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Poly-L-lysine modified cryogels for efficient bilirubin removal from human plasma

Mehmet Emin Çorman

Sinop University, Faculty of Sciences and Arts, Department of Chemistry, 57000 Sinop, Turkey

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ABSTRACT

In this study, poly-L-lysine (PLL) immobilized PHEMA cryogel was designed for the specific bilirubin removal from human plasma. The surface of PHEMA cryogels is surrounded by PLL chains to enhance specific binding of bilirubin molecules via specific complementary electrostatic interactions. The functionalization of the PHEMA cryogel was carried out by direct coupling of PLL to pre-activated OH-group of the HEMA alcohol units via carbodiimide activation. Prior to removal of bilirubin from human plasma, the optimization parameters including, initial bilirubin concentration, flow rate, temperature, ionic strength, and adsorption rates on adsorption of PLL-PHEMA cryogel were investigated from the aqueous medium. According to the elemental analyses results, the incorporation of PLL was 690.0 μmol/g cryogel. The cryogel has highly interconnected supermacroporous structure sized between 20 and 100 μm with a positive surface charge value. The maximum bilirubin adsorption capacity was found as 59.9 mg/g of the dry weight of PLL-PHEMA cryogel. Reusability study explored that PLL-PHEMA could be used with a negligible loss in the bilirubin adsorption capacity after successive ten adsorption-desorption cycles using the same adsorbent. The results of the study demonstrated that the PLL-PHEMA cryogel exhibited high specificity that can be used as an efficient column for removing the bilirubin from the human plasma.

1. Introduction

Bilirubin (BR) is a metabolic breakdown of hemoglobin occurring in blood serum and present in three major forms: conjugated bilirubin (direct bilirubin), unconjugated bilirubin (indirect bilirubin) and unconjugated-unbound (free bilirubin) [1,2]. The detection of BR is of great value regarding diagnostic for neonatal jaundice and the metabolic disorder causing hyperbilirubinemia classified as, Dubin-Johnson, Rotor, Gilbert and Crigler-Najar syndrome that originate from ineffective erythropoiesis [3]. BR is transported by human serum albumin in the blood to the liver and then converted into water-soluble form after conjugated with glucuronic acid in the liver. Finally, the conjugated form is excreted into the bile [4]. Hyperbilirubinemia should be considered when bilirubin levels exceed 2×10^{-5} mol L^{-1}. If free unconjugated BR (UCBR-free) is increased in extracellular fluid and bloodstream, it accumulates in the eye, skin and mucose to leads jaundice. UCBR-free is associated with kernicterus and another bilirubin neurotoxicity that is more specificity and sensitivity predictor in preterm and full-term infants with unconjugated hyperbilirubinemia when compared to total serum bilirubin (TSB) [5,6]. The BR is clinically significant because levels of UCBR-free are directly related to neurotoxicity risk of in preterm and full-term infants. Especially relatively low bilirubin levels of 150 μmol/L (9 mg/dL, 17.1 μmol/L = 1 mg/dL bilirubin) in extreme low-birth-weight (ELBW) cause the UCBR-free to cross the blood-brain barrier resulted in mental deficiency and spasticity in an infant. The recent researches have shown that BR plays a fundamental role in metabolism as well. BR has been recognized as a potent antioxidant that serves protective effect against to atherosclerosis and coronary artery disease due to reducing plaque formation by preventing oxidation of lipids and lipoproteins [7]. Furthermore plays an active role in the regeneration of liver cell and has clinical value.

HPLC would appear to be a mostly used technique for studying bilirubin in serum, plasma, and bile [8]. Then, Chang et al. have also reported accurate quantification of bilirubin in pig livers using a reversed-phase column in HPLC method after extraction of samples from pig liver samples by dimethyl sulfoxide and acetonitrile with a recovery of 75.7–84.9% [9]. Zelenka et al. reported a highly sensitive method for tissue unconjugated bilirubin (UCB) determination using a reverse phase column in HPLC. The extraction of UCB was

E-mail address: mecorman@sinop.edu.tr

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performed by chloroform/methanol/hexane at pH 6.2 with 75 ± 5% of recoveries efficiency [10]. In general, these methods could be applied to quantify bilirubin species after extraction procedures. The separation and quantification of bilirubin in biological fluids are problematic because of the instability of the pigments. These methods have required extensive pre-separation prior to chromatography. Conventional pre-separation methods are tedious, require large volumes of organic solvent that lead to remarkable decreasing of bilirubin recovery [11,12]. Difficulties with these requirements, rapid, and easy pre-separation procedures are needed for bilirubin pretreatment from samples. Therefore, many methods have been established and still being continuously developed for bilirubin extraction as solid-phase prior to the quantitative measurement. The selection of affinity ligand plays the critical role in the separation for a specific interaction between target molecule and adsorbents. L-Lysine is an interesting candidate for the bilirubin extraction. There is a specific interaction between the positively charged ε-amino group of a lysine residue and negatively charged propionic acid side chains of BR that provide selective BR removal. Shi et al. used lysine as a model ligand to develop affinity based membrane for the removal of bilirubin [13]. Wang et al. reported amine-functionalized nanofibrous membrane for selective adsorption of bilirubin [14]. Denizli et al. have reported the approach of Alkali Blue 6B and Cibacron Blue F3GA (CB F3GA) immobilized on poly(EGDMA-HEMA) or p(GMA) microbeads as affinity sorbents for bilirubin removal [15–17]. Baydemir et al. have suggested the application of bilirubin imprinted particles for the removal of bilirubin [18]. The novel teflon microporous membranes have been prepared with CB F3GA and albumin as a model ligands for removal of bilirubin from human plasma [19,20]. To overcome the limitation of HPLC for the analysis of bilirubin species in serum, we decided to generate cryogel which was demonsrated to able to use as efficient solid-phase extraction adsorbents for a wide range of biomolecules separation in the various application.

