COLORIMETRIC NANOSENSOR FOR THE DETECTION OF BOVINE SERUM ALBUMIN

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RESUMO – A detecção de albumina do soro bovino (BSA) é importante nas áreas alimentícia, farmacêutica e médica. Entretanto, as técnicas utilizadas para sua detecção são demoradas, caras e pouco sensíveis. Este trabalho descreve as propriedades de nanossensores de polidiacetileno (PDA) - copolímero tribloco L64 para detecção de BSA. A eficiência dos sensores foi avaliada em função da composição do nanossensor. Os nanossensores detectaram pequenas concentrações de BSA (0,04 mM) por meio de transição colorimétrica visível. A variação da energia livre de Gibbs padrão, a estequiometria e a constante de interação do complexo BSA-nanossensor mostrou relação direta entre resposta do sensor e interação BSA-nanossensor, sendo esta interação dirigida entropica ou entalpicamente, quando o nanossensor continha ou não colesterol, respectivamente. Os nanossensores de PDA-L64 são rápidos e de baixo custo para detecção de pequenas quantidades de BSA nativa.

ABSTRACT – The bovine serum albumin (BSA) detection is important in food, pharmaceutical and medical areas. However, traditional techniques used to detect BSA are often time-consuming, expensive, and show limited sensitivity. This work describes properties of polydiacetylene (PDA)-triblock copolymer (L64) nanosensors, synthesized to detect small amounts of BSA. Sensor efficiency was studied as a function of nanosensor composition. Nanosensors detected small concentration of BSA (0.04 mM) through naked-eye detectable blue-to-red transition. The standard Gibbs free energy change (−10.4±ΔG°<−49.52 kJ.M⁻¹), stoichiometry complex (1<n<3), and binding constant (6.7 × 10⁶ <K₅<4.79×10⁹ M⁻¹) of BSA-nanosensor complex formation established a direct relationship between nanosensor response and BSA-nanosensor interaction. BSA-nanosensor interaction was entropically (without cholesterol), and enthalpically (with cholesterol) driven. PDA-L64 nanosensors are potential low-cost sensors for rapid detection of small amounts of BSA.

PALAVRAS-CHAVE: polidiacetileno; fluorescência; microcalorimetria; proteínas do leite.

KEYWORDS: polydiacetylene; fluorescence; microcalorimetry; milk proteins.
1. INTRODUCTION

Bovine serum albumin (BSA) detection has attracted increasing attention due to its application in different fields such as food, biochemical and immunological sciences. It is a very useful marker to follow mastitis. However, traditional techniques used to detect BSA are often based on spectrometric methods (Wang et al., 2015); are time-consuming, use expensive equipments and show limited sensitivity and narrow linear range. Therefore, it is necessary to develop alternative ways to fast detect (in real time) small amounts of BSA in different matrices, such as milk.

Polydiacetylenes (PDAs) are conjugated polymers able to respond to different stimuli, undergoing blue-to-red transition. Systems based on PDAs detect bacteria, enzymes, surfactants, solvents, and others by colorimetric changes, making PDA suitable for use in sensor systems, with several merits such as simple, rapid and label-free detection, and easy naked-eye recognition (Pires et al., 2010).

PDAs self-assemble in different structures, such as vesicles and films; and colorimetric transition depends on PDA aggregation form. The application of PDA vesicles for detection of different molecules in suspension has been widely studied. However, there are some limitations, such as low stability for long periods, and restricted colorimetric transition for some conditions, hiding the visual color change (Pattanatornchai et al., 2013). Thus, it would be useful to develop new PDA-based templates for the efficient use of this polymer as a sensor to detect different molecules in suspensions. Gou et al. (2010) showed that PDAs interact with amphiphilic molecules such as triblock copolymers (TCs), which are formed by arrays of ethylene oxide (EO) and propylene oxide (PO) units (symbolized as \((EO)_n(PO)_m(EO)_n\), where “n” and “m” mean the number of ethylene oxide and propylene oxide unit segments, respectively).

