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Cysteinylhexapeptide Attached Poly(2-Hydroxyethyl Methacrylate) Beads for Cd(II) Removal from Human Plasma in a Packed-Bed Column

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ABSTRACT

Poly(hydroxyethyl methacrylate) (PHEMA) beads (in the size range of 150–200 μm) with good mechanical properties were prepared and crosslinked with ethylene glycol dimethacrylate (EGDMA) to increase their chemical stability. Because of their hydroxyl groups, they can serve as affinity adsorbent and can be employed for medical applications. Cibacron Blue F3GA was succesfully immobilized onto the beads. The maximum dye attachment was 16.5 μmol/g. Then, metallopeptide-ligand cysteinylhexapeptide (CysHP) was incorporated onto these beads and they were used for removal of cadmium ions [Cd(II)] from human plasma in a packed-bed column. The maximum amount of CysHP attached was 3.2 mg/g. Non specific Cd(II) adsorption from human plasma on

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the PHEMA beads was 0.32 mg/g. The adsorption capacity of the beads decreased from 11.8 to 3.7 mg/g with the raise of the flow-rate from 1.0 to 5.0 ml/min. It has been found that the CysHP loading has a great effect on the capacity of beads for adsorbing Cd(II) ions from human plasma. This suggests that these modified beads could be used for removal of Cd(II) ions from human plasma.

Key Words: Cd(II) removal; Poly(2-hydroxyethyl methacrylate); Metal detoxification; Cibacron Blue F3GA; Affinity beads.

INTRODUCTION

Distinct biological roles for most metal ions are now well recognized. Metal ions play roles in catalysis, hormone action, gene and other regulatory functions, structural stabilization of macromolecules, muscle contraction, nerve conduction, and transport. The alkali and alkaline earth metal ions as well as cobalt, copper, iron, manganese, molybdenum, nickel, zinc, and others are known to be essential, many of them in the mechanism of action of specific enzymes. However, the adverse effects of metal ions will manifest when a metal ion level exceeds a certain threshold level. The toxicities of heavy metals may be caused by the following mechanisms: blocking the essential functional groups of biomolecules; displacing essential metal ions from biomolecules; modifying the active conformation of biomolecules, and disrupting the integrity of biomembranes and modifying some other biologically active agents.[1] Toxic metals in food and drinking water are absorbed through the air passages and alimentary canal and they disturb the economy of endogenous metals and biochemical equilibrium. They have an etiological effect on hypertension, cancer, decrease the ventila of the lungs and other lung diseases. Toxic metal ions are the source of degeneration, reduction of pancreatic efficiency and disfunction of kidneys.[2]

No specific treatments for acute or chronic cadmium poisoning are available. However, in addition to supportive therapy and hemodialysis, heavy metal poisoning is often treated with a chelating agent. Different chelating agents that are available commercially for the treatment of cadmium poisoning are British anti-lewisite (BAL) and calcium disodium ethylene diamine tetraacetic acid (EDTA). But there is histopathological evidence for increased toxicity in animals when calcium disodium EDTA is utilized.[3] Recently, one of the most promising techniques for blood detoxification is extracorporeal affinity adsorption.[4] So far, only a few affinity adsorbents were reported for metal detoxification.[5,6]

Recently, we have focused our attention on poly(2-hydroxyethyl methacrylate) (PHEMA) beads carrying different ligands for removal of toxic substances including pathogenic biomolecules and toxic metal ions from human plasma.[7–9] We have also studied cadmium [Cd(II)] removal from human plasma by using thionein carrying PHEMA adsorbents in microsphere and membrane form in batch system.[10,11] In this study, we used metal chelate-forming beads for Cd(II) removal from human plasma in a packed-bed column. Monochlorotriazinyl dye ligand Cibacron Blue F3GA was immobilized onto the PHEMA beads. Later, a metallo-peptide (i.e., cysteinylhexapeptide) was attached to the dye-immobilized beads. Then they were used for cadmium removal from human plasma poisoned with Cd(II) in a continuous column system. PHEMA beads were selected as the basic polymer matrix which carries functional hydroxyl groups for further modification. PHEMA beads have hydrophilic character, good blood-compatibility properties, minimal non specific protein interactions, high chemical and mechanical stabilities for column applications, and resistance toward microbial and enzymatic attack.[12–14]

