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Affinity membrane chromatography: relationship of dye-ligand type to surface polarity and their effect on lysozyme separation and purification

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Abstract

Two different dye-ligands, i.e. Procion Brown MX-5BR (RB-10) and Procion Green H-4G (RG-5) were immobilised onto poly(2-hydroxyethylmethacrylate) (pHEMA) membranes. The polarities of the affinity membranes were determined by contact angle measurements. Separation and purification of lysozyme from solution and egg white were investigated. The adsorption data was analysed using two adsorption kinetic models the first order and the second order to determine the best-fit equation for the separation of lysozyme using affinity membranes. The second-order equation for the adsorption of lysozyme on the RB-10 and RG-5 immobilised membranes systems is the most appropriate equation to predict the adsorption capacity for the affinity membranes. The reversible lysozyme adsorption on the RB-10 and RG-5 did not follow the Langmuir model, but obeyed the Temkin and Freundlich isotherm model. Separation and purification were monitored by determining the lysozyme activity using *Micrococcus lysodeikticus* as substrate. The purities of the eluted lysozyme, as determined by HPLC, were 76 and 92% with recovery 63 and 77% for RB-10 and RG-5 membranes, respectively. For the separation and purification of lysozyme the RG-5 immobilised membrane provided the best results. The affinity membranes are stable when subjected to sanitization with sodium hydroxide after repeated adsorption–elution cycles.

Keywords: Surface polarity; Lysozymes; Dye-ligands

1. Introduction

In the affinity membrane chromatography, pseudo-specific ligands, e.g. dyes, amino acids and chelated metal ions have been used instead of biospecific ligands [1–5]. The change in surface properties was found to affect the interaction of surface with the surroundings [6–9]. In particular, protein adsorption depends on the surface composition and morphology of the membrane. There are many reports that adsorption of cells and proteins on the polymer membrane surface are quantitatively changed, depending on the type of ligands immobilised [10–14]. The adsorption of protein is a very complex process, which can be determined by several factors. Among these, the chemical structure, surface roughness, the degree of hydrophilicity of surface, electrostatic interactions of the protein molecules with each other and with surface, and the structural stability of protein molecules are the most important [6,14–17]. Therefore, the surface polarity of an adsorbent should be characterized to explain its interactions with proteins. The surface polarity of solids can be obtained from contact angles measurements using different test liquids [18,19].

Equilibrium binding studies have shown that a simple Langmuir-type may not adequately describe protein binding to affinity supports: binding heterogeneity as evidenced by non-linear Scatchard plots is often observed for binding proteins. The protein adsorption on different functional heterogeneous surface could be better described by the Temkin isotherm model [5,20]. The Temkin isotherm offers two advantages over other isotherm models to explain the heterogeneous nature of protein adsorption on various ion exchange and affinity adsorbents [20]. First, for low to moderate coverage, adsorption is described by two physically meaningful parameters, which can be determined by equilibrium adsorption experiments. Second, the Temkin isotherm satisfies Henry's law, a necessary requirement in employing local equilibrium theory to predict chromatographic behaviour from equilibrium adsorption expressions [21].

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Lysozyme (E.C.3.2.1.17) is a commercially important enzyme and is currently used in food technology and in pharmacological technology as a drug for treatment of ulcers and infections. The potential use of lysozyme as an anticancer drug and in the treatment of HIV infection has also been discussed [22,23].

In the present study, two different dye-ligands immobilised affinity membranes surfaces were tested for its propensity to adsorb lysozyme in a batch system. The adsorption conditions (i.e. concentration of lysozyme, temperature, medium pH, ionic strength) were varied to evaluate their effects on the performances of the affinity membranes. The adsorption kinetics of lysozyme on the different dye-ligands immobilised affinity membranes were studied to characterize of the surface complexation reaction. The adsorption isotherm was measured in order to evaluate the discrepancy between the experimental data and the theoretical equilibrium capacity predicted from the kinetic equations. Experiments have been carried out to determine the surface polarities of the affinity membranes after ligands attachment by contact angles measurements. Finally, these affinity membranes were used for the purification of lysozyme from egg white. The purity of the eluted lysozyme was determined by HPLC and the activity of the purified lysozyme was measured using *Micrococcus lysodeikticus* as a substrate microorganism.

