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Silk fibroin organization induced by chitosan in layer-by-layer films: Application as a matrix in a biosensor

Jorge A.M. Delezuk[a,*], Adriana Pavinatto[a], Marli L. Moraes[b], Flávio M. Shimizu[a], Valquíria C. Rodrigues[a], Sérgio P. Campana-Filho[c], Sidney J.L. Ribeiro[d], Osvaldo N. Oliveira Jr.[a]

[a] São Carlos Institute of Physics, University of São Paulo (USP), CP 369, 13560-970, São Carlos, SP, Brazil
[b] Institute of Science and Technology at the Federal University of São Paulo (UNIFESP), 330 Talim Street, São José dos Campos, SP, Brazil
[c] São Carlos Institute of Chemistry, University of São Paulo (USP), CP 780, 135660-970, São Carlos, SP, Brazil
[d] Institute of Chemistry, São Paulo State University (UNESP), CP 355, 14801-970, Araraquara, SP, Brazil

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ABSTRACT

In this paper, we show that chitosan may induce conformation changes in silk fibroin (SF) in layer-by-layer (LbL) films, which were used as matrix for immobilization of the enzyme phytase to detect phytic acid. Three chitosan (CH) samples possessing distinct molecular weights were used to build CH/SF LbL films, and a larger change in conformation from random coils to β-sheets for SF was observed for high molecular weight chitosan (CHH). The CHH/SF LbL films deposited onto interdigitated gold electrodes were coated with a layer of phytase, with which phytic acid could be detected down to 10^{-9} M using impedance spectroscopy as the principle of detection and treating the data with a multidimensional projection technique. This high sensitivity may be ascribed to the suitability of the CHH/SF matrix, thus indicating that the molecular-level interactions between chitosan and SF may be exploited in other biosensors and biodevices.

1. Introduction

The immobilization of active biomolecules on solid surfaces is one of the most important challenges in the design and fabrication of biosensors, (Putzbach & Ronkainen, 2013) since a high performance in terms of sensitivity and selectivity depends on preserved bioactivity. In this context, the layer-by-layer (LbL) film-forming method has been proven excellent because it may lead to a suitable matrix as well as an active layer (Ariga, Hill, & Ji, 2007), mostly because entrained water in the film assists in keeping the native structure of the biomolecules (Siqueira, Caseli, Crespilho, Zucolotto, & Oliveira, 2010). The LbL film-forming method was originally conceived for the alternate deposition of oppositely charged polyelectrolytes (Decher & Schlenoff, 2002), but it has now been extended to a whole variety of other materials, and it may also be based on hydrophobic (Wong et al., 2012), hydrogen bonds (Kharlampieva, Kozlovskaya, & Sukhishvili, 2009) and covalent bonds (Fadeev & McCarthy, 2000). Many have been the materials used as matrix for immobilizing biomolecules in LbL films, but perhaps one could single out natural polymers and biomaterials (Li, Wang, & Sun, 2012), especially because they tend to be suitable scaffolds for the biomolecules. For instance, silk fibroin in β-sheet conformation has been used to enhance the analyte adsorption in biosensors produced with LbL films (Moraes, Lima, Silva, Cavicchioli, & Ribeiro, 2013).

Silk fibroin (SF), derived from *Bombyx mori* cocoons, is a widely used protein with remarkable mechanical properties (Bhardwaj & Kundu, 2011), in addition to being biocompatible (Rockwood et al., 2011). In contrast to other proteins, SF is characterized by Ala-Gly-X primary sequence, leading to regular conformations at its primary level (Altman et al., 2003). The repeating region for silk fibroin comprises various units, including highly repetitive GAGAGS hexamer and less repetitive GAGAGY (the less organized sequence) or/and AGVGYGAG motifs (Ha, Gracz, Tonelli, & Hudson, 2005). It may adopt different conformations, including random coils and β-sheets (Magoshi, Magoshi, & Nakamura, 1993). The latter (β-sheets) display improved properties in terms of degradation rate (Hu, Zhang, You, Wang, & Li, 2012), thermal stability (Moraes, Nogueira, Weska, & Beppu, 2010) and mechanical performance (Numata & Kaplan, 2010). SF has already been used in LbL films of different

* Corresponding author.
E-mail address: delezuk@gmail.com (J.A.M. Delezuk).

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materials (Altman et al., 2003; Moraes et al., 2013; Shang, Zhu, & Fan, 2013), including with chitosan (Nogueira et al., 2010) which is a polycation in slightly acidic solutions, non-toxic, biocompatible and biodegradable. Chitosans have been exploited in biomedical and pharmaceutical applications as biomaterials as well as components of biodevices (Bégin & Van Calsteren, 1999; Kumar, 2000; Rinaudo, 2006). The solubility in water and biological activity of chitosans are governed by structural and physicochemical characteristics. Because the latter can be varied and tuned by changing molecular weight and average degree of acetylation, chitosans can actually be tailored for specific applications. In biosensors, for instance, chitosans have been shown excellent as matrices (Suginta, Khunkaewla, & Schulte, 2013). It is clear therefore that combining SF and chitosan in a matrix may result in improved performance in biosensing.