EXPERIMENTAL

Preparation of Dye-Immobilized Beads

2-Hydroxyethyl methacrylate (HEMA) was purchased from Sigma (St Louis, MO) and purified by passing through active alumina. Ethylene glycol dimethacrylate (EGDMA) supplied from Rohm and Haas (Darmstadt, Germany) was purified by the same procedure. Benzoyl peroxide (BPO) and polyvinylalcohol (PVAL) (M_n: 100,000, 98% hydrolyzed, Aldrich, Rockford IL) were utilized as the initiator and the stabilizer, respectively, and were used without further purification. Toluene (Merck, Darmstadt, Germany) was selected as the diluent and used as received. All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system. The resulting purified water (deionized water) has a specific conductivity of 18 ms. All glassware was soaked overnight in 4 M nitric acid.

PHEMA beads were prepared by the method described previously.[7] The reactor was flushed by bubbling nitrogen and then was sealed. The EGDMA/HEMA ratio, the amounts of BPO, PVAL, and toluene, and the agitation speed were 8 ml/4 ml, 0.06 g, 0.2 g, 12 ml, and 600 rpm,

respectively. Polymerizations were carried out 65°C for 4 h and 90°C for 2 h. In order to remove the diluent (i.e., toluene), unreacted monomers and the physically adsorbed poly(vinyl alcohol) (i.e., stabilizer) molecules, an extensive cleaning procedure was applied which was as follows: the beads were separated from the aqueous phase by filtration of the polymerization mixture through 5 μm filter papers. The beads were first washed with water, and the polymeric aggregates were removed by filtration (sieving). The beads were dispersed in water, and the dispersion was sonicated for 30 min in an ultrasonic bath (200 Watt, Bransonic 200, Danbury, CT, USA). The water phase was removed and the beads were resuspended in ethyl alcohol. This dispersion was sonicated for 1 h. Ethyl alcohol was removed and the beads were transferred into toluene and were kept there by stirring about 30 min. Toluene was removed, then beads were left within ethyl alcohol for about 30 min. Ethyl alcohol was removed, and the beads were washed with distilled water once again, then were filtered and dried in a vacuum oven at 60°C for 48 h. Note that, about 90% of the beads obtained with the recipe and conditions given above were in the size range of 150-200 μm (i.e., the swollen size).

Cibacron Blue F3GA was supplied from Sigma (St. Louis, MO, USA) and used as received. It was covalently attached to the beads as follows. Three grams of PHEMA beads was magnetically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80°C for 4 h with 100 ml of the dye aqueous solution. This was followed by the addition of 4.0 g NaOH in order to stimulate the deposition of the dye on the beads. Under these conditions, a nucleophilic substitution reaction takes place between the group of the Cibacron Blue F3GA having chloride and hydroxyl group of the PHEMA, with the elimination of NaCl, resulting in the coupling of dye to the PHEMA beads. The initial concentration of the dye in the medium was 3.0 mg/ml. After incubation the solution cooled down to the room temperature and then the dye-immobilized beads were filtered, and then washed with distilled water and methanol several times until all the physically adsorbed dye molecules were removed.

The dye content on the beads was determined by using an elemental analysis instrument (Leco, CHNS-932, Chicago, IL, USA) by considering the nitrogen and sulfur stoichiometry. The leakage of the dye from the modified beads was followed by treating the beads with fresh human plasma samples for 24 h at room temperature. Dye released after this treatment was measured in the liquid phase spectrophotometrically at 630 nm.

When not in use, the resulting adsorbents were kept under refrigeration in 0.02% NaN_3 solution for preventing of microbial contamination.

