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Emulsion templated open porous membranes for protein purification

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ABSTRACT

Approximately 25 cm × 25 cm large sheets of crosslinked highly porous poly(glycidyl methacrylate-co-ethyleneglycol dimethacrylate-co-ethylhexyl methacrylate) membranes with an average thicknesses between 285 and 565 μm were prepared by casting a high internal phase emulsion (HIPE) containing monomers onto glass substrates and subsequent polymerisation. Open cellular porous polyHIPE type membranes were obtained with large pores (cavity) sizes between 3 and 10 μm while interconnecting pores were between 1 and 3 μm. The percentage of ethylhexyl acrylate and ethyleneglycol dimethacrylate influenced the flexibility and morphology of the resulting membranes. Porous membranes were chemically modified with diethylamine to yield functionalised supports for ion exchange chromatography. Cylindrical housings were used for positioning of the membranes and allowing flow of the mobile phase. Pulse experiments were used to study the flow characteristics and a homogeneous flow through the entire area of the membrane was found. Bovine serum albumin was purified by a 8 ml column containing functional membrane in modular shape; dynamic binding capacity was measured to be as high as 45 mg/ml.

1. Introduction

Membranes can offer an attractive solution for downstream technology including separation and purification of proteins. A large amount of research has been invested into membranes for separation via diffusion mechanism in the last decades. On the other hand open porous polymeric membranes are produced on the commercial scale mostly for filtration purposes [1]. In recent years there were several reports on the applications where functionalized membranes were used for purification of various macromolecules, e.g., monoclonal antibodies [2], plasmid DNA [3] or viruses [4] and especially for DNA and viral clearance [5]. All membranes are very thin, typically few tens of μm and to obtain required thickness they are stacked or wrapped in several layers. An alternative convective media are monoliths [6] which are to some extend similar to the membranes regarding their microscopic structure but can be cast in various geometries and thicknesses [7–9]. While this results minimal dispersion inside the bed [10] their dimensions are fixed and because of their rigid structure tailor made dimensions are difficult to obtain. Because of that it seems advantageous to prepare membranes providing enough elasticity for wrapping and with thicknesses of several hundred μm or even a millimeter.

Open porosity of membranes can be achieved through various techniques, like using dissolvable particles, porogens or various templating methods [11,12]. Common polymer based materials used so far for the membrane preparation include cellulose, polysulphone, polyamide, poly(hydroxyethyl methacryate), chitosan and also various composite materials [11]. Glycidyl methacrylate (GMA) is one of the monomers of choice for the preparation of polymeric chromatographic media because poly(glycidyl methacrylate) contains free epoxy groups which are easily functionalized [13]. In the form of bulk polymerized monolithic columns the material has been commercialised (CIM columns, BIA Separations, Ljubljana, Slovenia).

Apart from mentioned techiques for pore generation, open porous morphology of the polymeric material can be also achieved via an emulsion templating procedure. This approach utilises the internal phase of an emulsion to serve as a porogenic phase while the continuous phase contains monomers. If the volume ratio of the internal phase exceeds 74% (volume of densely packed equally sized droplets), the emulsion is termed HIPE (high internal phase emulsion) and the monolithic polymer, derived by the polymerisation of the continuous phase, a polyHIPE [14]. Various applications

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Table 1
Preparation data for polyHIPE membranes with thickness and cavity sizes of the membranes.

Membrane	mol%			Thickness (μm)	Average cavity (μm)	Surface area[b] (m^2/g)	
	GMA	EGDMA	EHA			BET	Hg
M0[a]	98	2	/				
M1	98	2	/	476	4.5	5.00	–
M2	95	5	/	446	4.6	6.67	13.10
M3	90	10	/	487	–	8.33	–
M4	90	5	5	495	7.31	3.45	11.27
M5	85	5	10	503	5.43	2.29	–
M6	80	5	15	409	8.12	2.75	8.54
M7	75	5	20	567	9.60	2.4	–
M8	65	5	30	421	3.28	–	8.89
M9	80	10	10	479	8.39	3.86	–

[a] Cured at 60 °C using potassium persulfate as initiator.
[b] BET– determined by nitrogen adsorption using BET model; Hg– determined using mercury porosimetry.