2. Experimental

2.1. Materials

Lysozyme (chicken egg white, EC 3.2.1.7) was supplied from Sigma (St. Louis, MO, USA) and used as received. 2-Hydroxyethylmethacrylate (HEMA) was obtained from Fluka AG (Switzerland), distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. α-α′-Azoisobutyronitrile (AIBN), Procion Green H-4G (Reactive Green 5), Procion Brown MX 5BR (Reactive Brown 10), glycerol, diiodomethane 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. Dye-ligand immobilisation onto pHEMA membrane

The synthesis of the pHEMA membrane was carried out as previously described method [1]. RB-10 and RG-5 were covalently immobilised onto pHEMA membrane via the nucleophilic reaction under alkaline conditions. RB-10 or RG-5 (300 mg) was dissolved in distilled water (10 ml), and transferred to NaCl solution (1.0 M, 60 ml) in which pHEMA membrane disks (6 g) were equilibrated for 1 h. After this period, sodium carbonate solution (3.2 M, 30 ml) was added to the medium (about pH 10) and heated at 80 °C for 4 h in a sealed reactor. After the reaction, the pHEMA membranes were washed several times with distilled water, 2.0 M NaCl and 10% methanol.

2.3. Optimisation of lysozyme separation conditions

Separation of lysozyme from solution was studied at various pHs, in either acetate (7.5 ml, 50 mM, pH 4.0–5.5) or in phosphate buffer (7.5 ml, 50 mM, pH 6.0–8.0). The adsorption experiments were conducted at 25 °C, for 2 h while continuous stirring. The effect of temperature and ionic strength on lysozyme adsorption was carried out in phosphate buffer (7.5 ml, 50 mM, pH 7.0, containing 1.0 mg ml^{-1} lysozyme) at four different temperatures (i.e. 5, 15, 25 and 35 °C) and at three different NaCl concentrations (i.e. 0.1, 0.5 and 1.0 M), respectively. In all these experiment, the initial concentration of lysozyme was 1.0 mg ml^{-1}. To determine the adsorption capacities of the affinity membranes, the initial concentration of lysozyme was changed between 0.05 and 3.0 mg ml^{-1}. The time to reach equilibrium adsorption was found to be 90 min and in the rest of the study 120 min adsorption duration time was employed. The concentration of lysozyme was measured at 280 nm by using a double beam UV-Vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601). A calibration curve was prepared using lysozyme as a standard (0.05–3 mg ml^{-1}).

2.4. Lysozyme purification from egg-white by affinity membranes

Chicken egg white was separeted from fresh eggs and diluted to 50% (v/v) with phosphate buffer (50 mM, pH 7.0). The diluted egg-white was homogenised in an ice-bath and centrifuged at 4 °C, at 12,000 rpm for 15 min. The supernatant fluid was used as a lysozyme source. The elution of proteins from affinity membrane was performed with phosphate buffer (50 mM, pH 8.0) containing 0.5 M NaCl.

In the purification experiments, the activity of lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of o/n culture of *M. lysodeikticus* cells suspended in phospate buffer (0.1 M, pH 7.0) was followed for 6 min after addition of lysozyme and/or elution. One unit lysozyme activity was defined as the amount of enzyme causing a decrease of 0.001 o.d. value per min at 25 °C and pH 7.0. The purity of lysozyme in the purified samples was analysed using HPLC.

2.5. Lysozyme elution and stability of affinity membranes in repeated use

The lysozyme elution was performed in a buffer solution containing 1.0 M NaCl at pH 8.0. The elution ratio was

calculated from the amount of lysozyme adsorbed on the affinity pHEMA membranes and the amount of lysozyme desorbed. To determine the reusability of the dye-ligands immobilised pHEMA membranes the adsorption and desorption cycle was repeated five times by using the same affinity pHEMA membranes.

2.6. Instrumentation and chromatographic conditions

A Dionex HPLC system (Dionex Co., Germering, Germany) was used for the chromatographic studies. The HPLC system consisted of a quaterary 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). Chromatographic 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 protein 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 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 detector 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.

2.7. First- and second-order equations

The large number and different chemical groups on the adsorbents surface were created during preparation of dye-ligand immobilized pHEMA membranes [i.e. $–NH_2$, =NH, $–SO_3H$, –OH, –COOH and hyrophobic groups], imply that there are many types of lysozyme–dye-matrix 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 [24]. It may be represented as follows:

$$\frac{dq_t}{dt} = k_1(q_{eq} - q_t) \quad (1)$$

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 lysozyme at equilibrium and at time t (mg ml^{-1}), respectively. After integration by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$, gives

$$\log\left(\frac{q_{eq}}{q_{eq} - q_t}\right) = \frac{k_1 t}{2.303} \quad (2)$$

Eq. (3) can be rearranged to obtain a linear form

$$\log(q_{eq} - q_t) = \log q_{eq} - \left(\frac{k_1 t}{2.303}\right) \quad (3)$$

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.

