Journal of the Taiwan Institute of Chemical Engineers 63 (2016) 101–106

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

Interactions of bovine serum albumin with humic acid–Cu(II) aggregates in poly(hydroxyethylmethacrylate) cryogel column

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ARTICLE INFO

Article history:
Received 22 April 2015
Revised 7 March 2016
Accepted 15 March 2016
Available online 2 April 2016

Keywords:
Humic acid fouling
Protein
Cryogel

ABSTRACT

Understanding the interactions of proteins with humic acid in different media is essential for many applications. This paper investigates the interactions of bovine serum albumin with humic acid–Cu(II) complexes in a poly(hydroxyethylmethacrylate) cryogel column. Humic aggregates were immobilized within the cryogenic matrix. Protein sorption studies were conducted at different temperature, ionic strength, protein concentration, pH and flow-rate. Protein adsorption increased at lower pHs (close to the isoelectric point of the protein; 4.7) and higher temperatures but decreased at higher ionic strength and flow-rates. Both electrostatic and hydrophobic interactions played roles in the sorptive behavior of the protein molecules. Isotherm analysis showed the monolayer protein adsorption onto humic molecules immobilized column.

1. Introduction

Assessments of protein adsorption under various conditions are essential in many fields including biomedical applications [1], membrane research [2], environmental studies [3] and pharmaceuticals [4]. Proteins are large macromolecules with a tendency to accumulate at solution/solid interfaces. Evaluation of protein adsorption onto various solid surfaces in the presence of humic acid (HA) is necessary [5].

Humic acids are ubiquitous and biogenic molecules occurring in all terrestrial and aquatic environments [6] and cited as a major foulant in filtration studies [2]. The acidic groups on humic molecules are strongly pH-dependent and can undergo de/protonation depending on the nature of the solution. Especially in weakly acidic or alkaline solutions, deprotonated carboxyl and hydroxyl groups can interact with metal cations via complexation and electrostatic interactions [7]. Protein-HA interaction in the presence of metal ions is particularly important [8]. Since metal ions can form stable complexes with both HA molecules and proteins [9], HA fouling is a major problem in membrane studies [2]. HA molecules could also change the secondary structure of protein molecules; facilitating development of fouling cake [8]. HA forms stable complexes with proteins by affecting their activity and stability in soil, so HA-protein interactions in soil are also important [10, 11].

Sorption of proteins is a complex phenomenon; it can be difficult to reveal the exact characteristics contributing to the adsorption [12]. Various surface and interfacial interactions should be considered while studying protein adsorption surfaces. Some studies have shown that hydrogen bonding, electrostatic and hydrophobic interactions play roles in protein adsorption. Yet, the interactions governing charge and conformational changes of proteins, orientation of the adsorbed protein molecules and surface characteristics should be evaluated to reveal relative contribution of each interaction [13]. It appears that more protein adsorption studies in different systems can be helpful to evaluate specific roles of these interactions and the mechanisms involved in protein sorption characteristics [1].

Supermacroporous poly(hydroxyethylmethacrylate), p(HEMA), cryogel columns are widely utilized as an efficient media for separation and recovery of biomolecules. These matrices have sponge-like structure with interconnected macropores and are produced by free radical polymerization under cryogenic conditions [14].

Bovine serum albumin (BSA) is a soft, globular protein and changes its conformation following the binding onto surfaces [15]. Charged pendant groups make it hydrophilic and soluble in water. Both BSA and HA have distinct physicochemical properties and behave differently under different conditions [16].

Due to their recalcitrant nature, studies on HA can be challenging. Earlier workers investigated HA fouling in protein filtration studies by adding HA to the protein solutions or by immersing the

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http://dx.doi.org/10.1016/j.jtice.2016.03.032
1876-1070/

membranes in humic solutions before the experiments [17,18,8]. Yuan and Zydney [17] reported that the aggregates had a role in HA fouling and humic aggregates served as binding sites for the subsequent deposition. Katsoufidou et al. [18] reported that rapid, irreversible and persisting HA fouling occured in the pores of the membranes resulting from stable multivalent metal aggregates.

This study investigates the interactions of BSA with immobilized humic aggregates rather than free humic molecules. It also describes the preparation of HA–Cu^{2+} aggregates immobilized p(HEMA) column. Immobilization of HA within the polymeric matrix made it possible to observe the interactions of BSA with HA under different conditions. The findings of this present study may provide some insights into the interactions of blood proteins with humic substances for separation and membrane studies and soil science.

2. Material and methods

2.1. Materials

N,*N*′-methylene-*bis*-acrylamide (MBAAm), ammonium persulfate (APS), BSA and humic acid (Aldrich 53680; Pcode: 53609184) were obtained from Sigma-Aldrich. Aldrich HA was used as provided and its characterization was presented as Supplementary Material. 2-hydroxyethylmethacrylate (HEMA) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Fluka.

