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Preparation and characterisation of surfaces properties of poly(hydroxyethylmethacrylate-co-methacrylolyamido-histidine) membranes: application for purification of human immunoglobulin G

M. Yakup Arıca*, Emine Yalçın, Gülay Bayramoğlu

Biochemical Processing and Biomaterial Research Laboratory, Faculty of Science, Kırıkkale University, Yahşihan-Kırıkkale, 71450, Turkey

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Abstract

In this study, an affinity membrane containing L-histidine as an amino acid ligand was used in separation and purification of human immunoglobulin G (HIgG) from solution and human serum. The polarities and the surface free energies of the affinity membranes were determined by contact angle measurements. HIgG adsorption and purification onto the affinity membranes from aqueous solution and human serum were investigated in a batch and a continuous system. Effect of different system parameters such as ligand density, adsorbent dosage, pH, temperature, ionic strength and HIgG initial concentration on HIgG adsorption were investigated. The maximum adsorption capacity of p(HEMA-MAAH-4) membranes for HIgG was 13.06 mg ml^{-1}. The reversible HIgG adsorption on the affinity membrane obeyed both the Langmuir and Freundlich isotherm models. The adsorption data was analysed using the first- and second-order kinetic model and the experimental data was well described by the first-order equations. In the continuous system, the purity of the eluted HIgG, as determined by HPLC, was 93% with recovery 58% for p(HEMA-MAAH-4) membrane. The affinity membranes are stable when subjected to sanitization with sodium hydroxide after repeated adsorption-elution cycles.

Keywords: Poly(hydroxyethylmethacrylate-co-methacrylolyamido-histidine); Immunoglobulin G

1. Introduction

The recently introduced affinity membrane chromatography offers some apparent advantages over conventional bead-packed column chromatography in bioseparation such as higher flow rate, low pressures drop, faster binding kinetics, and simple scale-up [1,2]. An affinity separation relies on the highly specific binding between a target protein in solution and an immobilized specific ligand to achieve a high degree of protein purification.

The purification of IgG is generally required for the purposes of immuno-diagnostics, immunochromatography and immunotherapy. Moreover, IgG removal from human serum is employed for the treatment of immuno disorders, allo-immunization and cancer [3,4]. Different types of affinity ligand molecules have been used for purification of IgG (such as protein A, protein G, phenyl group, amino acids and thiophilic [5–12]) from human serum and other sources. The L-histidine ligand has been incorporated on different matrices (membranes, microspheres and silica particles) to purify IgG and presents similar binding capacities as protein A and protein G using mild elution conditions [13,14]. The L-histidine ligands are also much more stable than protein ligands because they do not require a specific tertiary structure for maintaining biological activity [15]. In addition, protein ligands are expensive and difficult to handle, sterilize and preserve its biological activity [16,17]. They can lose their activity by harsh elution and cleaning conditions. The L-histidine ligand is much more stable than protein ligands and it also offers several advantages over protein ligands in terms of economy, ease of immobilization and high adsorption capacity. It interacts with several proteins through its carboxyl, amino and imidazole group at near their isoelectric points and has shown particular efficacy in separating IgG from human serum and purification of antibodies from biological fluids [18].

* Correspondence author. Tel.: +90-318-357-2477; fax: +90-318-357-2329.
E-mail address: yakuparica@tnn.net (M.Y. Arıca).

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Affinity membranes find a wide range of applications in the field of biotechnology and biomedicine such as in bioaffinity chromatography, enzyme immobilization, biosensor, immunoassays, and others. Some of the methods employed to prepare affinity membranes are: (1) the attachment of specific ligand after derivatization of membrane surfaces with various activating agents (such as cyanogen bromide, epichlorohydrin or carbodiimide) and (2) the coupling of specific ligand on the surfaces of membrane carrying reactive group which was obtained after copolymerisation of two different monomers one of them contains reactive groups (such as poly(acrylonitrile-co-hydroxyalkyl methacrylate) or poly(hydroxymethacrylate-co-glycidyl methacrylate) [19–21].

In this study, preparation method of novel affinity membrane was investigated by copolymerisation of 2-hydroxyethylmethacrylate (HEMA) monomer with an amino acid ligand introduced co-monomer (2-methacryloly-amido-histidine; MAAH). This method is effective in that the ligand can be easily introduced in to the membrane at a desired density by adjusting the concentration of ligand carrying co-monomer in the polymerisation mixture. A series of p(HEMA-MAAH-1-4) membranes with different HEMA/MAAH ratios were prepared by UV-initiated photo-polymerisation in the presence of an initiator (α,α′-azobisisobutyronitrile; AIBN). The surfaces properties of p(HEMA-MAAH-1-4) membrane were determined by measuring the contact angle value to different test liquids and the surface free energy of membranes was calculated from contact angle using acid–base method. System parameters such as properties of membranes (i.e., effect of HEMA/MAAH ratio on the water content, contact angles and surfaces free energy of membranes), the adsorption conditions (i.e., sorbent dosage, initial concentration of HIgG, medium pH, ionic strength and temperature), the effect of ligand density on the HIgG adsorption capacities, and the adsorption-desorption behaviour are studied. Finally, the experimental data were analysed using different adsorption kinetics and isotherm models. The thermodynamic parameters for the adsorption of HIgG on the membrane systems were also investigated.

2. Experimental

2.1. Materials

Human immunoglobulin G and L-histidine methylester dihydrochloride were supplied from Sigma (St. Louis, MO, USA) and used as received. 2-Hydroxyethyl methacrylate (HEMA) and methacryloylchloride was obtained from Fluka AG (Switzerland), distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. α-α′-Azoisobisbutyronitrile (AIBN), trimethylamine, glycerol, diiodomethane (DIM) and trifluoroacetic acid (TFA) were obtained from Sigma. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in the following experiments was purified using a Barnstead (Dubuque, IA, USA) 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.

2.2. Preparation of methacryloly-amidohistidine monomer

L-Histidine methylester (5.0 g) and hydroquinone (0.2 g) were dissolved in 100 ml dichloromethane. The mixture was transferred in a 250 ml round-bottomed three-necked flask fitted a dropping funnel. After cooling to 0 °C, triethylamine (10 ml) was added and stirred magnetically under a nitrogen atmosphere. A 6.0 ml of methacryloylchloride was placed into the dropping funnel and was introduced drop wise to the reaction mixture in 10 min. The reaction chamber was then removed from the ice-water bath and the reaction was maintained at room temperature for 2 h. After reaction, unreacted methacryloylchloride was removed from the medium by extraction with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The product “methacryloly-amidohistidine” was dissolved in ethanol.