of this highly porous material are known [15–20]. Apart from well established water in oil type polyHIPEs, recently oil in water type of HIPEs have been used to synthesize more hydrophilic polyHIPEs [21–24]. Also, supercritical CO_2 has been used as the internal phase [25–27]. PolyHIPE materials based on GMA have already been prepared and utilised as separation media [28,29]. There are some advantages of polyHIPE type GMA based porous monoliths, most notably the possibility of preparing a material with a very high porosity and less problems regarding heat transfer during the polymerisation. On the other hand, there are only a few reports of the preparation and application of polyHIPE membranes. Ruckenstein and co-workers first described the application of HIPEs for the preparation of thin films of polymeric materials used for permselective separation of mixtures [30–32]. Recently, Cameron et al. described a sensor incorporating a VBC based polyHIPE membrane [33]. However, many problems regarding the homogeniety and open surface are mentioned. The problem was partly attributed to a stamping method is used to obtain thin films. We have shown that a casting method can be used to prepare styrene based polyHIPE membranes [34]. This produces surface open structure of polyHIPE morphology in the case of styrene, vinylbenzyl chloride and also poly(dicyclo pentadiene) membranes prepared by ring opening metathesis polymerisation of HIPE [35].

In this report, we describe a preparation of GMA based open cellular porous polyHIPE membranes with controlled thickness, porosity and morphology via a casting method. Influence of comonomers, namely ethyleneglycol dimethacrylate and ethylhexyl acrylate, on the mechanical properties (vis.) and morphology is also reported and the application of novel supports for the purification of protein is described.

2. Experimental

2.1. Materials

Glycidyl methacrylate (GMA; Aldrich), ethylhexyl acrylate (EHA; Aldrich) and ethylene glycol dimethacrylate (EGDMA; Aldrich) were passed through the layer of Al_2O_3 to remove the inhibitors. Potassium persulfate (KPS, Fluka), calcium chloride hexahydrate (Merck, Darmstadt, Germany), the surfactant Synperonic PEL 121 (ICI Chemical, London, UK), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, Fluka), ethanol(Fluka), acetone (Aldrich), bovine serum albumin (BSA; Fluka, Buchs, Switzerland), NaCl (Aldrich) and diethylamine (DEA; Fluka, Buchs, Switzerland) were used as received.

2.2. Preparation of high internal phase emulsion

HIPEs were prepared as follows: GMA, EGDMA, EHA and Synperonic PEL 121 (20 wt% of oil phase) were placed in a three-necked 250 ml round-bottomed flask, fitted with an overhead stirrer (glass rod fitted with a D-shaped PTFE paddle), and the mixture was purged with nitrogen gas for 15 min. The aqueous phase was prepared separately by dissolving 0.2 g of ammonium persulfate and 1.79 g of calcium chloride hexahydrate in deionized water, and the resulting solution was purged with nitrogen for 15 min (preparation data for polymer supports are in Table 1). The organic solution was stirred at ca. 300 rpm, and the appropriate amount of aqueous phase (75% according to total volume of emulsion) was added dropwise under constant stirring. After complete addition of the aqueous phase, stirring was continued for 1 h to produce a homogeneous emulsion. TEMED was added to the emulsion a few seconds before the end of mixing.

2.3. PolyHIPE membrane preparation

Emulsions were cast on the glass support using doctor blades with dimensioned slits between 300 and 1000 μm. Emulsions were covered with a glass plate, polymerised at room temperature (24 h) and washed with water and ethanol.

2.4. Membrane thicknesses

The membrane thickness was measured using SEM and Minimer HD1 (Seltron, Slovenia).

2.5. Membrane modifications

To introduce weak anion-exchange DEA (diethylamine) groups, membrane was immersed into 50% solution of diethylamine in ethanol for 24 h at 40 °C. After modification was completed, the membrane was extensively washed with distilled water to remove residual reagent.

2.6. Structural characterisation of the monoliths

FT-IR spectra were recorded on a Perkin-Elmer FT-IR 1650 (Fremont, USA) spectrometer and the morphologies of the materials were investigated using a FEI XL30 ESEM. Mercury intrusion porosimetry analysis was performed using a Pascal 440 (Thermo-Quest Italia, Rodano, Italy), with the highest applied pressure of 100 MPa.

2.7. Membrane module description

Membrane (M4; thickness 495 μm) was wrapped around stainless steel frit to provide additional mechanical stability to the membrane. The membrane itself was rather sticky therefore no additional fixation was needed (see Fig. 4). Total

volume of the membrane module was 8 ml. Geometrically wrapped membrane was similar to 8 ml CIM monolithic tube (www.biaseparations.com). Membrane was placed in appropriate 8 ml CIM stainless steel housing, fixed from the top and bottom between two pistons to avoid by-passing thus forcing the mobile phase to cross the membrane in a radial direction. The entire set-up was connected to the HPLC system. Details of the construction are described elsewhere [9]. Void volume of membrane column, taking into account the membrane pores, was 7 ml.

