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Evaluation of a chitosan membrane for removal of endotoxin from human IgG solutions

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Abstract

Cross-linked chitosan membranes – prepared with silica particles as porogen agent – were evaluated for the removal of endotoxin (ET) from human immunoglobulin G (IgG) preparations. The effects of solution conditions on the efficiency of ET removal and IgG recovery were studied. The depyrogenation studies were performed with Tris–HCl at pH 7.0 due to low IgG adsorption and significant degree of protonation (0.50) of the membrane in this buffer. Adsorption ET data were analyzed using the Langmuir model (maximum binding capacity and dissociation constant were 280 μg/mL and 4.0×10^{-11} mol/L, respectively). A high ET clearance (96%) and IgG recovery (99%) were obtained with ET and IgG initial concentration of 116.4 EU/mL and 1.0 mg/mL, respectively. Therefore, these results assure the potential of using chitosan membrane filtration for ET removal in the downstream processing of IgG solutions.

Keywords: Depyrogenation; Endotoxins; Human IgG; Chitosan; Adsorptive membranes

1. Introduction

Intravenous polyclonal human IgG is an alternative treatment in a wide range of medical disorders, including bone marrow and solid organ transplantations, chronic demyelinating inflammatory polyneuropathy, severe asthma, pediatric HIV, and multiple sclerosis [1]. Contamination of IgG products with endotoxins (ET) liberated by Gram-negative bacteria can take place if proper preventive measures are not satisfied during processing. ET are known to have potent biological effects in human and animals if administered systemically [2]. The adverse effects of intravenously administered ET range from a fever when present at concentration as low as 1 ng/kg of body mass to irreversible septic shock at higher doses. Therefore, it is essential that the downstream processing of IgG products accomplish absence or the reduction of ET concentration to acceptable levels [2].

ET are very stable molecules, negatively charged at a broad range of pH, resisting to extreme temperatures and pH values in comparison to protein [2]. The removal of ET from solutions of proteins [3], polyssacharides [4], and DNA [5,6] can be a challenge specially due to product loss [3]. Adsorption methods have proved to be very effective for the removal of ET from protein solutions [7]. Different ligands – cationic functional groups like chitosan and quaternized chitosan [8,9], polymixin B, poly-L-lysine, diethyl aminoethyl, poly(ethyleneimine), histamine, and histidine [7,10,11] – immobilized on particulate matrix of soft gels have been used for this purpose. However, operational problems like high pressure drops, internal diffusion limitation, compressibility, and clogging are often found when using soft beads. Some of these difficulties can be circumvented with the use of membranes as adsorption matrix. Adsorptive membrane chromatography resulted from technological advances in both membrane filtration and fixed-bed liquid chromatography. In these membranes, dissolved molecules are carried directly to adsorptive sites by convective flow, thus reducing the long diffusion time typical of soft gel-based chromatography. Other advantages of membranes over soft gels are the possibility of operating at high flow rates with

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low pressure drops, easy scale-up, and high mechanical stability [12,13].

Flat and hollow fiber microfiltration membranes, and supermacroporous monolithic matrix functionalized with cationic functional groups or small ligands (adsorptive membranes) were effective for ET removal in the presence of proteins [14–18]. Recently, cross-linked chitosan microfiltration membranes have been employed as supports for immobilization of various ligands or functional groups for proteins purification [19–21]. Due to their high amine residue content, the cross-linked chitosan membrane (pK_a between 6.5 and 7.0) can serve as a weak anion-exchanger. Consequently, it is not necessary to introduce cationic functional groups, which often deteriorate the structure and mechanical strength, in such materials [19,22]. Compared to the adsorptive membranes with immobilized functional cationic groups, the chitosan ion-exchange membranes are much cheaper and have a higher adsorption capacity [21,22]. Cross-linked chitosan membranes possess controlled pore sizes, good mechanical strength, and chemical stability, as well as hydrophilicity and biocompatibility [19,20].

In our previous work, cross-linked chitosan microfiltration membranes were used as anion-exchanger to efficiently remove ET from buffers and bothrops antivenom serum ($F(ab')_2$ fragments of equine antibodies) [23]. The presence of protein in the feed solution did not significantly affect the ET clearance by the membrane [23].

The present work extends the application of Freitas studies [23] of ET removal from protein solutions. The purpose of this study was to evaluate the feasibility of using cross-linked chitosan membrane as cationic adsorber for the removal of ET from human IgG solutions, in terms of capacity and selectivity. Experimental tests were conducted aiming to find a favourable condition – pH and buffer system – for an efficient depyrogenation with low IgG loss. An equilibrium binding study was also performed and the adsorption isotherm was analyzed with the Langmuir model for the determination of the dissociation constant (K_d) and maximum binding capacity (Q_m).