2.3. Preparations of p(HEMA-MAAH) membranes

The poly(2-hydroxyethylmethacrylate-co-methacryloly-amido-histidine), p(HEMA-MAAH-1-4), membranes were prepared by UV initiated photopolymerisation. To check the effect of ligand density on the adsorption capacity of the affinity membranes, in the initial polymerization mixture four different MAAH ratios were used (i.e., HEMA: MAAH ratios: 156:1, 68:1, 34:1 and 23:1). The membrane preparation mixture (5.0 ml) contained 2.0 ml HEMA, 25–150 mg MAAH, 20 mg AIBN as polymerisation initiator and 3.0 ml Tris–HCl buffer (50 mM, pH 7.0). The resulting mixtures were equilibrated at 25 °C for 30 min in a water bath. The mixtures were then poured into the moulds and exposed to long-wave ultraviolet radiation for 1.0 h. The poly(HEMA-MAAH-1-4) membranes were washed several times with distilled water, ethanol (70%, v/v) and Tris–HCl buffer in a sonicated waterbath and was cut into circular pieces (diameter: 0.75 cm) with a perforator.

2.4. Adsorption and purification studies of IgG

2.4.1. Effect of solid/liquid ratio, ligand density, pH, temperature and ionic strength on HIgG adsorption

Different quantities p(HEMA-MAAH-4) membrane, varying from 0.13 to 0.52 ml affinity membrane in each 7.5 ml of HIgG solution (1.0 mg ml^{-1}) were stirred 100 rpm for 3 h at 25 °C. Once the optimum affinity membrane

dosage was determined, the effect of ligand density, pH, temperature and ionic strength were conducted. Adsorption of HIgG on the p(HEMA-MAAH) membrane was studied at various pH's, in either acetate (50 mM pH 4.0–5.0), in phosphate buffer (50 mM, pH 6.0–6.5), in Tris–HCl buffer (50 mM, pH 7.0–8.0) or in in carbonate (50 mM, pH 9.0). The effect of temperature and ionic strength on HIgG adsorption were carried out in in phosphate buffer (50 mM, pH 6.0) and Tris–HCl buffer (50 mM, pH 8.0) for p(HEMA-MAAH-4) membranes at four different temperatures (i.e., 4, 15, 25 and 37 °C) and at three different KCl concentrations (i.e., 0.1, 0.25 and 0.5 M), respectively. All experiments were conducted in duplicates with 0.26 ml affinity membranes and initial concentration of HIgG was 1.0 mg ml^{-1} in each set experiments.

2.4.2. Effect of initial concentration of HIgG on adsorption capacity

To determine the adsorption capacities of the p(HEMA-MAAH-4) membranes, the initial concentration of HIgG was changed between 0.1 and 3.0 mg ml^{-1} in Tris–HCl buffer (7.5 ml, 50 mM, pH 7.0). A calibration curve was prepared using HIgG as a standard (0.05–3.00 mg ml^{-1}). The amount of HIgG adsorbed onto affinity membranes was determined by subtracting the UV absorbance at 280 nm after adsorption from the value before adsorptions using UV–vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601). Calibration curves were prepared using HIgG as standard (0.05–3.00 mg ml^{-1}).

2.4.3. HIgG purification from human serum

Purification of HIgG with p(HEMA-MAAH-4) membrane from human serum was studied in batch and continuous systems. Fresh human blood samples were obtained from healthy donor in the presence of sodium citrate, (Faculty of Medicine, Kırıkkale University). The samples were centrifuged for 10 min at 2000 rpm at 4 °C.

In the batch system, the purification of HIgG was carried out as described above except that 5-fold diluted human serum (Tris–HCl buffer; 50 mM, pH 7.0) used as adsorption medium instead of pure HIgG solution and eluent was analysed by HPLC.

The continuous system was made from Pyrex glass (length 6.0 cm, diameter 1.8 cm, total volume 15 ml). The poly(HEMA-MAAH-4) membranes (3.6 ml) were loaded into the continuous system yielding a void volume of about 11.0 ml. The five-fold diluted human serum was introduced to the system at a flow rate of 20 ml h^{-1} with a peristaltic pump (ISMATEC, IPC Model) though the lower inlet part. It was operated at 25 °C for each run for 4.0 h and the diluted serum samples leaving the system was collected and analysed by HPLC.

In the batch and continuous systems, the purities and quantities of adsorbed HIgG onto affinity membrane were analysed using HPLC after elution with Tris–HCl buffer (50 mM, pH 7.4) containing 0.5 M KCl.

2.5. Instrumentation and chromatographic conditions

A Dionex HPLC system (Dionex Co., Germering, Germany) was used for the chromatographic studies. The HPLC system consisted of a quarterary pump with an on-line vacuum degasser (Model P580 A), an autosampler with a variable injection capacity from 1 to 250 μl (Model ASI-100), a column oven (Model STH 585) and an UV–vis diode array dedector (Model 340 S). Chromatograpfic seperation of proteins was achieved on a VYDAC 259 VHP 5415 column (150 mm × 4.6 mm i.d.) protected by a guard column (20 mm, 4.6 i.d.). All serum solutions used in chromatographic studied were pre-filtered through a syringe membrane filter (0.2 μm, Millipore) to remove particles and large aggregates. HPLC mobile phase A and B were prepared by adding trifluoroacetic acid (TFA) (0.01%, v/v) in Milli Q water and 95% acetonitril and 5% Milli Q water, respectively. The mobile phases were filtered prior to use. The chromatographic separation was performed using a gradient at 1.0 ml min^{-1} flow-rate (0–20 min, phase B from 25 to 60% and 21–25 min, from 60 to 25%) and the sample injection volume of the autosampler was 20 μl. The UV–vis dedector was set at 220 nm and the temperature was maintained at 25 °C. Dionex CHROMELLEON® software was used and operated under Windows 98 for data acquisition and integration.

2.6. Repeated use studies

To determine the reusability of the p(HEMA-MAAH-4) membranes adsorption and elution cycle was repeated six times by using the same affinity membranes. HIgG desorption experiments were performed by immersing the membranes in Tris–HCl buffer (50 mM, pH 7.4) containing 0.5 M KCl. Desorption medium was stirred magnetically at 100 rpm at 25 °C for 3 h. The final HIgG concentration in desorption medium was determined by spectrophotometry.

2.7. Characterization studies

2.7.1. NMR spectra

A JEOL GX-400 instrument (300 MHz) was used to acquire the proton NMR spectra of polymer solutions in $CDCl_3$. The residual non-deuterated solvent ($CHCl_3$) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to $CHCl_3$.

2.7.2. FT-IR spectra

FTIR spectra of the p(HEMA-MAAH-4) membranes was obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry membrane (about 0.1 g) mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded.