Cryogels have been attempted as a powerful tool for designing the affinity-based chromatographic processes, especially for the removal of biomolecules over the presence methods [21]. The application of cryogel in separation field has provided enhanced mass transfer, short diffusion paths, and low-pressure drop according to traditional methods. Furthermore, some attempts have been demonstrated for the fabrication of composite cryogel with affinity-based ligand [22], molecular imprinting [23], and particle embedding [24] to separate and preconcentrate target molecules. The cryogel-based adsorbents have features of high and controlled large interconnected pores; however, they have low surface area and low affinity for a target molecule that lead to low and non-specific adsorption capacity. Firstly, in order to increase surface area, the composite cryogels were formed by incorporating some functionalized carriers that provide more binding sites exposed pore surface, resulting in improved and higher adsorption capability in contrast to plain cryogel. Secondly, the surface derivatization with reactively functionalized and tentacle ligands, i.e. poly-L-lysine, into cryogel backbone provides an enhanced affinity for the target molecules. By applying these approaches, cryogel has gained superior features such as higher affinity, higher density of reactive groups for specific binding capacibility for target molecules and lower biotoxicity in separation science [25]. On the other hand, one of the most critical properties of cryogel is the excellent biocompatibility which has shown great potential in various biotechnological applications, especially in biomedical and bioseparation field [26].

The aim of this study was to develop cryogel to direct removal of bilirubin from aqueous media, and human serum by poly-L-lysine immobilized cryogel which might be replaced the traditional SPE column with a rapid and easy procedure. First, HEMA cryogel was prepared to construct the biocompatible sorbent by bulk polymerization. Poly-L-lysine was used as an affinity ligand to provide a biocompatible microenvironment for the binding of BR as well as gaining favorable affinity and lower biotoxicity for the BR.

2. Experimental

2.1. Materials

The basic monomer (2-Hydroxyethyl methacrylate, HEMA) and the crosslinker [(*N, N'*-methylene-bis(acrylamide)), MBAAm] were obtained from Sigma-Aldrich (St. Louis, USA). Ammonium persulfate (APS), *N,N,N',N'*-tetramethylene diamine (TEMED), and coupling agent [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC] were provided from Sigma-Aldrich (St. Louis, USA). Target molecule (bilirubin) and affinity ligand, (Poly-L-lysine, PLL) were supplied from Sigma-Aldrich (St. Louis, USA). All of the other chemicals were of analytical grade and supplied from Merck AG (Darmstadt, Germany). All of the water was obtained from a Barnstead ROpure LP reverse osmosis unit (Dubuque, IA, USA) to use in the adsorption experiments.

2.2. Preparation of HEMA based cryogels

The procedure for preparing of cryogel was described as follows: Briefly, 1.3 mL of HEMA and 0.3 g of MBAAm were dissolved in 5 mL of deionized water under nitrogen vacuum for about 5 min. After, APS (20 mg) as an initiator and TEMED (25 μL) as an activator were added to the solution above, the reaction mixture was poured into a plastic syringe (5 mL, id. 0.8 cm) with a closed outlet at the bottom. The polymerization was proceeded at −12 °C for 24 h and then thawed at room temperature. The resulted cryogel was washed with deionized water and then stored at 4 °C until further use.

2.3. Immobilization of PLL

HEMA based cryogel has a large number of reactive —OH groups. Prior to covalent immobilization of PLL ligands to HEMA, —OH groups should be activated by CDI. Therefore, PHEMA cryogel was equilibrated with phosphate buffer (PBS, pH 7.4, 10 mM) before treating with CDI solution (1.0 mg/mL) in PBS buffer at room temperature for 24 h. After overnight treatment of PHEMA cryogel at room temperature, the PLL-immobilized cryogel (PLL-HEMA) was washed extensively with water and stored at 4 °C until further use. The schematic presentation of PLL-PHEMA cryogel is shown in Fig. 1.

2.4. Characterization of the cryogels

The swelling behaviors of the supermacroporous PHEMA and PLL-PHEMA cryogels have been measured as follows: Preweighed dry cryogel samples (m_{drygel}) in the oven at 60 °C were immersed in water until swelled to equilibrium. Then excess water was removed with filter paper, and swollen cryogel samples were weighed (m_{wetgel}). Equilibrium swelling behavior was calculated from the following equation,

$$S = \frac{m_{wetgel} - m_{drygel}}{m_{drygel}} \quad (1)$$

The Fourier transform infrared (FTIR) spectra of cryogels were measured using the FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). FTIR spectrum was recorded for pre-dried cryogel samples (0.1 g) that were mixed with KBr (0.1 g, IR Grade, Merck, Germany). The surface morphology of the dried cryogel samples was examined by scanning electron microscope (SEM). Each of dried samples was coated with gold-palladium alloy (40:60) by SEM sputter coaters, and then they were examined using a JEOL