3. HPLC analysis

3.1. Hardware

HPLC experiments were carried out on a preparative gradient HPLC system comprising of two preparative K-1800 pumps, an injection valve with a 1 ml SS sample loop, a preparative UV K-2500 detector set to 280 nm, preparative mixing chamber, all connected by 1.5 mm I.D. PEEK (polyetheretherketone) capillary tubes and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany).

3.2. Pulse response experiment

Pulse response was measured by injecting 1000 μl of 16.7% acetone solution in water (v/v) at the flow rate of 40 ml/min. Absorbance was measured at 280 nm.

3.3. Dynamic binding capacity measurements and calculation of surface coverage

The bovine serum albumin, BSA (Fluka, Buchs, Switzerland) was dissolved in a binding buffer (20 mM Tris–HCl, pH 7.4) to the concentration of 1 mg/ml. The solution was pumped through the monolithic column at a flow rate of 20 ml/min and the absorbency, set at 280 nm, of the outlet was measured. The capacity of the monolithic column was calculated on 50% of the final absorbance value of the break-through curve. The monolithic column was regenerated with 240 ml of 20 mM Tris–HCl, pH 7.4 containing 1 M NaCl.

To calculate the surface covered by BSA radius of gyration of 2.8 nm was used [36]. Dry membrane density was found to be 0.20 g/ml and BET surface area of 3.45 m^2/g was used in the calculation.

4. Results and discussion

To prepare thin films of GMA based polyHIPEs, simple casting of the emulsion (a part of the technique frequently used for the preparation of asymmetric membranes by phase separation) [37], was used. If the HIPE is not very viscous, a doctor blade can be used to spread the emulsion over the supporting substrate. Several custom made doctor blades were used, producing cast thicknesses of 500, 700 and 1000 μm, respectively (Fig. 1). A blade fitting piston was manufactured to facilitate the casting in the case of more viscous HIPEs. Cameron and co-workers described a stamping method for producing the sheets of polyHIPE [33]. Our approach is an alternative and we believe it has less impact on the structure of the polymer at the interface of emulsion-supporting substrate. Firstly, an emulsion with a composition optimised for monolithic GMA polyHIPEs, was used [28]. High internal phase emulsion was spread on the glass substrate using the doctor blade, covered with thin glass plate and polymerized at 60 °C. At elevated temperature stability of the emulsion is reduced, the individual droplets begin to coalesce and emulsion tends to collapse. The result is an inhomogeneous membrane, and relatively large pores are visible, which in some cases are extended over the entire thickness of the membrane (cf. Fig. 2, M0). To overcome the reduced stability of the emulsion at elevated temperature, polymerisation was performed at room temperature using a redox initiator system (potassium persulphate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine). Membrane thus obtained was significantly more homogeneous (cf. Fig. 2; M4 and M7) and the morphology resembles a characteristic polyHIPE structure with larger cavities and smaller interconnecting pores. PolyHIPE membranes with various compositions of precursor emulsions, namely monomer ratio (GMA, EHA), cross-linking degree and the amount of the internal phase were prepared (cf. Table 1). PolyHIPE membranes were characterised using FT-IR spectroscopy to confirm their chemical structure. Signal at around 1725 cm^{-1} indicates the presence of acrylate carbonyl group and the signal at 900 cm^{-1} signifies the epoxy group (Fig. 3).

Fig. 1. The doctor blade, used for emulsion casting.

Produced sheets of membrane with the molar ratio of GMA/EGDMA 9/1 and the nominal porosity of 75% (M3) were fairly rigid and could only be bent to approx. 40° in both directions. As the intended application required a much more flexible material, optimisation in terms of mechanical properties was needed. As the portion of cross-linker (EGDMA) was lowered, from 10% (M3) to 5% (M2) or 2% (M1), an improvement in flexibility was obvious, and the membrane could be bent to approximately 120°. However, the drawback of low cross-linking degree is excessive swelling of the membrane in the case of chemical modification and therefore the membrane is more prone to tearing. By adjusting only the cross-linking degree, the appropriate mechanical properties could therefore not be achieved.