2.7.3. Elemental analysis

The degree of MAAH incorporation in the synthesised p(HEMA-MAAH-1-4) membranes was determined by measuring the C, H, N, O contents with a Laco, Model CHNSO-932) Elemental Analyser.

2.7.4. Scanning electron microscopy

The dried p(HEMA-MAAH-4) membranes were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (JSM 5600) scanning electron microscope.

2.7.5. Water content of p(HEMA-MAAH-1-4) membranes

The water content of copolymer membrane prepared with different amounts of MAAH was determined at room temperature in Tris–HCl buffer (50 mM, pH 7.0) by using a gravimetric method. The water content was defined as the weight ratio of water contained within swollen to dry membrane. The water content of the p(HEMA-MAAH-1-4) membranes were calculated by using the following expression:

$$\text{Swelling ratio (\%)} = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

where W_d and W_s are the dry and swollen weights of p(HEMA-MAAH-1-4) membranes, respectively.

2.7.6. Contact angle measurements and calculation of surface energy

Contact angles to different test liquids (i.e., water, glycerol and diiodomethane) of the dried p(HEMA-MAAH-1-4) membranes samples were measured by sessile drop method at 25 °C by using a digital optical contact angle meter CAM 200 (KSV Instruments Ltd., Helsinki, Finland). The sessile drop was formed by depositing the liquid from the above using a manual micro-syringe on the membrane surfaces. Both the left- and right contact angles and drop dimension parameters were automatically calculated from the digitalized image. The measurements were the average of five contact angles at least operated on three membrane samples.

The free surface energy parameters of the p(HEMA-MAAH-1-4) membranes were calculated using the contact angle data of the probe liquids. The results are analysed according to acid–base method of van Oss et al. [22]. In this method, the contact angles against at least three liquids with known values of γ^{LW}, γ^+ and γ^- are measured and the superscripts (LW), (+) and (−) refers to dispersive, Lewis acid and base components, respectively. The values for each experiment are put into the following equation:

$$(1+\cos\theta)\gamma_l = 2[(\gamma_s^{LW} \times \gamma_l^{LW})^{1/2} + (\gamma_s^+ \times \gamma_l^-)^{1/2} + (\gamma_s^- \times \gamma_l^+) \quad (2)$$

The total surface energy γ^{TOT} is regarded as the sum of Lifschitz–van der Waals and the Lewis acid and base components.

$$\gamma^{TOT} = \gamma^{LW} + \gamma^{AB} \quad (3)$$

where γ^{LW} designated Lifschitz–van der Waals interaction, reflecting the long-range interactions (including the dispersive interaction, the dipole–dipole interaction, and dipole-induced dipole interaction, which is dominated by the dispersion), was calculated from the measured diiodomethane contact angles, and γ^{AB} designated such acid–base interactions as hydrogen bonding, and γ^+ and γ^- refer to proton and electron donating character, respectively. The detailed description of the used method can be found in the literatures [22] and only the basic equations are presented here. The method equations were solved using CAM 200 software package operated under Windows 98 (KSV Instruments Ltd.).

3. Results and discussion

3.1. Properties of p(HEMA-MAAH) membranes

In this study, we have suggested a novel method of introducing L-histidine ligand into the polymer membrane structure via co-polymerisation of HEMA monomer with a ligand carrying co-monomer methacrylolyamido-histidine. This method is effective in that the ligand readily introduced into the porous membrane at a required density by selecting the co-monomer ratio in the polymer preparation mixture. A two-step process was carried out for the preparation of p(HEMA-MAAH) membranes. In the first step, MAAH was synthesized from 2-methacryloylchloride and L-histidine. The second step consisted of the copolymerisation of HEMA monomer with MAAH co-monomer via UV initiated photo-polymerization in the presence of an initiator (AIBN).

^{1}H NMR was used to determine the MAAH structure. ^{1}H NMR spectrum of MAAH is shown to indicate the characteristic peaks from the groups in monomer unit. These characteristic peaks are as flows: ^{1}H NMR ($CDCl_3$) δ 1.99 (t; 3H, $J = 7.08$ Hz, CH_3), 1.42 (m; 2H, CH_2), 3.56 (m; 3H, –OCH_3), 4.82–487 (m; (1H, methin) 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86–7.82 (δ; 1H, $J = 7.4$ Hz, NH), 6.86–7.52 (m; 5H, aromatic).

The FTIR spectra of both MAAH and p(HEMA-MAAH-4) have the characteristic stretching vibration band of hydrogen-bonded alcohol, O–H, around 3540 cm^{-1}. The FTIR spectra of p(HEMA-MAAH) have characteristic amide I and amide II adsorption bands at 1650 and 1525 cm^{-1}, respectively. On the other hand hydrogen bonded alcohol O–H stretching band intensity of pHEMA membrane is higher than that of p(HEMA-MAAH-4) membrane due to the incorporation of MAAH into the polymer structure.

The amount of L-histidine in the p(HEMA-MAAH-1-4) membranes was determined by elemental analysis. The L-histidine content of the membranes were calculated from nitrogen stoichiometry and found to be between 59 and 253 μmol/ml membrane.

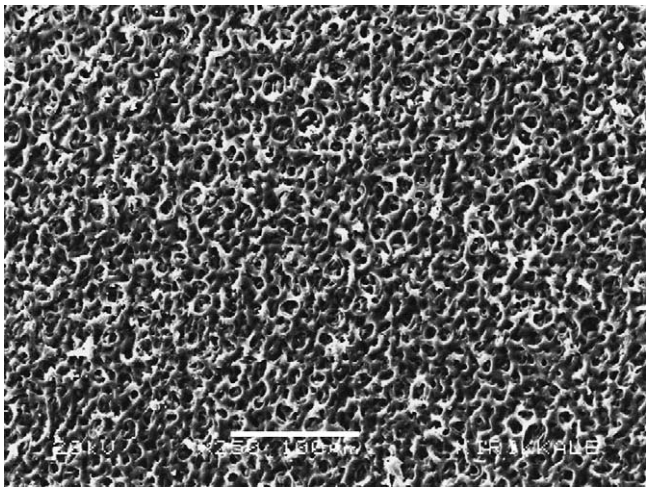

Fig. 1. The SEM micrograph of p(HEMA-MAAH) membrane surface.