Cysteinylhexapeptide Coupling Studies

Coupling of cysteinylhexapeptide acetate salt (CysHP) (Lys–Cys–Thr–Cys–Cys–Ala, molecular mass: 627.8, Sigma) on the dye-immobilized PHEMA beads was studied in batch system. The pH of the adsorption medium was adjusted to 7.4 by using 0.1 M phosphate buffer (K_2HPO_4–KH_2PO_4). The initial concentration of CysHP in the aqueous phase was changed between 0.1–1.0 mg/ml. In a typical adsorption experiment, CysHP was dissolved in 100 ml of buffer solution containing beads (1.0 g). The adsorption experiments were carried out for 1 h (i.e., equilibrium time) at 25°C at a stirring rate of 250 rpm. The beads were washed after being separated from the solution in order to remove weakly adsorbed CysHP molecules. The CysHP coupling capacity was determined from the mass balance.

Cd(II) Removal from Human Plasma

The-adsorption studies were carried out in a packed-bed column system, in which the adsorption column (with an internal diameter of 0.9 cm and height of 10 cm) equipped with a water jacket for temperature control was used. The column was filled with the unmodified or dye-immobilized PHEMA beads. The blood samples were obtained from the blood-bank of university hospital (4.0% sodium citrate). The plasma freshly separated from the human blood was overloaded with 20 ml of cadmium solution containing different amounts of Cd(II) to obtain different initial Cd(II) concentration. In a typical continuous column system, the plasma was recirculated through the column containing polymeric beads for 2 h. The amounts of Cd(II) removed (i.e, final plasma Cd(II) concentration) were determined by using an atomic absorption spectrophotometer (Unicam AA 939) equipped with a graphite furnace (Unicam GF-90). The cadmium hollow cathode lamp was operated at 10 mA and the 288.8 nm absorption line was employed in conjunction with a monochromator band-pass of 0.7 nm. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. For each sample, the mean of 10 replicate measurements was recorded. The amount of Cd(II) adsorption per unit mass of the modified beads was calculated by concentration difference.

Adsorption of plasma proteins (e.g., albumin, flbrinogen, and gamma-globulins) was also studied. PHEMA/CysHP beads were contacted with a human plasma containing albumin (40.7 mg/ml), fibrinogen (2.2 mg/ml), and gamma-globulins (17.3 mg/ml) in a column system at room temperature for 2 h. The flow-rate was kept constant at 1.0 ml/min. Total protein concentration

was measured by using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm, which was based on Biuret reaction. Chronometric determination of fibrinogen according to the Clauss method on plasma was performed by using Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-I'Etoile, France). Human serum albumin concentration was determined by using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 229241), which was based on bromocresol green dye method. Gamma globulin concentration was determined from the difference.

RESULTS AND DISCUSSION

Cibacron Blue F3GA was used as the dye-ligand for specific binding of CysHP molecules and Cd(II) ions. Dye molecules were covalently immobilized to the beads. It has been shown that ether bonds were formed between the reactive triazine ring of the dye and the hydroxyl groups of the adsorbent. Elemental analysis of the dye-immobilized PHEMA beads was performed, and the attachment of the dye was found to be 16.5 μmol/g from the nitrogen stoichiometry. After the dye immobilization, the size of the swollen beads did not change, but the color became dark blue, which is a clear indication of the attachment of the dye molecules in the structure of the beads. Dye leakage was also investigated in human plasma. Dye leakage was not observed from any of the dye-immobilized beads, even over a long period of time (more than 2 months). The visual observations (the color of the beads), elemental analysis, and FTIR spectra were already published in our previous papers.[7,10]

Cysteinylhexapeptide (CysHP) Coupling

Metallothioneins are a group of nonenzymatic (6–7 kilodalton) low molecular mass proteins with 61–68 of amino acid residues including 20 cysteins, bound to certain divalent ions such as Zn(II), Cd(II), Hg(II), Bi(II), Sn(II), Ni(II), Rb(II), or Tc(II) with high affinity.[15] The binding is also observed with univalent ions such as Cu(I), Ag(I), and Au(I). Metallothionein and its analogues are widely distributed among organisms, from bacteria and fungi to plants and mammals. Metalloproteins play an important role in the metabolism and kinetics of metals including transport of metals, removal of metals, protection from metal toxicity, free radical scavenger, storage of

metals, metabolism of essential metal ions, immune response, and genotoxicity and carcinogenecity. CysHP is a small metallo-peptide (molecular mass: 627.8) composed of six amino acids, three of them being cysteine residues. The thiol groups in the CysHP structure permit it to form metal clusters, including Cd(II), Zn(II), Hg(II), Cu(I), and some other bivalent and divalent metal ions. This makes them attractive ligands, and metalloproteins are among the most selective chelators known for metal ions for biomedical applications.[16]