2.2. Preparation of p(HEMA)–HA–Cu^{2+} cryogel column

Cryogels were produced by free radical polymerization reaction proceeding in an aqueous solution of polymeric precursors (in a plastic syringe 5 mL) in an ice bath; HA (20.0 mg) was dissolved in alkaline solution and pH was adjusted to 5.6 by adding a few drops of 0.1 M HCl solution. The solution was titrated into 50 mL of Cu^{2+} (110 mg L^{-1}) solution and agitated overnight. Then, metal-humate aggregates were separated by centrifugation to remove the uncomplexed metal ions and HA. Concentration of HA in the feed solution and the supernatant was determined with a UV–vis spectrophotometer at 240 nm (Shimadzu, Tokyo, Japan, Model 1601). The amount of HA aggregates was estimated to be 19.6 mg from the calibration curve. Then, the aggregates were blended with polymer precursors (i.e.; 300 μL of HEMA and 70 mg of MBAAm) in 4.5 mL of distilled water. The mixture was vortexed for 7 min. The polymerization was initiated by adding 100 μL APS solution (10% w/v) and 20 μL TEMED. Then, the mixture was vortexed and transferred into a plastic dead-end syringe. The polymerization mixture was kept in the fridge at −12 °C overnight and then left to thaw at room temperature. To remove unconverted monomers, initiator and uncomplexed HA molecules and Cu^{2+} ions, the column was extensively rinsed with water. The concentration of HA leached from the column was determined spectrophotometrically by assaying the resulting washing solution at 240 nm until no leakage was observed. Finally, the amount of HA aggregates entrapped within the cryogel column was found to be 14.7 mg. Also, Cu^{2+} leakage from the column was monitored using an atomic absorption spectrophotometer (GFAAS, Analyst 800/PerkinElmer, USA). The attempt of preparation of uncomplexed HA–p(HEMA) cryogels in the absence of copper ions failed due to the continuous leakage of HA during the washing, making further studies not possible because of the interferences of two UV absorption bands for HA and BSA.

2.3. BSA–HA–Cu^{2+} interaction studies

Protein adsorption studies were carried out in a 5 mL column in a recirculating system. Prior to each run, the column was rinsed first with distilled water (30 mL) and subsequently was equilibrated with corresponding buffer solution (50 mM) at desired pH and temperature. BSA solution in buffer (20 mL) was loaded onto the column (flow-rate 1.0 mL min^{-1}). The fractions were collected and assayed spectrophotometrically at 280 nm.

The effects of protein concentration (0.1–4.0 mg mL^{-1}), pH (4.0–8.0), flow-rate (0.5–3.0 mL min^{-1}), ionic strength (0.01–0.1 M NaCl solution) and temperature (5, 10, 14 and 25 °C) on sorption of BSA were investigated. For studies at pH 4.0 and 5.0, sodium acetate buffer was used; for the ones at pH 6.0, 7.1 and 8.0, sodium phosphate buffer was used. Unless otherwise mentioned, all studies were carried out at pH 5.0 at 25 °C for solution of 0.5 mg mL^{-1} BSA. Each protein adsorption study was followed by washing to remove the BSA molecules retained in the column but not specifically bound to the gel matrice. The washing steps were carried out under the same conditions as the corresponding adsorption studies were done. The volume of the washing buffer solution was in the range of 25–45 mL and loading of buffer solution onto the column was continued until a protein free buffer solution fraction was observed.

Desorption of the protein was achieved by running 30 mL of 1.0 M NaCl solution through the column (flow-rate 1.0 mL min^{-1}). BSA molecules eluted from the column was monitored spectrophotometrically. Upon achieving complete desorption of BSA, the column was rinsed with distilled water and then equilibrated with corresponding buffer solution.

In adsorption, washing and desorption studies, BSA concentration in the solution was determined spectrophotometrically. The amount of BSA adsorbed by the column was calculated using the expression:

$$q_e = (C_0 - C_e)V/m \quad (1)$$

where C_0 and C_e are the initial and equilibrium concentrations of BSA solution (mg mL^{-1}), respectively; q_e is the amount of BSA adsorbed (mg g^{-1}), V is the final volume of the protein solution, buffer solution or salt solution run through the column (mL), and m is the dry mass of the cryogel used (g). The amount of BSA specifically bound to the support was calculated by subtracting the amount of protein in washing buffer solution from the total amount of protein loaded onto the column.

BSA sorption studies on p(HEMA) column without humic-Cu(II) aggregate was also conducted to check whether the p(HEMA) column itself contributed to BSA sorption (pH 5.0, temperature 25 °C, 20 mL of 0.5 mg mL^{-1} BSA, flow-rate: 1.0 mL min^{-1}). It was observed that washing with buffer solution removed all the non-specifically bound proteins from the column (0.32 mg, average of three runs), indicating that washing procedure was effective in elution of unbound proteins from the column and there is no contribution of p(HEMA) itself to the protein sorption in the end.

2.4. Surface morphology

The surface morphologies of p(HEMA) and p(HEMA)–HA–Cu^{2+} cryogels were studied with scanning electron microscopy (SEM, EVO LS 10 ZEISS).

3. Results and discussion

3.1. Characterization of p(HEMA)–HA–Cu^{2+}cryogel

SEM images showed the inner walls of the p(HEMA) cryogel and p(HEMA)–HA–Cu^{2+} cryogel columns (Fig. 1). Both cryogel supports had large continuous interconnected pores. These macropores provided necessary channels for the protein solution to flow easily. The images also verified the presence of HA aggregates; Cu^{2+}–humate aggregates made the polymer surface rougher than that of pristine p(HEMA) surface.