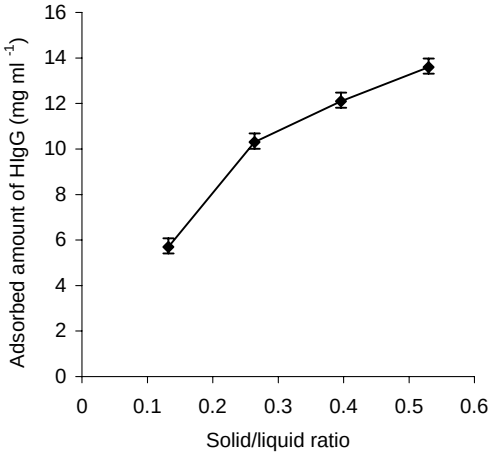

Fig. 2. Effect of solid/liquid ratio on the adsorption capacity of the p(HEMA-MAAH-4) membrane.

The effect of p(HEMA-MAAH) mole ratio on the water content of the membrane was in Tris–HCl buffer (50 mM, pH 7.0). Compared with pHEMA membrane (58%), the water content of p(HEMA-MAAH-4) membranes increased (up to 86%) with varying co-monomer ratios. When MAAH was introduced in the structure of the membrane, it was found that this increased the water content. This appears reasonable when it is remembered that HEMA is not highly polar compound and does not have fixed charge. The imidazole and carboxylic groups of the MAAH introduced positive and negative charges into the polymer structure, and should be caused more water uptake.

The scanning electron microscope (SEM) micrograph showing the surface sturucture of the p(HEMA-MAAH-4) membrane is presented in Fig. 1. The average pore size and the pore distribution are about 4 μm and 2.8×10^5 pore cm^{-2}, respectively. As seen from the figure, many pores were observed on the surface of the affinity membrane, which should contain available L-histidine ligand in the vicinity of pore space for interaction with protein. The porous surface structure should be considered as a factor providing an increase surface area. In addition, these pores reduce the mass transfer resistance and facilitate the diffusion of protein molecule because of high internal surface area with low diffusional resistance in the membrane (imply high adsorption capacity and rate).

3.2. HIgG adsorption studies in batch system

3.2.1. Solid/liquid ratio

The effect of the solid/liquid ratio on the adsorption capacity of the p(HEMA-MAAH-4) membrane was studied for an initial concentration of HIgG (2.0 mg ml^{-1}) and the content of solid was varied between 0.13 and 0.53 ml (affinity membranes) per 7.5 ml adsorption medium and is presented in Fig. 2. The resulted increase in the amount of HIgG removed from medium with the increase of the solid/liquid ratio can be explained by the augment of the number of active sites of the affinity membranes. An increase solid ratio from 0.13 to 0.53 ml affinity membrane in 7.5 ml adsorption medium leads to increase up to 51% in HIgG removal from the medium. In the remaining study, 0.39 ml affinity membrane was used in 7.5 ml adsorption medium.

3.2.2. Effect of ligand density

The effect of ligand density on the HIgG adsorption is shown in Fig. 3. As observed in this figure, an increase in ligand density led to an increase in adsorption capacity of the affinity membrane to HIgG but this relation levelled of at around 180 μmol L-histidine per ml membrane structure. At higher ligand density, however, the increase in HIgG adsorption capacity was slightly less proportional. This observation can be explained either to the overcrowded ligand density resulting in binding difficulties for relatively large HIgG molecules or to the mass-transfer resistance of the liquid film on the solid membrane surface further

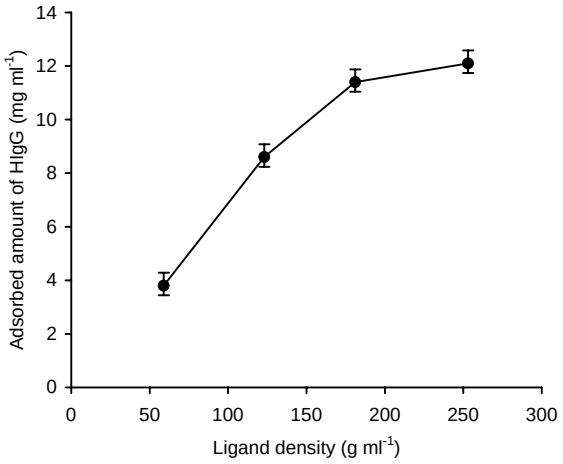

Fig. 3. Effect of ligand density on the adsorption capacity of the p(HEMA-MAAH-1-4) membranes.

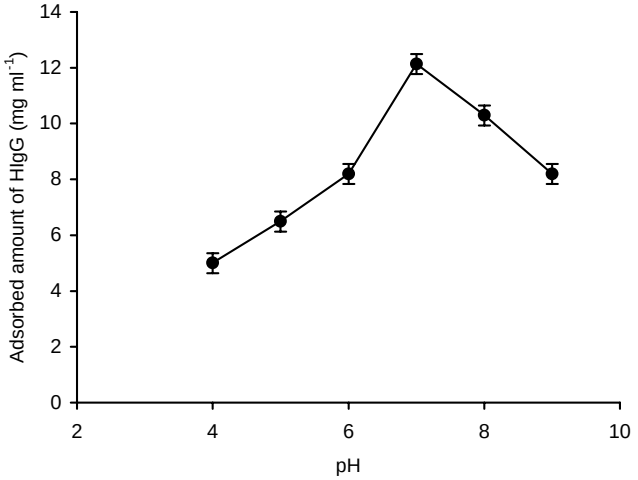

Fig. 4. Effects of pH on HIgG adsorption capacity of the p(HEMA-MAAH-4) membrane; initial concentration of l HIgG: 2.0 mg ml^{-1}; temperature: 25 °C.

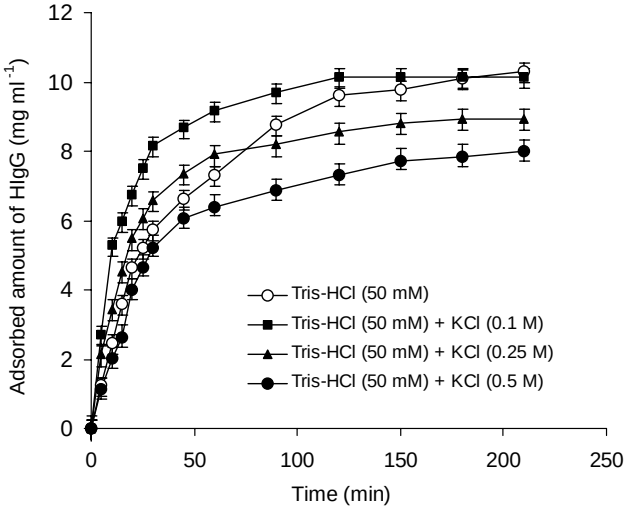

Fig. 5. Effects of ionic strength on HIgG adsorption on the p(HEMA-MAAH-4) membrane; initial concentration of HIgG: 2.0 mg ml^{-1}; temperature: 25 °C, pH: 7.0.

hindering HIgG molecules from reaching the ligand [23]. A maximum adsorption value (12.14 mg m^{-1}) was obtained with the p(HEMA-MAAH-4) membrane (containing 253 μmol L-histidine ml membrane, at 2.0 mg ml^{-1} HIgG concentration). The rest of the study was carried out with p(HEMA-MAAH-4) membranes.