Figure 1 shows coupling of CysHP molecules onto the unmodified and PHEMA/Cibacron Blue F3GA beads. Note that one of the main requirements in biochromatography is the specificity of the adsorbent. The nonspecific interaction between the support, which is the PHEMA beads in the present case, and the molecules to be adsorbed, which are the CysHP molecules here, should be minimum in order to consider the interaction as specific. As seen in this figure, negligible amount of CysHP was adsorbed nonspecifically on the plain PHEMA beads, which was 0.19 mg/g, while dye-immobilization significantly increased the CysHP coupling capacity of the beads (up to 3.2 mg/g). This increase in the CysHP coupling capacity may have resulted from cooperative effect of different interaction mechanisms such as hydrophobic, electrostatic, and hydrogen bonding caused by the acidic

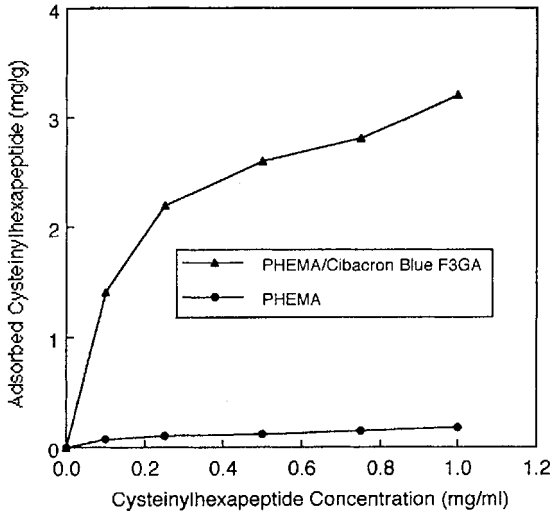

Figure 1. Effect of CysHP initial concentration on CysHP coupling: Dye loading: 16.5 μmol/g; and pH: 7.4; T:25 °C.

groups and aromatic structures on the Cibacron Blue F3GA and by groups on the side chains of amino acids on the CysHP molecules. It should be mentioned that Cibacron Blue F3GA is not very hydrophobic overall, but it has planar aromatic surfaces that prefer to interact with hydrophobic groups in CysHP structure.

In order to verify the dye attachment conditions on CysHP coupling, PHEMA beads exposed to dye-attachment conditions without dye were also used for CysHP coupling. The CysHP coupling observed was in the same amount (0.19 mg/g) as that detected for plain beads. It can be said that the cross-linked PHEMA beads are resistant to the harsh alkaline conditions used for dye-attachment. No carboxylic moieties are likely to be formed by hydrolysis of the ester bond of HEMA molecules in the polymer chain and hence these undesired moities do not contribute to CysHP coupling. Therefore, it can be concluded that CysHP molecules were coupling on the beads via immobilized dye molecules.

Cd(II) Removal from Human Plasma

Effects of flow-rate on adsorption: In this group of experiments, the flow-rate of the plasma was changed between 1.0–5.0 ml/min, and other parameters were kept constant as given in the figure legend. The adsorption capacity (the amount of Cd(II) adsorbed per gram of the beads in 120 min) at different flow-rates are given in Fig. 2. The adsorption capacity decreased significantly from 11.8 to 3.7 mg Cd(II)/g polymer with the increase of the flow-rate from 1.0 ml/min to 5.0 ml/min.