Therefore, to overcome limitations of PDA vesicles, we developed nanoaggregates formed by a mixture of PDA and TC L64 to detect BSA. However, for a successful future application of these nanosensors is fundamental to understand the mechanisms of BSA-nanosensor interaction that leads to protein detection. Thus, besides detecting the protein, we were also interested in investigating the effects of nanoaggregate composition, and in determining the thermodynamic parameters associated with nanosensor-protein interaction.

2. MATERIAL AND METHODS

2.1. Materials

BSA (98% wt. pure), α-lactalbumin (85% wt. pure), β-lactoglobulin (90% wt. pure), lactoferrin (85% wt. pure), αS1-casein (70% wt. pure), β-casein (98% wt. pure), 10,12-pentacosadiynoic acid (PCDA, 97% wt.), and cholesterol (99% wt. pure) were purchased from Sigma-Aldrich (USA). TC L64 ([EO]_{13}(PO)_{30}(EO)_{13}], with average molar mass of 2900 g.mol\(^{-1}\), acquired from Aldrich (USA), was used. Polyvinylidene difluoride (PVDF) syringe filters (0.33 mm of diameter and 0.45 μm pore) were purchased from Millipore (USA), and Millipore water, were used in all experiments (R ≥ 18.2 MΩ.cm).

2.2. Nanosensor Production

Nanosensors of PCDA and L64 were prepared by solubilizing L64 in water, at concentration of 1.0% (w/w). PCDA (1 mM) was dissolved in the TC solution, the mixture was sonicated for 10 min (Sonics & Materials Inc., Model VC750, USA), and immediately filtrated through PVDF filter. The
suspension was kept overnight at 4°C, with the aim of orientating PCDA monomers in order to promote polymerization. Then, photopolymerization was carried out by exposing the suspension to UV radiation (254 nm), for 6 min, until the suspension turned blue in color.

To evaluate the effect of a lipid insertion on color transition caused by BSA, nanosensors containing cholesterol were manufactured. Cholesterol (1, 2 or 3 mM) was dissolved into L64 solution (1% wt.) and the mixture was sonicated for 5 min; then PCDA (1 mM) was added and the synthesis followed as described above for nanosensors without cholesterol.

2.3. Colorimetric Response (CR)

BSA solution (0.75 mM) was prepared by solubilizing the protein in L64 1% (wt.). Aliquots of BSA solution were added to the nanosensor suspension, at increasing concentrations up to a final concentration of 0.20 mM. The mixtures were stirred for 30 s, and maintained at 25 °C for 1 h, to allow the system to achieve thermodynamic equilibrium. Spectra were obtained between 350 and 700 nm (Shimadzu UV-2550, Japan), at 25 °C. To quantify the percentage of blue-to-red conversion of polyydiacetylenes, a parameter termed “colorimetric response (CR)” was calculated (Charych et al. 1993).

2.4. Light Scattering, Electrokinetic and Fluorescence Experiments

Size and zeta potential of nanostructures were measured at 25 °C, with a Zetasizer nano ZS90 (Malvern, UK). Samples were diluted 20 times to avoid blue color interference on the measurements. Each experiment was repeated 3 times, and each result was presented as the average of 10 measurements.

Fluorescence spectra of protein were recorded in a CaryEclipse Fluorescence Spectrometer (Agilent, USA), using a 1/cm path length quartz cuvette. Nanosensor aliquots were added to the protein (BSA or denatured-BSA) solution (0.75 mM), and the fluorescence emission spectra were recorded between 296 and 500 nm, at the excitation wavelength of 295 nm, which is specific for tryptophan residue excitation.

2.5. Isothermal Titration Microcalorimetric (ITμC) Experiments

Titration analyses were performed in an isothermal titration microcalorimeter (ITμC), model CSC 4200 (TA Instruments Inc., USA), controlled by ITCTRun software. The microreaction system (1.8-mL stainless steel vessels for sample and reference), containing PCDA/L64 nanosensors, was maintained under constant stirring at 300 rpm. When thermal equilibrium between the vessel and the heat sink was reached, 10-μL aliquots of BSA solution were titrated at 500 s intervals, until protein concentration of 0.20 mM was achieved.

