Separation and Purification Technology 115 (2013) 20–26

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

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

# A metal ion charged mixed matrix membrane for selective adsorption of hemoglobin

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ARTICLE INFO

*Article history:*
Received 12 March 2013
Received in revised form 17 April 2013
Accepted 19 April 2013
Available online 1 May 2013

*Keywords:*
Mixed matrix membrane
Porous beads
Iminodiacetic acid–copper
Bovine hemoglobin
Bovine serum albumin

ABSTRACT

In this work, we developed a mixed matrix membrane by incorporating 20–40 μm size iminodiacetic acid modified immobeads within porous Ethylene vinyl alcohol (EVAL) polymer matrix. The MMM were charged with copper ions for selective adsorption of bovine hemoglobin in presence of bovine serum albumin. EVAL membrane without immobead and immobead alone showed low non-specific adsorption towards bovine hemoglobin and bovine serum albumin. Use of buffers with pH $\leqslant$6.8 i.e. pI of bovine hemoglobin resulted in high bovine hemoglobin adsorption. The developed mixed matrix membrane has a maximum static adsorption capacity of 219.5 mg bovine hemoglobin per g bead and the adsorption isotherm curve is of a Langmuir type. Under similar flow rates, the mixed matrix membrane showed significant low pressure drop compared to a packed bed column containing equal amount of beads. Maximum and faster (6 h) adsorption of bovine hemoglobin can be achieved through dynamic mode only using mixed matrix membrane and not by packed bed column and static mode of mixed matrix membrane. The mixed matrix membrane has three times selective adsorption of bovine hemoglobin than bovine serum albumin, from a binary mixture containing equivalent amounts of both proteins.



## 1. Introduction

Selective adsorption of proteins with similar size for e.g. Hemoglobin (Hb) and Albumin is of particular interest in protein research and biotechnology. Hb, the oxygen transporter biomolecule embedded with iron in its interior structure contains 2α and 2β identical globin chains, is one of those high abundant proteins that is present in red blood cells. In hereditary diseases such as hemoglobinopathies, sickle cell diseases and thalassaemia rupture of the red blood cell membrane i.e. intravascular hemolysis results in plasma with high concentration of Hb [1–3]. This excess of Hb in there makes the detection of low abundant potential biomarker proteins difficult.

In the literature, one finds several studies to selectively deplete high abundant Hb using techniques like e.g. molecular recognition, ion exchange chromatography, immobilized metal affinity chromatography (IMAC) in which various materials like polymer beads or gels, membranes, and nanoparticles are widely employed [4–17]. However, the use of small beads in columns and membranes have certain disadvantages such as high pressure drop and low mass transfer of packed bed columns, and low surface area and low adsorption capacities of membranes. In this work, we report for the first time the development of a mixed matrix membrane (MMM) charged with copper ($Cu^{2+}$) ion for selective adsorption of BHb over similar sized protein i.e. bovine serum albumin (BSA). The MMM concept combines the advantages of both conventional packed bed columns (high surface area and high adsorption capacity) and membranes (low pressure drop, high mass transfer and easy scale-up) [18–22].

The membranes were prepared by incorporating 20–40 μm size epoxy group immobeads (Ib) within porous Ethylene vinyl alcohol (EVAL) polymer matrix. The Ib were functionalized with iminodiacetic acid (IDA)–$Cu^{2+}$ complex for protein adsorption. The prepared MMM was evaluated for its non-specific and specific adsorption of two proteins (BHb and BSA), influence of pH and salt concentration on BHb adsorption, and its maximum adsorption capacity. Subsequently, their performance was compared with packed bed columns in terms of pressure drop and adsorption kinetics (static vs dynamic mode). Finally, the membrane was successfully employed for selective adsorption of BHb from a binary mixture containing equivalent amounts of BHb and BSA.

## 2. Materials and methods

### 2.1. Materials

Iminodiacetic acid (IDA), EVAL (a random copolymer of ethylene and vinyl alcohol) with an average ethylene content of

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http://dx.doi.org/10.1016/j.seppur.2013.04.036

44 mol%, 1-octanol, dimethyl sulfoxide, monopotassium phosphate, dipotassium phosphate, bovine hemoglobin (BHb), bovine serum albumin (BSA), sodium thiosulfate, sodium chloride, hydrochloric acid, copper (II) sulfate pentahydrate and ethylene diamine tetraacetic acid (EDTA) were all purchased from Sigma Aldrich, The Netherlands. Immobeads (Ib) (150–300 μm; macroporous acrylic polymer) were purchased from Chiralvision, The Netherlands. SPARTAN 30/0.2 RC membrane filter unit was purchased from VWR International, Netherlands. Fresh MilliQ water was used in preparation of all solutions. All experiments in this study were performed in triplicates.

### 2.2. Preparation and characterization of $Cu^{2+}$ ion charged Immobeads (CIb)

Ib of 150–300 μm size were grinded and fractionated to 20–40 μm in size using a Fritsch analysette shaker stacked with 20 μm and 40 μm sieves. The scheme for coupling of IDA and copper ions is shown in Fig. 1.