It is known that the addition of some acrylates into the polymer matrix results in improved mechanical properties of the material with regards to flexibility [38]. This can be attributed to the decrease of glass transition temperature due to the increase of polymer free volume. Ethylhexyl acrylate was thus added to the oil phase of the emulsion. No significant decrease of emulsion stability was observed (up to 30% of EHA in the oil phase) and the resulting membranes (M4–M7) were much more flexible while still keeping the cross-linking degree at 5%. The membranes could be wrapped around a frit (cf. Fig. 4) numerous times, completely reversible. With the addition of ethylhexyl acrylate even the thickest membranes were flexible enough for wrapping around a frit with a diameter of 6 mm.

The addition of EHA did not compromise the emulsion stability visually, although an effect on the morphology of the resulting membrane was found. The higher the proportion of EHA, the more closed structure, in terms of reducing interconnecting pore sizes, was obtained. By incorporation of 30% of EHA (M8) interconnecting

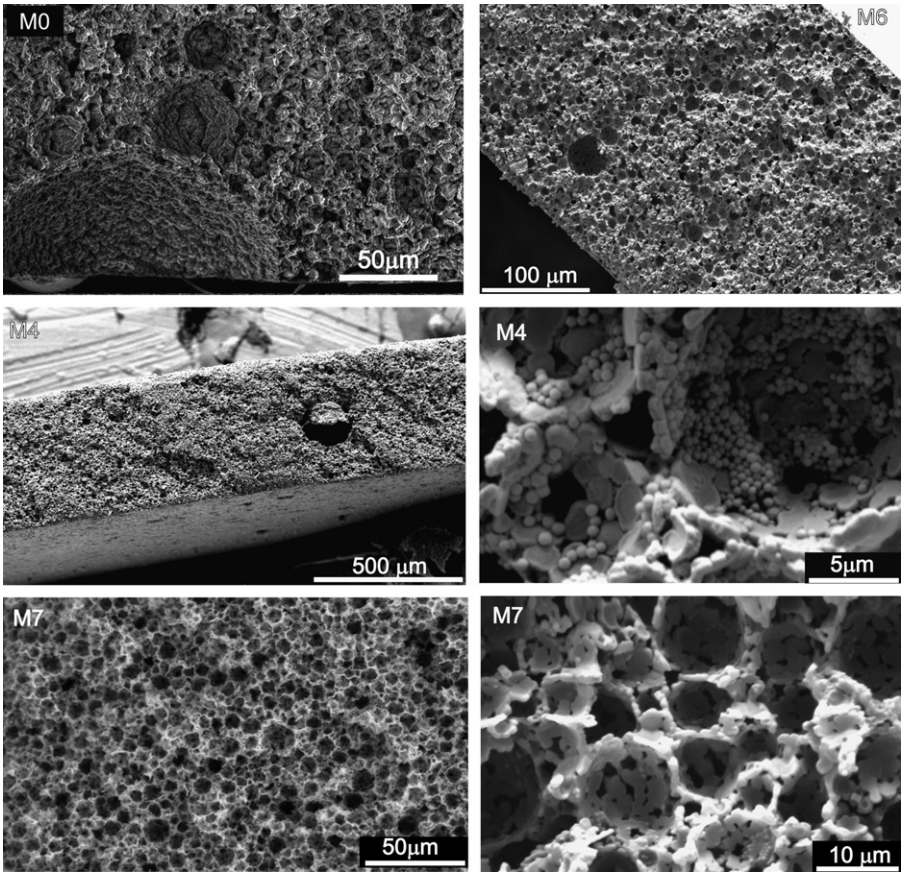

Fig. 2. SEM images of the membrane prepared with potassium persulfate and cured at 60 °C (M0: 2 mol% EGDMA, 98 mol% GMA), and the membranes prepared with ammonium persulfate + TEMED initiator system and cured at room temperature (M4: 5 mol% EGDMA, 5 mol% EHA, 90 mol% GMA; M6: 5 mol% EGDMA, 15 mol% EHA, 80 mol% GMA; M7: 5 mol% EGDMA, 20 mol% EHA, 75 mol% GMA).

pores are virtually no longer present.Another finding with regards to morphology is the incorporation of small spherical polymer particles inside the cavities, ranging in size from 0.4 to 0.6 μm (Fig. 2, M4). Similar observations were reported in regards to the synthesis of poly-pickering-HIPEs [39]. Authors assume that due to the partly hydrophilic nature of the organic phase, part of monomers is dissolved in aqueous phase, where it polymerises and remains trapped within the cavities. This interpretation is consistent with our findings; increasing the content of EHA in the organic phase contributes to less hydrophilic nature, solubility in aqueous phase is reduced and in membranes with 30% of the EHA spherical particles inside cavities are no longer present (Fig. 2, M8).