The main goal in this study is to verify whether synergy may be established with SF and chitosan of different molecular weights. The working hypothesis is that by using chitosan one may induce order in SF molecules, which would then be an optimized matrix for a biosensor. This was tested by fabricating multilayered SF/chitosan LbL films onto which a layer of the enzyme phytase was adsorbed to detect phytic acid using impedance spectroscopy as the principle of detection. The most common procedures to determine phytic acid concentration are precipitation with iron (III) followed by titration analysis (Wu, Tian, Walker, & Wang, 2009), nuclear magnetic resonance spectroscopy (O'Neill, Sargent, & Trimble, 1980) and high-performance liquid chromatography (Lehrfeld, 1994). Phytic acid has also been detected with biosensors containing immobilized phytase in layer-by-layer (LbL) films (Moraes, Oliveira, Filho, & Ferreira, 2008). Amperometric biosensors have reached a detection limit of 2.0×10^{-3} mmol L^{-1} (Mak, Ng, Chan, Kwong, & Renneberg, 2004), while a biosensor based on impedance spectroscopy has allowed a detection limit for phytic acid of 10^{-6} mol L^{-1} (Moraes, Maki et al., 2010). Our sensing data were obtained with a selected film architecture, which was determined in a systematic characterization of SF/chitosan films using UV–vis spectroscopy, fluorescence spectroscopy, circular dichroism, contact angle measurements and polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS).

2. Experimental

2.1. Silk fibroin

Silk fibroin (SF) was extracted from the cocoons of *Bombyx mori* silkworm supplied by Bratac SA, Brazil. 10 g of cocoons were boiled during 30 min in 2 L of 0.02 M Na_2CO_3 solution to remove sericin. For each 10 g of the silk yarn, 100 mL of $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8) solution were added and heated to 60 °C for dissolution. This solution was then dialyzed against deionized water using a cellulose acetate membrane at room temperature for 48 h. SF was then centrifuged three times at 20,000 rpm for 30 min at 5 °C to remove impurities and aggregates (Rockwood et al., 2011). The final concentration of SF in solution was 3.5% in weight.

2.2. Chitosans

Three chitosans with distinct weight average molecular weights were used to build up chitosan/silk fibroin films: (i) high-molecular weight (CHH) obtained by deacetylation of β-chitin by using ultrasound irradiation (Delezuk, Cardoso, Domard, & Campana-Filho, 2011), (ii) medium-molecular weight (CHM) purchased from Polymar-Brazil and iii) chitosan oligosaccharide (CHL) from Kitto Life-South Korea. The average degree of acetylation (DA) of the chitosans was determined using ^{1}H NMR spectroscopy while the weight average (Mw) was determined by size exclusion chromatography (SEC) in a Shimadzu (CTO-10A), RID – 6A equipment, using Shodex Ohpak SB-G (50 mm × 6 mm – pre column) + Shodex Ohpak SB-803-HQ (8 mm DI × 300 mm) + Shodex Ohpak SB-805-HQ (8 mm DI × 300 mm) columns, refractive index detector, flow rate 0.6 mL min-^{1} and sample concentration of 4 mg mL^{-1} in acetic acid buffer as solvent at 35 °C (Pavinatto et al., 2013). The results of DA and Mw chitosans are given in Table 1.

Table 1
Weight average molecular weight (Mw) and average degree of acetylation (DA) of chitosan samples used to build LbL films.

Chitosan	Mw (g mol^{-1})	DA (%)
CHH	470,900 ± 2000	13.6 ± 0.3
CHM	136,000 ± 1200	14.0 ± 0.5
CHL	2561 ± 110	11.0 ± 0.6

2.3. Layer-by-layer films

SF was found to adopt β-sheet structures in chitosan/SF films (Chen, Li, & Yu, 1997) for a 1:9 SF:chitosan ratio, which was also used here with an aqueous SF solution (pH 5.6) at a concentration of 0.025% (w/v) and chitosan solution in 0.3 M acetic acid/0.2 M sodium acetate buffer (pH 4.5) at 0.225% (w/v). The choice of a 1:9 SF:chitosan ratio does not mean that this is the final composition in the LbL films, since adsorption governed by electrostatic interactions ceases when there is charge compensation. Chitosan/SF films were deposited onto quartz substrates previously treated with 1:1:5 solution of NH_4OH:H_2O_2:H_2O for 10 min at 70 °C, and then with a 1:1:6 solution of HCl: H_2O_2:H_2O for 10 min at 70 °C. The deposition process was carried out by immersing the substrate in chitosan solution for 10 min and in SF solution for 10 min. After each step of deposition, the film was washed with deonized water (twice) to remove poorly adsorbed molecules and dried gently with constant flowing nitrogen. The multilayer deposition was monitored by UV–vis and fluorescence spectroscopy, performed with a U-2900 UV–vis spectrophotometer from Hitachi and RF-5301PC spectrofluorimeter from Shimadzu, respectively. The film thickness was measured using Veeco Dektak 150 Surface Profilometer, and the values reported are taken as the average from four measurements.

Contact angle measurements were carried out in a KSV system (KSV, Finland). A drop of water (10 μL) was deposited on the film surface and the drop shape was recorded by a digital CCD camera (LG). The acquired image was analyzed using KSV software, from which the evolution of the contact angle as a function of time was determined. The value of the contact angle was taken as the average from at least three measurements, after 15 s of the water being dripped to reach equilibrium, made on different areas of the film surface. The PM-IRRAS analysis was carried out using a KSV PMI550 instrument (KSV, Finland), with spectral resolution of 8 cm^{-1}. The light beam reached the film at 81°, being continuously modulated between *s*- and *p*-polarizations at a high frequency. This allows for the simultaneous measurement of the spectra for the two polarizations. The difference spectrum provides surface-specific information on oriented moieties, while the sum gives the reference spectrum. In addition, with the simultaneous measurements, the effect of water vapor is reduced. Circular dichroism (CD) spectra of SF aqueous solutions were collected with a quartz cell of 1 mm optical path length. The CD spectra of chitosan/SF films were measured directly over the films deposited on quartz substrates, with the optical path being given by the film thickness. Measurements were performed on a J-815 Circular Dichroism Spectrometer (Jasco Inc., Tokyo, Japan), with the bandwidth of 1 nm, a response