2. Materials and methods

2.1. Chemicals

Chitosan (from crab shells, minimum 85% deacetylated), particulate silica gel (particle diameter in the range of 15–40 μm) and crystalline bovine serum albumin were from Sigma (USA) and epichlorohydrin was from Merck (Germany). Pyrogen-free human IgG (purity of approximately 95%, prepared from Cohn Fraction II, III) was from Aventis Behring (Germany). All other chemicals were of analytical grade. Ultrapure water was obtained using the Millipore Milli-Q system (Millipore, USA). The QCL-1000 *Limulus Amoebocyte Lysate* (LAL) Endotoxin kit and purified endotoxins from *Escherichia coli* 05:B55 were purchased from BioWhittaker (USA). Pyrogen-free water (Mesquita or Ariston, Brazil) was used for solutions preparation and adsorber rinsing.

2.2. Preparation of cross-linked chitosan macroporous membranes

The chitosan macroporous membranes were prepared as described by Zeng and Ruckenstein [19]. Briefly, 1 g of chitosan was dissolved in 100 mL of aqueous acetic acid solution (1%, v/v). A mass of 12 g of silica particles (an inorganic porogen agent) was added to the chitosan solution and the resulting suspension was vigorous mixed to uniformly disperse the particles. The suspension was then poured into a rimmed glass plate which was placed in an oven at 80 °C for 5 h to allow water evaporation. The dried membrane was then immersed in a 1.25 mol/L NaOH solution for 2 h at 80 °C in order to form macroporous as result of the silica particles dissolution. Finally, the porous membrane was washed with distilled water to remove any remaining NaOH.

The chitosan membranes were cross-linked to avoid their dissolution in acidic solutions. In order to maintain the number of amine groups, epichlorohydrin was selected as the cross-linking agent, since it reacts only with the OH groups under weak basic conditions [19–21]. In order to accomplish this cross-linking, the membranes were immersed in 20 mL of 0.010 mol/L epichlorohydrin aqueous solutions at pH 10 and 50 °C for 2 h followed by extensive washing with distilled water [19,20].

2.3. Tensile testing

The tensile testing of the chitosan macroporous membranes (8 mm of width, wet state) were determined at 20 °C using an Universal Testing Instrument (Model DL-2000, USA), employing a cell load of 10N. The gauge length was 20 mm and the extension rate was set as 10 mm/min. Five independent samples were used for each measurement.

2.4. Porosity measurements

The porosity of the chitosan membranes was evaluated by their capacity of water absorption [19]. The porosity was calculate with the expression:

$$\text{porosity}\ (\%) = \frac{[(W_1 - W_2)/d_{\text{water}}]}{V} 100 \quad (1)$$

where W_1 and W_2 are the mass of the membrane in the wet and dry states, respectively, d_{water} the density of water at 20 °C, and V is the volume of the membrane in the wet state.

2.5. Scanning electronic microscopy

The morphology (pore size and pore size distribution) of the chitosan membranes was observed with a scanning electron microscopy (MEV Leica, Leo 440i, USA). The wet membrane was wiped with a filter paper to remove the excess water present on the its surface, then framed on a Petri dish to prevent shirinkage along the surface and lyophilized for 24 h at −60 °C (freeze drier Savant-Novalyphe, model NL150, USA). The membranes samples were fractured under liquid nitrogen and the fractured surfaces were coated with a thin layer of metal (gold, 92 Å) before scanning.

2.6. Analytical methods

Protein concentration was determined with the Bradford's method [24] using crystalline bovine serum albumin as a reference protein. Endotoxin concentration (1 EU correspond to 100 pg ET) was quantified with the chromogenic LAL test method (sensitivity of 0.01 EU/mL), according to the manufacturer's instructions. All samples were measured in duplicate.

2.7. ET decontamination of apparatus and membrane

All glass materials were ET decontaminated with overnight immersion in 1.5 mol/L NaOH, followed by washing with pyrogen-free water and heating at 200 °C for 2 h, as described by Nakata [25]. The ultrafiltration cell (model 8050, Amicon, USA) was first decontaminated with washing with 2 mol/L KOH and pyrogen-free water, as recommended by Petsch et al. [15]. Single membranes (a disk of 13.4 cm^2) were decontaminated with sequential washing with 0.1 mol/L NaOH containing 20% ethanol, 1.5 mol/L NaCl solution, pyrogen-free water and pyrogen-free equilibration buffer [15]. The same procedure was used for membrane regeneration.