For each injection, by raw data peak integration, the experimental enthalpy change (ΔH) was obtained. The observed molar enthalpy change (Δ\textsubscript{Obs}H) was obtained by dividing ΔH by the number of moles of BSA added in each injection. A control experiment was carried out in each run to determine the dilution enthalpy change (Δ\textsubscript{dil}H), by injecting BSA solution into nanosensor solvent (copolymer L64). As the amount of BSA molecules bound per PCDA was not known, it was not possible to calculate the exact molar enthalpy change of interaction. Therefore, an apparent enthalpy change of interaction (Δ\textsubscript{ap-int}H) was determined, calculated from the difference between Δ\textsubscript{Obs}H and Δ\textsubscript{dil}H curves.
3. RESULTS AND DISCUSSION

The presence of either L64 or cholesterol did not inhibit diacetylene polymerization, and the electronic spectra of all nanosensors were found to present similar profiles, with maximum absorption band at 640 nm, and a shoulder at 590 nm. However, the intensity of both bands increased with cholesterol concentration, indicating that the presence of lipid increased the polymerization yield. As the PCDA polymerization process is dependent on the orientated packaging of PCDA monomers, this increase in intensity demonstrated that cholesterol improved PCDA self-aggregation. The cholesterol added to the PCDA/L64 mixture could be dissolved in the hydrophobic region, and/or at interface of the nanostructure, interacting with PO segments and PCDA monomers, and possibly altering the polymerization yield and/or the sensitivity of nanosensor.

Cholesterol (CHO) also affected nanosensor hydrodynamic diameter (Dh), and zeta potential (ξ) (p<0.05). Nanostructures without CHO presented Dh and ξ equal to 78.2 ± 1.39 nm and −22.2 ± 0.12 mV, respectively. The addition of CHO resulted in the nanostructure being smaller (65.8 ± 0.94, 62.9 ± 1.28 and 67.4 ± 1.4 nm), and more negatively charged (−29.5 ± 0.66, −29.5 ± 0.58 and −28.3 ± 0.75 mV) (p<0.05), for CHO concentrations equal to 1, 2, and 3 mM, respectively, which did not change between them (p≥0.05), corroborating with the hypothesis that CHO molecules are located at hydrophobic/hydrophilic interface and in the hydrophobic core of nanostructures.

To evaluate the effect of milk proteins on colorimetric transition of PCDA/L64 nanosensors, increasing concentrations of BSA, α-lactalbumin or β-lactoglobulin or lactoferrin or αS1-casein or β-casein was added into nanostructure suspension. Red-to-blue transition of nanosensors only occurred in the presence of BSA. The extension of PCDA conversion due to interaction with BSA was determined using the colorimetric response (CR) parameter. Figure 1a illustrates the CR of PCDA/L64 1.0% (w/w), with or without CHO, as a function of BSA concentration at 25 °C.

CR was enhanced as BSA concentration increased, achieving a plateau of maximum CR at BSA concentration of 0.18 mM, with maximum CR of 34%, 65%, 60%, and 43% for PDA/L64 nanosensor containing 0, 1, 2, and 3 mM CHO, respectively. It was verified color change by naked eye for only PCDA/L64/CHO 1 mM and PCDA/L64/CHO 2 mM nanosensors (Figure 1b).

At 1 and 2 mM, the presence of CHO improved the sensitivity of PCDA nanosensor for BSA detection, suggesting a specific interaction between the protein and lipid. Nevertheless, CR reduced when CHO concentration increased to 3 mM, which may be related to PCDA stabilization, caused by...
CHO concentration increasing, since it is known that at higher CHO concentration the lipid segregation process may limit the conformational freedom of PCDA (Kolusheva et al., 2003).

Fluorescence spectroscopy is a powerful technique to study the intermolecular interaction between BSA and PCDA/L64 nanosensors, by measuring the fluorescence quenching of the biopolymer when protein-nanostructure complexes are formed. The BSA fluorescence intensity reduced and there was a displacement in the maximum peak (around 5 nm) as PCDA concentration increased, indicating BSA-PCDA complex formation. To determine BSA-nanosensor binding constant (Ka) and binding stoichiometry (n) we followed Stern-Volmer approach, and after using Gibbs relation, we determine standard Gibbs free energy (ΔG°) for the complex formation (Bourassa et al., 2010).

