgin, Lee, & Huang, 2013). Thus it is of importance to consider the fabrication of ECIN nano-thin films using food grade polyelectrolytes.

Chitosan is a positively charged biopolymer carrying amine groups in its structure. It is obtained by the deacetylation of chitin

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(an exoskeleton structure-forming polysaccharide obtained during krill, shrimp, and crab processing as a by-product) under alkaline conditions and it is the second most abundant renewable organic resource after cellulose (Shumilina & Shchipunov, 2002). At a deacetylation degree of 85%, chitosan holds one ammonium group for approximately each 1.2 saccharide unit, which allows it to form complexes with negatively charged polymers (Kujawa, Moraille, Sanchez, Badia, & Winnik, 2005).

Carrageenan is a generic name for high sulfate containing, linear, anionic heteropolysaccharide, commercially extracted from red marine algae (Bartkowiak & Hunkeler, 2001). This family of sulfated galactans are categorized by their charge densities; as lambda, iota, kappa carrageenans and furcelleran (aka Danish Agar, a polysaccharide very similar to kappa carrageenans in structure) having 2.07, 1.53, 0.92 and 0.69 sulfate groups per disaccharide unit, respectively (Hugerth & Sundelöf, 2001).

Pectins are polysaccharides made up of D-galacturonic acid units connected via the α-(1–4) linkages. Carboxyl groups are commonly esterified as methoxyl groups in nature, and the percentage of esterified groups, a.k.a. degree of esterification, is an important parameter in their classification as either high methoxyl (HM, >50%) or low methoxyl (LM, < 50%) pectins (Worth, 1967). Thus, the aims of this work were to: (a) investigate the potential of chitosan, pectin and carrageenan ability to form food grade multilayer systems via the LbL deposition technique, with ECINs adsorbed onto the multilayer system; and (b) evaluate the ice nucleation activity of the nano-thin film via the cooling bath experiments.

2. Materials and methods

2.1. Materials

Chitosans of 1 kDa and 10 kDa molecular weight were purchased from Kitto Life Co. Ltd. (Seoul, Korea) and 44, 130 and 330 kDa from Kunpoong Bio. Co. Ltd. (South Korea). Degree of deacetylation (DOD) for all chitosans was in the range of 98–99%. Pectins with different degrees of esterification (DE) and amidation (DA) were acquired from Danisco (Grindsted, Denmark) and Sigma Aldrich (St. Louis, MO) (Danisco Grindsted LA 410-DE:29–33%,DA:20%, LC 950-DE:31–33% and Sigma P9561-DE:90%). Furcellaran was a gift from FMC Biopolymer (Princeton, NJ). Kappa carrageenan (type I, CAS# 9000-07-1), iota carrageenan (type II, CAS# 9062-07-1), lambda carrageenan (type IV, 9064-57-7) were purchased from Sigma Aldrich (St. Louis, MO). ECINs produced by *Erwinia herbicola* subsp. *ananas*, (Cat. No. 11530, ATCC, Rockville, MD) was kindly provided by Prof. T.C. Lee's group. H_2O_2 and H_2SO_4 were purchased from Sigma-Aldrich (Milwaukee, WI), and all chemicals were used as received without further purification. Silicon wafers were purchased from Montco Silicon Technologies (Spring City, PA). Ultrapure water (Milli-Q plus system, Millipore) with a resistivity of 18.2 MΩ cm was used in all applications.

2.2. Production and isolation of extracellular ice nucleators

ECIN's were produced and isolated as described in (Li & Lee, 1998). *Erwinia herbicola* was grown at 18 °C in a yeast extract. This was followed by the purification of ECINs using centrifugation, sonication, filtration and ultracentrifugation. Then the pellet was suspended in Tris-buffer, freeze-dried and stored at −20 °C (Zhu & Lee, 2007). To evaluate the effect of ultracentrifugation speed on ice nucleators, supernatants were centrifuged using a Beckman ultracentrifuge equipped with a 60Ti rotor for 1 h cumulatively at speeds starting from 10,000 rpm to 50,000 rpm. For instance, the 30,000 rpm sample was centrifuged for a total of 3 h, starting with 1 h at 10,000 rpm, another hour at 20,000 rpm with the supernatant removed from the first step, and similarly another hour at 30,000 rpm.

2.3. Nanofilm preparation

1 mg/mL (0.1%) solutions of chitosans, carrageenans and pectins were prepared in 0,01 M NaCl and 0,01 M acetate buffer of pH 3.74. Carrageenans and pectins were heated at 80 °C and stirred at this temperature using an impeller for half an hour. Chitosans were stirred overnight and filtered through a 0,45 μm sized filter. Incremental bilayers of $(Chitosan/Carrageenan)_n$ and $(Chitosan/Pectin)_n$ (n from 1 to 6) were fabricated on silicon wafers, to serve as the foundation for the attachment of ECINs. Different molecular weights (1–330 kDa) of chitosan were used to interact with carrageenans of different charge densities (furcellaran, kappa, iota and lambda, in the respective order of low to high charge density). In the second system, carrageenans were replaced with pectins of different degrees of esterification (D.E.) and amidation (D.A.). Multilayer films were deposited onto silicon wafers to act as the positively charged base for ECIN absorption. Silicon wafers were cleaned earlier in a slightly boiled piranha solution (7:3 mixture of 98% H_2SO_4 and 30% H_2O_2) for 30 min, then rinsed with copious amount of Milli-Q water and dried with the nitrogen gas. The cleaned silicon wafers of 1 × 1 cm size were immersed consecutively in 1 mg/mL polymer solutions, and finally in different concentrations of ECIN solutions.

For the fabrication of the first layer, wafers were kept in the chitosan solution for one hour, assuring a complete layer of the positively charged polyelectrolyte. For successive layers of pectin/carrageenan, chitosan and ECIN as the top layer, dipping time was 20 min. Wafers were cleaned with Milli-Q water and flushed with gaseous nitrogen each time before dipping in successive solutions. Wafers to be analyzed for surface morphology were placed in a petri dish and covered with parafilm (punched earlier to allow the removal of excessive moisture) and vacuum dried overnight prior to AFM measurement.

2.4. Atomic force microscopy (AFM)

Surface morphology was investigated with the Multimode Nanoscope IIIA™ AFM (Digital Instruments, Veeco Metrology, Santa Barbara, CA, USA), operated in the tapping mode. Silicon tip with an average drive frequency of 265 kHz was used. 1 × 1, 2 × 2 and 5 × 5 micron images of the layers of interest were collected. Section analysis tool was used for the measurements of feature sizes, as the

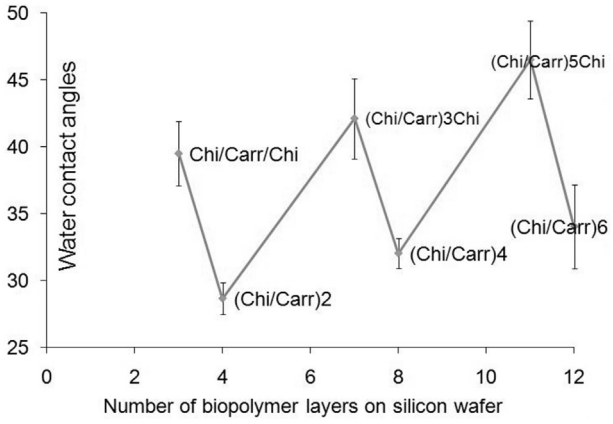

Fig. 1. Variation in water contact angles as multilayers are fabricated using chitosan of 330 kDa molecular weight and iota-carrageenan.