3.2.3. Effect of pH

The distribution of charged amino acid residues on the protein surface is very important factor in ion-exchange interactions with the ligand on the affinity membrane. In the aqueous medium, polar or charged residues tend to be on the surface and non-polar residues in the interior. However, each amino acid residue cannot be distributed independently owing to the primary structure of protein, the distribution varies from one protein to another. The optimal pH value for adsorption of IgG onto affinity membrane was investigated in the pH range 4.0–9.0. As shown in Fig. 4, the maximum IgG adsorption onto p(HEMA-MAAH-4) membrane was 12.14 mg ml^{-1} at pH 7.0. Significantly, lower adsorption was obtained in the other tested pH regions. The pK_a value of carboxyl group of the L-histidine molecules is around 1.8. At pH 7.0, more than 90% of R group of L-histidine molecule is the imidazole form and only 10% imidazolium form is positively charged at this pH value. Therefore, L-histidine ligand has net negative charge at pH 7.0. The isoelectric point (p*I*) of IgG is 6.95. The proteins have no net electrical charge at their isoelectric points, and therefore, the maximum adsorption of protein is usually observed at their isoelectric points. Specific interactions (hydrophobic, electrostatic and hydrogen bonding) between HIgG and L-histidine molecules at pH 7.0 may result from both the ionisation states of several groups on both p(HEMA-MAAH-4) affinity membrane (i.e. carboxyl, imidazole, imidazolium, carbonyl and hydroxyl groups) and amino acid side chains in HIgG. Proteins that change conformation as a function of their environment (pH, salt, temperature), such as HIgG, it has a molecular mass of 150 kDa and could change conformation upon binding functional surface. Thus, HIgG molecules would expand and contact according to the variation of the ionizable groups on the surfaces. At pH 7.0, the resulting HIgG adsorption may be also due to suitable conformation of HIgG molecules within the native medium for interaction with L-histidine.

3.2.4. Effect of ionic strength

The enthalpy of adsorption would be affected not only by the pH value on the electron donating capability, but also by the salt concentration on the hydrophobic and electrostatic interaction between HIgG and L-histidine ligand. The adsorption capacity of p(HEMA-MAAH-4) membranes to HIgG was reduced about 39% with increasing KCl concentration from zero to 0.5 M (Fig. 5). The decrease in HIgG adsorption capacities of affinity membrane with increasing ionic strength should be resulted from decrease in the electrostatic interactions between HIgG and L-histidine ligand. As the ionic strength increased in the adsorption medium, the electrical double layer around the molecules, given by the Debye–Hückel length, decreased. van Oss reported that with increasing electrolyte concentration in the medium the surface charge are screened [24]. These effects cause the electrostatic force between molecules to decrease. Consequently, the total electrostatic energy between the molecules decreases and the system becomes aggregating.

3.2.5. Effect of temperature

At higher temperature, the protein in the adsorption medium would be expected to progressively commence to unfold and expose buried hydrophobic amino acid residues on the surfaces. If the interaction between the protein and the ligand involved a significant hydrophobic interaction, the contact surface between the protein and immobilized

Table 1
Langmuir and Freundlich constants and correlation coefficients for adsorption of HIgG on p(HEMA-MAAH-4) membranes at different temperatures

Temperature (°C)	Langmuir constants				Freundlich constants		
	q_{ex} (mg ml^{-1})	q_m	k_d ($\times 10^6$) (M)	R^2	n	K_F	R^2
4	7.96	8.85	1.94	0.998	2.76	6.30	0.955
15	10.83	12.67	3.55	0.999	2.04	7.53	0.972
25	13.06	15.93	4.38	0.978	1.77	8.77	0.998
37	16.04	20.73	6.52	0.974	1.66	9.87	0.982

histidine should increase at higher temperatures, resulting in an increase in the affinity of protein for the adsorbent [25]. As temperature increase, the contact area between the protein and the ligand on the matrix should increase, resulting in an increase in the affinity of proteins for the adsorbent at higher temperature. The HIgG showed an increased adsorption capacity with p(HEMA-MAAH-4) membrane at higher temperature and the results are presented in Table 1. From 4 to 37 °C, the absorption capacity of the affinity membrane for HIgG increased by 40%. The increase in the adsorption capacity of the affinity membrane for HIgG at higher temperature indicates that there is a significant hydrophobic interaction between the HIgG and the ligand L-histidine.

3.2.6. Effect of initial HIgG concentration

Fig. 6 shows the effect of initial HIgG concentration on HIgG adsorption. As presented in this figure, with increasing HIgG concentration, the amount of HIgG adsorbed on p(HEMA-MAAH-4) membrane increased. But this relation levelled off at around 2.0 mg ml^{-1}. This could be explained by saturation of interacting groups of p(HEMA-MAAH) membrane with the adsorbed HIgG molecules, as a result of which maximum adsorption capacity is achieved. Very low non-specific adsorption of HIgG onto the pHEMA membrane was obtained (0.17 mg ml^{-1}).

3.2.7. Adsorption isotherms

The adsorption isotherm was obtained from batch experiment at different temperatures and the results are presented in Fig. 7. Two theoretical isotherm models (Langmuir and Freundlich) were used to analyse the experimental data.

The Langmuir model described by the following equation:

$$\frac{dq}{dt} = k_1 C(q_m - q) - k_2 q \quad (4)$$

where C is the concentration of protein in solution, q is the amount of protein adsorbed on the membrane and q_m is the maximum adsorption capacity of the membrane. At equilibrium, Eq. (4) leads to

$$q = \frac{q_m C}{k_d + C} \quad (5)$$

The Freundlich isotherm is frequently used to describe the adsorption. It relates the adsorbed concentration as the power function of solute concentration. This empirical equation takes the form:

$$q = K_F(C)^{1/n} \quad (6)$$

where K_F and n are the Freundlich constants characteristic of the system. K_F and n are indicator of the adsorption capacity and adsorption intensity, respectively. The slope and the intercept of the linear Freundlich equation are equal to *1/n* and ln K_F, respectively.