Ka, “n” and ΔG° values (Table 1) depended on nanostructure composition, with the following order for their modulus: PCDA/L64 < PCDA/L64/CHO 3 mM < PCDA/L64/CHO 2 mM < PCDA/L64/CHO 1 mM. The more intense the BSA-nanostructure interaction (lower ΔG° values), and the greater the number of PCDA molecules per protein (higher “n”), the more sensitive the nanosensor. The magnitude of ΔG° values shows that BSA-nanosensor interactions should occur through dispersive interactions (van der Waals and London forces), electrostatic forces, and hydrogen bonding between PCDA carboxylic groups present at the nanostructure hydrophobic/hydrophilic interface and amino and carboxyl groups of BSA amino acid residues.

Table 1 – Binding constant (K_a), complex stoichiometry (n), and standard free Gibbs energy change (ΔG°) for BSA-PCDA complex formation, at 25 °C.

<table>
<thead>
<tr>
<th>Nanosensor</th>
<th>K_a (10^8 L mol⁻¹)</th>
<th>n</th>
<th>R²</th>
<th>ΔG° (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCDA/L64</td>
<td>0.22±0.01</td>
<td>1.6</td>
<td>0.99</td>
<td>-30.52±1.22</td>
</tr>
<tr>
<td>PCDA/L64/CHO 1 mM</td>
<td>4.79±0.19</td>
<td>2.7</td>
<td>0.99</td>
<td>-49.52±1.74</td>
</tr>
<tr>
<td>PCDA/L64/CHO 2 mM</td>
<td>1.35±0.05</td>
<td>2.3</td>
<td>0.99</td>
<td>-40.68±1.43</td>
</tr>
<tr>
<td>PCDA/L64/CHO 3 mM</td>
<td>0.69±0.02</td>
<td>1.8</td>
<td>0.98</td>
<td>-33.31±1.27</td>
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To characterize the BSA-nanosensor complex formation process in greater detail, the enthalpic contribution for BSA-nanosensor interaction was determined by microcalorimetric assays. Figure 2 shows the apparent interaction enthalpy change (Δap-intH) between BSA and PCDA/L64 nanosensors, containing or not containing CHO, versus BSA concentration.

Figure 2 - Δap-intH between BSA and PCDA/L64 nanosensors containing (■) 0 mM, (○) 1 mM, and (▼) 3 mM cholesterol, as a function of BSA concentration.

In the absence of CHO, interaction between BSA and the nanostructure was found to be an endothermic process, with Δap-intH of 22.82 kJ mol⁻¹, at a concentration of 9.11 μM BSA (first injection).
However, at both CHO concentration (1 or 3 mM), the interaction process between BSA and nanostructure was exothermic, releasing \(-2.68\,\text{kJ.mol}^{-1}\) (at 3 mM CHO) and \(-42.20\,\text{kJ.mol}^{-1}\) (at 1 mM CHO). The more exothermic process of BSA-nanosensor (1 mM CHO) interaction may be attributed to an intense and specific interaction between CHO and BSA that releases more energy than is absorbed to break CHO-PCDA, CHO-L64, and CHO-CHO interactions. On the other hand, for the BSA-nanosensor (3 mM CHO) interaction, less energy was released, possibly due to a segregation process involving CHO molecules forming nano-domains of pure lipids inside the nanostructure, which demands a higher energy cost to break CHO-CHO interaction that is released by BSA-CHO bonds.

4. CONCLUSION

An optical nanosensor was synthesized by controlled self-assembly of polydiacetylene and copolymer molecules, and its efficiency of detection of small BSA concentrations was demonstrated. The PCDA/L64 colorimetric transition was induced by a direct interaction between BSA and nanostructures, driven by an increase in entropy, when CHO was absent from the nanosensor composition, and driven by enthalpy in the presence of the lipid.

5. ACKNOWLEDGMENTS

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6. REFERENCES