Surface area of polyHIPE membranes was determined by nitrogen adsorption and mercury intrusion porosimetry. Both methods reveal rather low surface area in the range of a few m^2/g (Table 1), namely 1.31–13.10 m^2/g, and is slightly growing by increasing the amount of cross-linker (from 5.00 to 8.33 m^2/g by changing cross-linker amount from 2 to 10 mol%). Incorporation of ethylhexyl

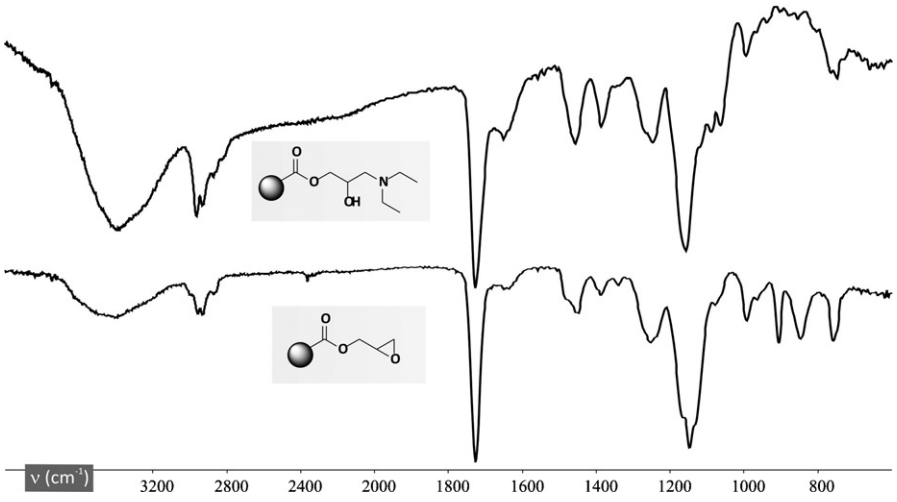

Fig. 3. FTIR spectra of membrane M4 before and after the functionalisation with diethylamine.

Fig. 4. Membrane M4 wrapped around the frit in a module.

acrylate into the polymer matrix does not effect the surface area to a high extent, however the effect on pore size distribution is more pronounced. At 5% addition of cross-linker the narrowest pore size distribution is obtained by incorporation of 5 mol% of ethylhexyl acrylate (Fig. 5, M4). Increasing plasticiser amount to 15 mol% (M2) slightly broadens pore size distribution and by incorporation of 30 mol% of EHA into the membrane (M8), pore size distribution is spread in very broad area of radius and this membrane was not useful for protein separations. It is to be noted that by the use of mercury porosimetry it is likely that some compression of the material occurs, especially in the case of samples with a higher amount of EHA. Specific morphology of the polyHIPE material (bigger pores with smaller interconnecting pores) adds to the limitations of mercury porosimetry in this case. Similar limitations have been noted and described in the case of polyHIPE GMA monoliths [28].

To evaluate membrane chromatographic properties membrane was functionalised using diethylamine to obtain weak anion-exchange supports. Transformation is evident on FTIR spectra: signal for epoxy group disappears and a new signal at around 1065 cm^{-1} confirmes presence of tertiary amine (Fig. 3). Membrane (M4) was inserted into a convective interaction media (CIM) 8 ml housing and connected to an HPLC system. Uniformity of the flow profile through the membrane was checked by pulse response experiment. Data are shown in Fig. 6; curve A. When comparing

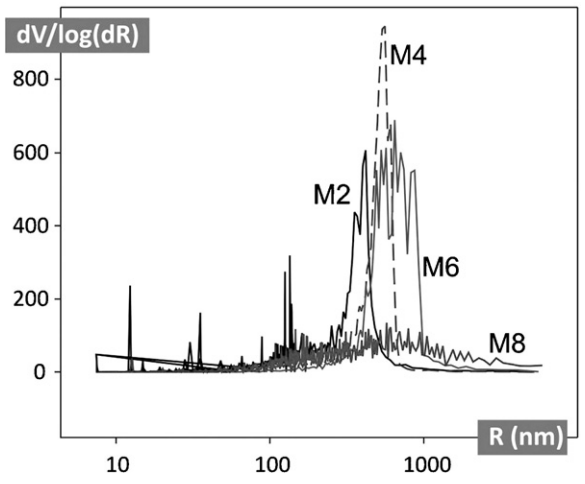

Fig. 5. Pore size distribution for polyHIPE membranes. Content of ethyl-hexyl acrylate: M2 – 0 mol%, M4 – 5 mol%, M6 – 15 mol%, M8 – 30 mol%.