#### 2.2.1. IDA immobilization on Ib

Preparation of IDA immobilized Ib was carried out according to an earlier reported method on poly(glycidyl methacrylate) beads [12]. In short, 20–40 μm size Ib (20 g) were suspended in disodium salt of iminodiacetic acid (IDA, 11 g) solution (pH 10) at 60 °C for 12 h. Then the beads were washed extensively with MilliQ water and dried in vacuum for 2 days before further analysis and modification. The amount of IDA immobilized was tested using sodium thiosulfate test. The protocol is as follows [23]: Either Ib without IDA (300 mg) or IDA immobilized Ib (300 mg) were suspended in 15 mL 1.3 M $Na_2S_2O_3$ solution (pH 7.2) for 2 h. Then the amount of hydroxyl ($OH^-$) groups released in the solution were determined by back titration using 0.1 M hydrochloric acid. The volume of HCl added relates to the number of hydroxyl groups (in moles) present in the solution, which in turn relates to the exact amount of epoxy groups (in moles) per g bead. See Table S1 supplementary information for more practical details.

#### 2.2.2. $Cu^{2+}$ ion charged Ib (CIb)

The dried IDA immobilized Ib (10 g) were suspended in a 250 mL aqueous copper (II) sulfate solution (2.5 mg/mL; pH 5–5.5) for 24 h on a shaker at room temperature. The beads charged with $Cu^{2+}$ ions (CIb) were washed extensively with MilliQ water and dried in vacuum for 2 days. The amount of $Cu^{2+}$ ions charged on the beads was determined according to a previous protocol [24]: 1 mL of 50 mM EDTA solution was added to 1 mL of copper solution (before and after reaction) and the absorbance of the $Cu^{2+}$–EDTA complex was measured directly at a wavelength of 740 nm (the visible region) using an UV–VIS spectrophotometer (Varian Cary 350).

### 2.3. Preparation and characterization of flat sheet MMM

Membrane containing IDA immobilized Ib were prepared based on earlier protocol [18]. A polymer dope solution containing EVAL (10 g), 1-octanol (10 g) and DMSO (80 g) was prepared at 45 °C for 24 h. To this solution, 20–40 μm size IDA immobilized Ib (10 g) was added while stirring the solution vigorously. The vigorous stirring of the dope solution continued for further 24 h to have a homogeneous distribution of the beads. This dope solution was cast on a glass plate using 500 μm casting knife and immediately immersed in a water coagulation bath at 45 °C to result in the desired membrane. The formed membranes were washed continuously with MilliQ water for more than 72 h to remove any remaining solvent traces. Charging of $Cu^{2+}$ ions on the membranes was carried out according to the protocol mentioned in Section 2.2.2. EVAL membranes without Ib were also prepared to study their non-specific adsorption of BHb and BSA.

#### 2.3.1. Scanning electron microscope (SEM)

The surface morphology and distribution of IDA immobilized Ib in the membrane were examined with a Jeol JSM-5600 LV scanning electron microscope. Cross-sectional samples were prepared by freeze drying wet membrane pieces in liquid nitrogen. All membrane samples were gold coated using Balzers Union SCD 040 coater before analysis with SEM.

#### 2.3.2. Packed bed column vs MMM

The pressure drop of packed bed column and MMM was investigated by pumping a buffer solution (20 mM PBS buffer solution (pH 6.8, 0.5 M NaCl)) at various flow rates (0.5–3 mL/min). The packed bed column contained 148 mg CIb (20–40 μm) packed in a Omnifit column (6.6 mm × 11 mm; effective column length 12 mm). In case of MMM, 8 membranes (296 mg i.e. 148 mg CIb) were stacked together in a stainless steel membrane holder with an effective diameter of 23 mm per membrane.

### 2.4. Determination of surface exposed histidine molecules of BHb and BSA

The complexation of copper with BHb and BSA is achieved via the histidine groups present on their surface. Therefore it is important to know the exact total number of surface exposed histidine groups on both BHb and BSA. These were estimated using the three-dimensional crystal structures of BHb (PDB ID: 2qsp) [25] and BSA (PDB ID: 3v03) from the Protein Data Bank (PDB) (www.pdb.org). More information about the analysis can be found in Supplementary Information.

### 2.5. Non-specific and specific protein adsorption

To determine the non-specific and specific adsorption of BHb and BSA, experiments were executed by suspending Ib (20 mg), EVAL membrane (20 mg), CIb (20 mg), and MMM (40 mg) in 20 mM PBS buffer (pH 6.8, 0.5 M NaCl) solutions containing either BHb or BSA (0.2 mg/mL). In case of BHb adsorption studies, 25 mL sample volume was used and for BSA adsorption studies 10 mL sample volume was used. The samples were left on a shaker for 24 h at room temperature and afterwards the solutions were filtered using SPARTAN membrane filters (0.2 μm pore size) and

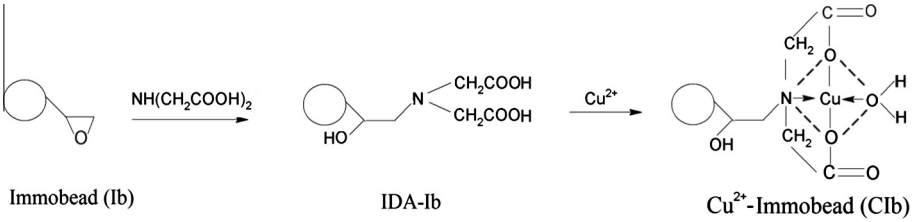


**Fig. 1.** Schematic representation of surface modification of immobead (Ib) with IDA and charging with copper ions.