The corresponding semi-reciprocal plots and Scatchard plots gave rise to linear plots for the p(HEMA-MAAH-4) membrane at different temperature and the correlation coefficients of semi-reciprocal plots (R^2) was greater than 0.978 for all the temperatures, indicating that the Langmuir

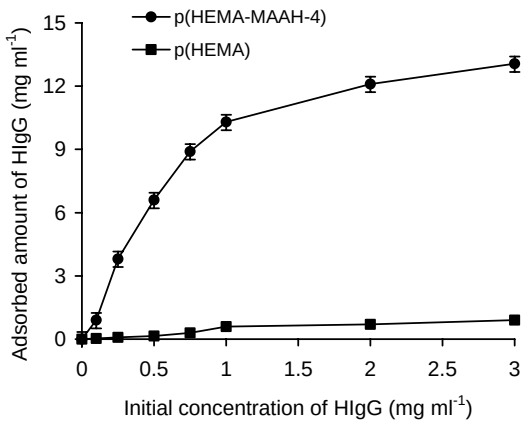

Fig. 6. Effects of HIgG initial concentration on HIgG adsorption on the p(HEMA-MAAH-4) membrane. pH: 7.0; temperature: 25 °C.

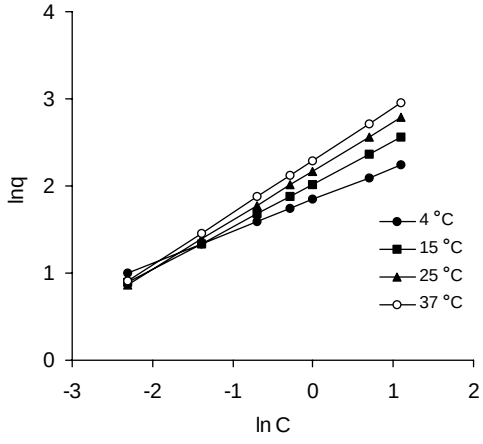

Fig. 7. The Freundlich isotherm plots for adsorption of HIgG on p(HEMA-MAAH-4) membrane at different temperatures.

Table 2
First-order rate constants and thermodynamic parameters for adsorption of HIgG on p(HEMA-MAAH-4) membranes at different temperatures

Temperature (K)	First-order rate constants				Thermodynamic parameters		
	q_{ex} (mg ml^{-1})	q_{eq}	k_1 ($\times 10^2$) (min^{-1})	R^2	ΔG° (kcal mol^{-1})	ΔS° (cal mol K^{-1})	ΔH° (kcal mol^{-1})
277	7.96	7.92	3.31	0.996	−7.20	47.55	
288	10.83	9.67	3.13	0.990	−7.25	45.94	5.92
288	13.06	12.99	2.99	0.995	−7.31	44.56	
310	16.04	16.87	2.84	0.985	−7.37	42.82	

model could be applied in this system. From the slopes, the maximum capacities (q_m) were found to be between 8.85 and 20.73 mg ml^{-1} for HIgG on the p(HEMA-MAAH-4) membrane at different temperatures and these calculated q_m values were very close to the experimental q_{ex} values (Table 1). Therefore, the adsorption of HIgG onto p(HEMA-MAH-4) membranes could be described in terms of the Langmuir model. The apparent dissociation constant (k_d) estimated from the intercept is a measure of the stability of the complex formed between a protein and an adsorbent under specified experimental conditions. For example, a large k_d value indicates that the protein has a low binding affinity for the adsorbent. At different temperatures, the k_d values were found to be between 1.94 and 6.52 × 10^{-6} M for the p(HEMA-MAH-4) membrane and, a high binding affinity was obtained for L-histidine ligand.

The Freundlich plots for HIgG adsorption on the p(HEMA-MAH-4) membrane at different temperatures are presented in Table 2. The Freundlich constants, K_F and n were found to be between 6.30 and 8.77 and, 1.66 and 2.76, respectively. Values of $n > 1$ for the L-histidine ligand indicates positive co-operativity in binding and a heterogeneous nature of adsorption. The magnitude of K_F showed easy adsorption of HIgG from the adsorption medium.

3.3. Kinetics modelling

The large number and different chemical groups on the adsorbents surface were created during preparation of the affinity membranes [i.e., secondary amino, carboxyl, carbonyl, hydroxyl and hyrophobic groups], imply that there are many types of HIgG and ligand interactions. The kinetic models (the first-order and second-order equations) can be used in this case assuming that measured concentrations are equal to adsorbents surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$\frac{dq_t}{dt} = k_1(q_{eq} - q_t) \quad (7)$$

where k_1 is the rate constant of the first-order biosorption (min^{-1}) and q_{eq} and q_t denote the amounts of adsorbed HIgG at equilibrium and at time t (mg ml^{-1}), respectively.Eq. (7) can be rearranged to obtain a linear form

$$\log(q_{eq} - q_t) = \log q_{eq} - (k_1 t)/2.303 \quad (8)$$

A plot of $\log(q_{eq} - q_t)$ against t should give a straight line to confirm the applicability of the kinetic model. In a true first order process $\log q_{eq}$ should be equal to the intercept of a plot of $\log(q_{eq} - q_t)$ against t.

The second-order equation was applied for adsorption of solutes on the adsorbents [26,27]. The second-order equation based on adsorption equilibrium capacity may be experesed in the form:

$$q_m/(q_m - q_t) = k_2 t + 1 \quad (9)$$

The linear form of equation is

$$\frac{1}{q_t} = \frac{1}{k_2 q_m t} + \frac{1}{q_m} \quad (10)$$

From Eq. (10), a plot $1/q_t$ versus $1/t$ should give a straight line and the sorption capacity q_m and the rate constant k_2 can be calculated from the intercept and the slope of the linear second-order equation, respectively.

In order to analyse the adsorption kinetics of HIgG, the first-order and the second-order kinetics models were applied to the experimental data. The first-order equation fitted well with the experimental data. The comparison of experimental adsorption capacities and the theoretical values estimated from the first-order equation are presented in Table 2. The theoretical q_{eq} values for the p(HEMA-MAAH-4) membrane were very close to the experimental q_{eq} values in the case of first-order kinetics. The first-order kinetics best described the data.

In addition, Arrhenius plots in the temperature range from 5 to 37 °C obtained from $1/T$ versus $\ln k_1$ (k_1; first-order rate constant) appear linear; activation energy (E_a) was found to be −3.31 for p(HEMA-MAAH-4) membrane. The lower activation energy calculated for L-histidine ligand indicates that the adsorption of HIgG on the p(HEMA-MAAH-4) ligand is favourable. The binding of HIgG on the p(HEMA-MAAH-4) may not require a large conformational deformation, thereby resulting in lower activation energy for the molecule to reorganise and attain the proper conformation for binding to the L-histidine-ligand.