This decrease may be explained as follows: the residence time in the column decreases with increasing flow-rate, which does not give enough time for the Cd(II) ions to contact with the chelating cysteine groups on the adsorbent. In addition, the increase in the flow-rate may cause channeling in the adsorbent bed, which results in a decrease in the effective use of the adsorbent beads in the column. Therefore, low adsorption capacities are observed at high flow-rates. Note that at flow-rates lower than 1.0 ml/min, we faced some technical problems in our experimental set-up, such as difficulties in sampling, therefore, we performed all other adsorption tests at a flow-rate of 1.0 ml/min.

Effects of Cd(II) initial concentration: Figure 3 shows the adsorption of Cd(II) ions onto the plain PHEMA and PHEMA/CysHP beads. As seen in this figure, Cd(II) adsorption was increased with an increase in the initial concentration of Cd(II) ions within the aqueous phase. CysHP coupling resulted in significant increases in the Cd(II) adsorption capacity

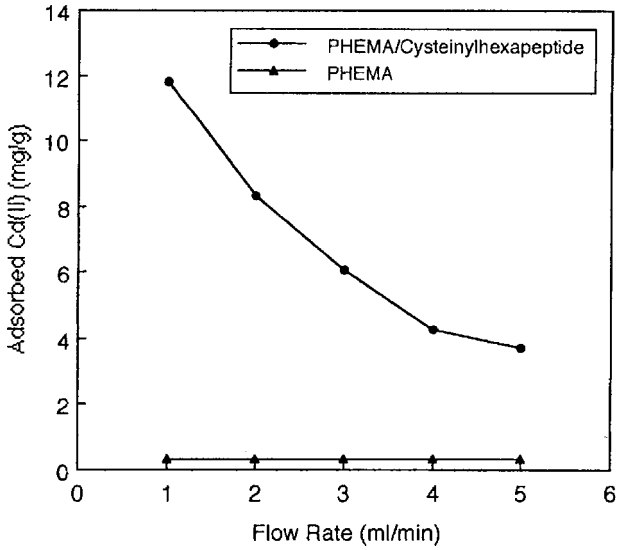

Figure 2. Cd(II) adsorption at different flow-rates: Dye loading: 16.5 μmol/g; CysHP loading: 3.2 mg/g; Cd(II) initial concentration; 50 mg/L, T:25°C.

(up to 11.8 mg/g polymer). Although different mechanisms of metal affinity interactions with proteins and peptides/polypeptides have been proposed,[17] the macromolecular recognition of proteins and peptides with metal ions remains unclear. In one proposed mechanism, the formation of a coordination complex structure between protein/peptide and metal ion is considered to be the major binding mode. However, more than one type of interaction mechanism is operational.[18] The major functional groups on protein contributing toward the interaction with metal ion consist of the histidine residue and the sulphur atom of the sulphidryl group of the free cysteine residue. Cd(II) ions are tetrahedrally co-ordinated with cysteine groups. While maintaining a free cysteine residue in a natural protein is rare,[19] the exposed histidine residue is the dominant binding site in protein-metal ion adsorption.[20] In the present case, CysHP molecules do not carry histidine but contain three cysteine amino acid groups, and these molecules are dominant in the Cd(II) adsorption studies.

In order to show PHEMA/CysHP specificity, competitive protein adsorption was also studied. Interesting results were obtained in these studies. Adsorption capacities were obtained as 4.96 mg/g for albumin, 2.75 mg/g for flbrinogen, and 1.36 mg/g for γ-globulin. The total protein adsorption was