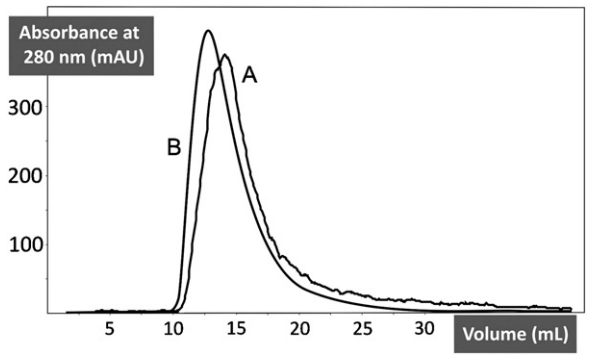

Fig. 6. Pulse response of polyHIPE membrane (stationary phase: DEAE 8 ml polyHIPE membrane; curve A) and CIM 8 ml tube (stationary phase: 8 ml CIM DEAE monolithic column; curve B). Flow rate: 40 ml/min. Sample: 16.7% acetone in distilled water; detection: UV at 280 nm.

this data with the pulse response of the 8 ml monolithic column (Fig. 6; curve B) it can be concluded that slightly higher dispersion is present with pronounced tailing. This might be due to the particular polyHIPE structure of the membrane where cavities might act like small mixing reactors but further investigation is needed to verify this hypothesis. Besides, slightly longer retention time is observed what is in accordance with a higher open porosity of the polyHIPE membrane as already found for polyHIPE methacrylate monoliths [28]. This is further confirmed with comparable pressure drop for membrane and monolith at a low flow-rate (data not shown). However, in contrast to rigid monolith structure, pressure drop on the membrane started to exponentially increase with the increase of the flow-rate, indicating membrane compression. This behavior limits its productivity when used in real purification processes. Another important chromatographic property is the capacity for macromolecules. Resins used in biochromatography are commonly characterized with protein bovine serum albumin (BSA) having a molecular mass of 66 kDa. Dynamic binding capacity (DBC) was determined with a frontal analysis experiment and found to be around 45 mg/ml. From Fig. 7 it is evident that the slope of the break-through curve is lower than that of commercially available monoliths, again confirming slightly higher dispersion as already noticed from the pulse response experiment. On the other hand, the value exceeds DBC of comparable monoliths [40] substantially (slightly above 20 mg/ml for monoliths) – demonstrating a considerable potential of polyHIPE membranes application for the purification of macromolecules. Taken into account the available membrane surface area and the average area covered by a single

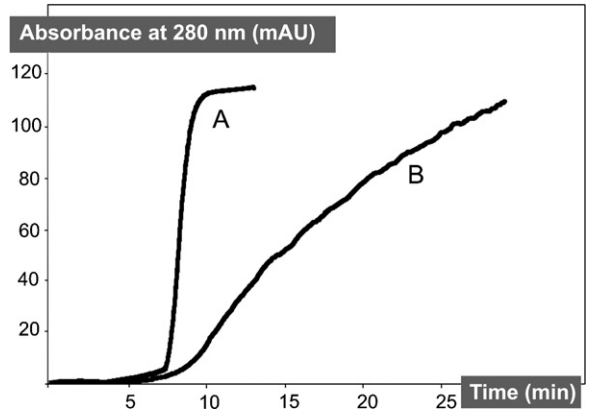

Fig. 7. Comparison of CIM tube 8 ml monolithic column (A) and polyHIPE membrane break-through curves (B). Conditions: sample: BSA in concentration of 1 mg/ml dissolved in a binding buffer (20 mM Tris–HCl, pH 7.4); flow rate: 20 ml/min.

BSA molecule, we can conclude that multilayer adsorption of BSA occurs.

5. Conclusions

Emulsion templating combined by doctor blading can produce highly porous membranes with excellent mechanical properties and functionalizable to yield ion-exchange groups for protein separation. Appropriate addition of a plasticizing comonomer enables flexibility for the use in module without sacrifising porous interconnecting structure. Using such membrane module with functionalised crosslinked poly(glycidyl methacrylate-co-ethylhexyl acrylate) porous polyHIPE membrane, a high binding capacity for bovine serum albumine was found.

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