3.4. Thermodynamic parameters

The thermodynamic parameters such as free energy changes (ΔG°), enthalpy change (ΔH°), and entropy change

(ΔS°) for the adsorption process can be estimated using the following equations:

$$\Delta G^\circ = -RT \ln K_a \quad (11)$$

$$\ln K_a = \frac{\Delta G^\circ}{R} - \frac{\Delta H^\circ}{RT} \quad (12)$$

The dependency of the equilibrium association constant, K_a ($K_a = 1/K_d$, from Langmuir constant) versus $1/T$ for the binding of HIgG on the p(HEMA-MAAH-4) membrane was analysed in terms of Van't Hoff plots. The ΔG° values for HIgG adsorbed on the p(HEMA-MAAH-4) membrane were calculated for each temperature and tabulated in Table 2. The negative ΔG° values for each temperature indicated that adsorption of HIgG on the p(HEMA-MAAH-4) membrane was a favourable process and those were ranged between -7.20 and $-7.37\,\text{kcal mol}^{-1}$. The ΔS° values for the adsorption of HIgG to p(HEMA-MAAH-4) membrane is presented in Table 2. At lower temperatures, due to lower kinetic motion of the L-histidine ligand, the value of the phase ratio will be different to that at higher temperatures. The positive values of ΔS° show the increased randomness at the solid/solution interface during adsorption. The relatively small positive ΔS° values in the system under investigation indicate that as a result of adsorption no significant structural change occurs in the affinity membrane material. The calculated ΔH° value of the system for the interaction for the HIgG with the L-histidine ligand was $5.92\,\text{kcal mol}^{-1}$. The positive value of ΔH° indicates the endothermic nature of adsorption.

3.5. Purification of HIgG from human serum

Purification of HIgG from human serum was studied in batch and continuous systems. The purity and amount of adsorbed HIgG on the p(HEMA-MAAH-4) affinity membrane were determined by HPLC using the eluent obtained from the affinity membranes. The chromatogram of the standard HIgG sample with a retention time 3.7 min was presented in Fig. 8. The initial and remaining concentration of HIgG in serum samples were quantitated by integration of peak areas (Fig. 8).

The continuous system operation parameters is presented in Section 2.4.3 and, residence time corresponding to the given flow rate ($20\,\text{ml h}^{-1}$) is calculated by the following equation:

$$D = \frac{\nu_o}{\varepsilon V} \quad (13)$$

where D is the dilution rate (h^{-1}), ν_o is the volumetric flow rate of the five-fold diluted serum (ml h^{-1}), V is the total volume of the continuous system (ml) and ε is the void fraction of given as ratio of void volume to the total volume of the continuous system. Residence time (τ) is the reciprocal of dilution rate.

The residence time was calculated as 0.55 h (or 33 min) for the continuous system and, this was 3.0 h for the batch system. In both systems, a five-fold diluted human serum was used and, the concentration of HIgG in the diluted serum was about $1.85\,\text{mg ml}^{-1}$. In the continuous system, the percent purity and recovery of HIgG were determined as 93 and 58%, respectively. On the other hand, the obtained purity and recovery of HIgG were decreased about 6 and 4% for the batch system compared to the continuous system (Fig. 7). The observed lower recoveries of affinity membrane in both systems may be resulted of the low concentration of HIgG in the diluted serum sample. When whole serum was used in the batch and continuous systems, a decrease in the purity of HIgG was observed (data not shown). Therefore, a five-fold diluted serum sample was employed through the purification studies. From these observations, low residence time (0.55 h) in the continuous system led to an increase in both percent purity and recovery of HIgG from diluted serum with respect to the batch system [28].

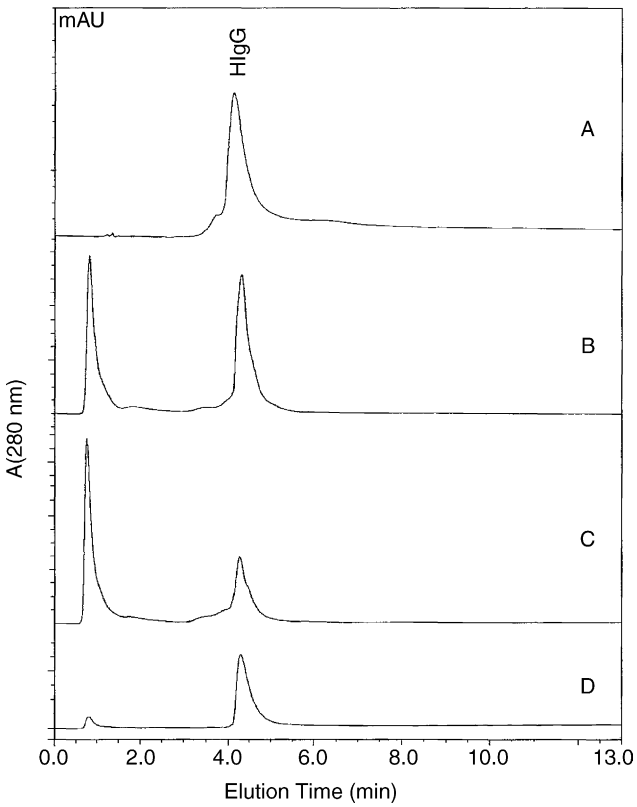

Fig. 8. HPLC chromatogram showing: (A) commercial HIgG, (B) human serum diluted with Tris–HCl buffer 1:5 ratio, (C) after interactions of diluted human serum with p(HEMA-MAAH-4), (D) the elution profiles of p(HEMA-MAAH-4) membranes after interactions with five-folds diluted human serum in the continuous system.

The p(HEMA-MAAH-4) affinity membrane provided an efficient single step method to purify HIgG from diluted serum sample, showing high binding capacity and high selectivity for HIgG under given experimental conditions. It should be noted that albumin (pI value 4.9) did not adsorbed to the affinity membrane under given experimental conditions. The purification experiments were carried out at pH 7.0 and affinity membrane has net negative charges at this

Table 3
Contact angles of water, glycerol and diiodomethane on the pHEMA and p(HEMA-MAAH-1-4) membranes

Properties of membrane samples	Test liquids and their surface tension (γ_l)		
	Water ($\gamma_l = 71.3$) (θ°)	Glycerol ($\gamma_l = 64.0$) (θ°)	Diiodomethane ($\gamma_l = 50.8$) (θ°)
pHEMA	55.6	53.6	39.3
p(HEMA-MAAH-1)	52.6	43.5	32.6
p(HEMA-MAAH-2)	48.9	41.9	33.8
p(HEMA-MAAH-3)	42.7	39.6	37.2
p(HEMA-MAAH-4)	40.6	38.2	38.7

pH value. The albumin molecules also have net negative charge at pH values above 4.9. From this point of view, the binding of albumin on the affinity membrane should be prevented by charge–charge repulsions.