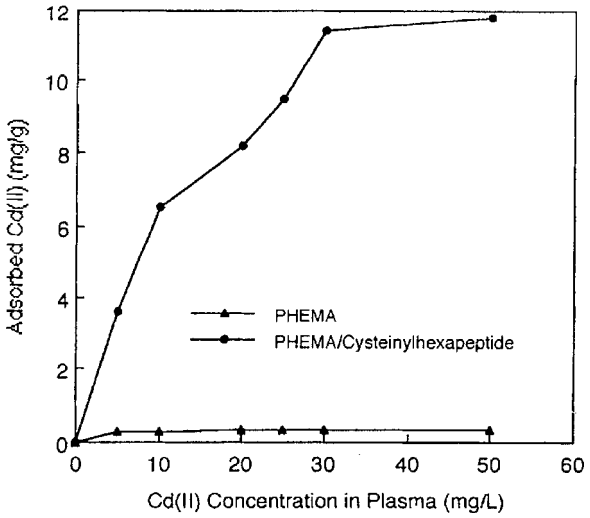

Figure 3. Effect of Cd(II) initial concentration on Cd(II) removal from human plasma: Dye loading: 16.5 μmol/g; CysHP loading: 3.2 mg/g; flow-rate: 1.0 ml/min; T:25°C.

determined as 10.1 mg/g. It is worth noting that adsorption of plasma proteins (i.e., albumin, fibrinogen, and gamma-globulin) on the PHEMA/CysHP beads is negligible. It may be resulted that this low adsorption of albumin, fibrinogen, and gamma-globulins is due to the high specificity of attached CysHP molecules. This result shows that Cd(II) is the only major component from plasma that binds PHEMA/CysHP beads.

Effects of CysHP Loading

The ligand content is an important parameter in the bioaffinity chromatography. Figure 4 provides the relationship between the Cd(II) removal capacity and the CysHP content in PHEMA beads. It has been found that CysHP loading has a great effect in the capacity of beads for adsorbing Cd(II) ions from human plasma. When the number of CysHP molecules on the beads increased (means at higher CysHP loadings) the amount of Cd(II) adsorbed onto the metallopeptide-incorporated beads increased rapidly, as expected due to the number of available active points on the CysHP ligand for interaction with Cd(II) ions. The maximum Cd(II) adsorption was 11.8 mg Cd(II)/g.

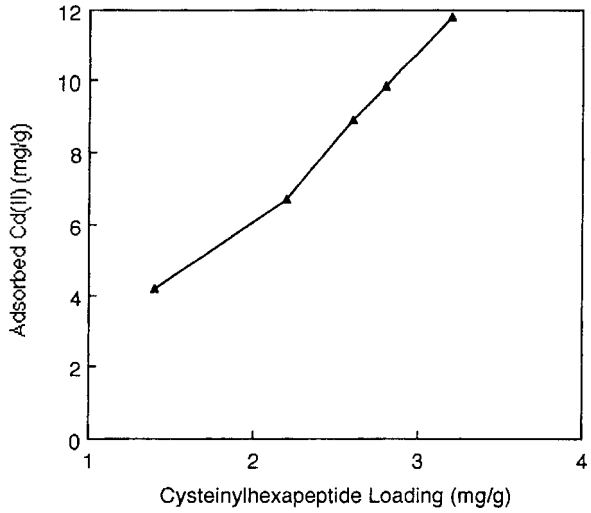

Figure 4. Effects of CysHP loading on Cd(II) Adsorption: Cibacron Blue F3GA loading: 16.5 μmol/g; flow-rate: 1.0 ml/min; T:25°C.

CONCLUSIONS

The experimental results obtained proved the existence of good adsorptive properties of CysHP-attached PHEMA beads. The packed-bed column-based adsorption technique has several potential advantages over the batch system. The packed-bed columns require high flow-rates with a much lower operating pressure than a batch system. In this method, the molecule to be removed can be directly transported by convection to the ligand immobilized on the inner surface of the beads, and higher throughput and faster processing times onto the affinity beads can be achieved. PHEMA beads were prepared by radical suspension polymerization of HEMA. Cibacron Blue F3GA was then successfully immobilized onto these beads (16.5 μmol/g). Then, CysHP was attached onto the Cibacron Blue F3GA-carrying beads. The loading amount of CysHP attached was 3.2 mg/g. These hydrophilic PHEMA beads carrying CysHP were used in the removal of Cd(II) from human plasma. The maximum amount of Cd(II) ions removed from human plasma by CysHP-attached beads was 11.8 mg/g. These results are encouraging and suggest that further studies should be done to develop CysHP-carrying PHEMA adsorbents.

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