3.6. Contact angle measurements and surface energy

Characterization of surface chemistry is a crucial issue in affinity technology. Contact angle measurements are used in the characterization of materials surfaces to describe hydrophilicity or to estimate surface free energy. The results of the contact angle measurements of water, glycerol and diiodomethane on the pHEMA and p(HEMA-MAAH-1-4) membrane surfaces are presented in Table 3. All the investigated membrane samples yielded a different contact angle value. The highest contact angles were obtained with water, whereas diiodomethane gave the lowest contact angles. The wettability of the polymer surfaces can be examined by comparing the contact angles for water and diiodomethane since these two solvents are often used as reference liquids in analyses of interaction of polar and apolar solvents with solid surfaces. As expected, pHEMA has a polar surface and shows a water contact angle of 55.6°. As can be seen in Table 3, for p(HEMA-MAAH) copolymer series contact angles of three liquids depend on the L-histidine ligand density of the membranes. As the amount of MAAH increases from 59 to 253 μmol ml^{-1}, water contact angles decrease from 52.6 to 40.6, respectively. This was opposite when diiodomethane used as a test liquid. This can be due to increase the density of polar histidine molecules on the copolymer surfaces. It was interesting to observe that the decrease in water contact angle value for p(HEMA-MAAH-4) membrane was not more pronounced with respect to p(HEMA-MAAH-3) membrane. This result also supports the IgG adsorption data related with the L-histidine ligand density, where an increase in the L-histidine density does lead a proportional increases in the amount of adsorbed IgG on the membrane as is predicted for p(HEMA-MAAH-2) compared to p(HEMA-MAAH-1) membrane (Fig. 3). These results indicated that the hydrophilicity of the surfaces of the p(HEMA-MAAH) membranes series significantly increased with increasing the MAAH ratio in the polymer structure.

The determined overall surface free energy (γ^{TOT}) calculated using the acid base method of van Oss et al.', consisting of the sum of the Lifschitz–van der Waals (γ^{LW}) and the acid–base components (γ^{AB}) applies for all investigated membranes at different values (Table 4). As can be seen in table that p(HEMA-MAAH) series seem to exhibit slightly "amphoteric" character. However, the basic parameter (γ^-) is significantly larger compared to the acidic parameter (γ^+). The relative high basic (γ^-) component of the surface energy is caused by the electron ion pairs of oxygen atoms contained in the copolymer (hydroxyl, carbonyl and carboxyl functionalities), which are effective in Lewis base sites [29,30]. As expected, all the investigated membranes exhibit different acid base components (γ^{AB}) of the surface free energy due to various amount functional groups of the copolymers. It should be noted that the contact angle degree decrease for water on the p(HEMA-MAAH) membranes series accompanied by an increase in (γ^{AB}) value and polarity of the membrane. On the other hand, the Lifschitz–van der Waals component of the surface free energy of the p(HEMA-MAAH) membranes series was also slightly increased as the L-histidine ligand density increases in the membrane structure. It is interesting to observe that the base parameter of the p(HEMA-MAAH) membranes series increased from 4.13 to 5.23 mN m^{-2} whereas acid parameter slightly decreased from 1.22 to 1.06 mN m^{-2}. Thus, all these parameters should be effective in determining the

Table 4
Surface free energy parameters (mN m^{-2}) of the pHEMA and p(HEMA-MAAH-1-4) membranes according to the van Oss et al. method

Properties of membrane samples	γ^{LW} (mN m^{-2})	γ^+ (mN m^{-2})	γ^- (mN m^{-2})	γ^{AB} (mN m^{-2})	γ^{Total} (mN m^{-2})	Polarity (%)
pHEMA	39.41	0.51	4.79	4.93	44.34	11.12
p(HEMA-MAAH-1)	42.01	1.22	4.13	10.09	52.10	19.37
p(HEMA-MAAH-2)	42.58	1.17	4.49	10.51	53.09	19.79
p(HEMA-MAAH-3)	42.98	1.11	5.07	11.23	54.22	20.71
p(HEMA-MAAH-4)	44.04	1.06	5.23	12.04	56.08	21.46

interactions of HIgG with affinity membranes. The amount of adsorbed HIgG on these affinity membranes should be also determined from these effective parameters.

3.7. Reusability of the affinity membrane

In order to show the reusability of the L-histidine ligand containing membrane; adsorption–desorption cycle of HIgG was repeated six times by using the same affinity membrane. The adsorption capacity of the affinity membrane did not noticeably change during the repeated adsorption–desorption operations. These results showed that p(HEMA-MAAH) affinity membranes could be repeatedly used in HIgG purification studies without detectable losses in their initial adsorption capacities. The affinity membrane is stable when subjected to sanitization with sodium hydroxide (0.1 M) after repeated adsorption–elution cycles.

4. Conclusions

An affinity membrane series containing amino acid ligand based on 2-hydroxyethylmethacrylate and L-histidine introduced co-monomer (i.e. 2-methacrylolyamido-histidine) were prepared via UV initiated photopolymerisation. The distinctive feature of this method is the elimination of activation and ligand-coupling step during preparation of affinity membrane. Some other advantages over other methods include the use of known amount of ligand in the polymer preparation mixture and the good reproducibility of the affinity membrane matrix. In this way, the method offers a simple affinity membrane preparation technique with a desired ligand density. The polarities and the surface free energies of the affinity membranes were determined by contact angle measurements. The adsorption behaviours of HIgG onto p(HEMA-MAAH-1-4) membranes were investigated using various ligand densities and various reaction conditions. It has been shown that the liquid/solid ratio, ligand density, pH, ionic strength and temperature can have important effects on the adsorption equilibrium. The pH of the medium has an important effect on the adsorption equilibrium of HIgG, and there is a preferential interaction between HIgG and p(HEMA-MAAH) membrane at pH 7.0. The equilibrium adsorption of HIgG onto all the tested adsorbents significant1y increased with increasing temperature. The HIgG adsorption capacity of affinity membrane was decreased with increasing ionic strength. The theoretical q_{eq} values for all the tested adsorbent systems were very close to the experimenta1 q_{eq} values in the ease of first-order kinetics. The first-order kinetics best described the data. Finally, the characterised affinity membrane was used for the purification of HIgG from diluted human serum in the batch and continuous system. The purity of the eluted HIgG, as determined by HPLC, was 87 and 93% with recovery 54 and 58% for batch and continuous system, respectively. Thus, the properties of the affinity membrane seem to provide an adequate approach to HIgG separation and purification based on the ion-exchange properties and, will be useful for purifying HIgG and monoclonal antibodies from various biological sources.

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