Ritchie proposed a second-order rate equation for the kinetic adsorption of gases on solids [25]. The second-order equation was applied for adsorption of solutes on the adsorbents [26]. The second-order equation based on adsorption equilibrium capacity may be expressed in the form:

$$\frac{q_m}{q_m - q_t} = k_2 t + 1 \quad (4)$$

The linear form of equation is

$$\frac{1}{q_t} = \frac{1}{k_2 q_m t} + \frac{1}{q_m} \quad (5)$$

From the Eq. (5), 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.

2.8. Contact angle studies

The contact angle values to different test liquids (i.e. water, glycerol and diiodomethane) of the membrane 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). Both the left- and right contact angles and drop dimension parameters (i.e. size of spherical sample drop) were automatically calculated from the digitalized image. The measurements were the average of 15 contact angles at least operated on three membrane samples.

3. Results and discussion

3.1. Properties of dye-ligands immobilised pHEMA membranes

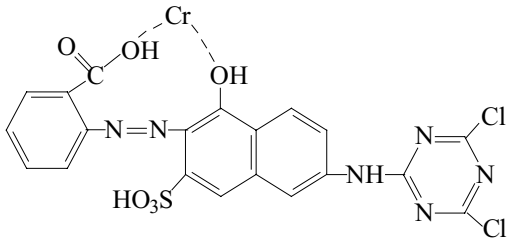
The macroporous poly(2-hydroxyethylmethacrylate) (pHEMA), membrane was prepared according to a previous methods [1]. The main physical and morphological properties of the dye-ligands immobilized pHEMA membrane were as follows: the water content of the dye-ligand immobilized membrane was 58.5%, the thickness in the wet state of the membrane was 0.06 cm; the density of the pHEMA membrane in the dry state was 1.26 g cm^{-3}, the plain surface area (smooth surface of the membranes without pores)

of the 1.0 ml wet membrane was 36.7 cm^2. The surface area of the membrane (include the pores of the membrane) was found to be 6.12 g m^2 by BET method. Elemental analysis of the RB-10 and RG-5 immobilised pHEMA membrane was performed, and the dye content on the membrane was found to be 0.312 μmol ml^{-1} for RB-10 and 0.254 μmol ml^{-1} RG-5 from the sulphur and nitrogen stoichiometry.

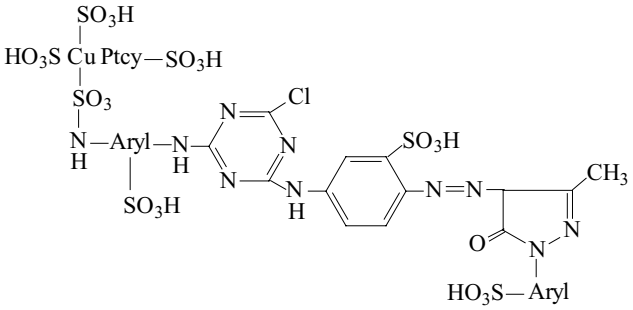
3.2. Adsorption studies

3.2.1. Effect of pH

Fig. 1 shows the effect of pH on the amount lysozyme adsorbed on dye-ligands immobilised affinity pHEMA membranes. The maximum lysozyme adsorption was obtained at pH 7.0 with RB-10 (32.6 mg ml^{-1}) and RG-5 (45.0 mg ml^{-1}) immobilised pHEMA affinity membranes. These results indicate that the pH of the medium has an important effect on the adsorption equilibrium of lysozyme, and there are preferential interaction between lysozyme and both dye-ligands immobilised onto pHEMA membrane at pH 7.0. The isoelectronic (p*I*) value of lysozyme is 11.2. The lysozyme molecules would be cationic at pH values below 11.2. Since proteins are amphoteric, the number of charges on the surface of a protein will vary with the pH of the medium. It should be noted that ion-exchange properties of a protein could not be satisfactorily explained by the 'net charge' concept and that the process is primarily governed by the distribution of charges on the proteins. Most of the lysozyme variants were resolved by a strong cation exchanger, indicating that cation exchange of lysozyme involves nearly the whole external surface of the protein [27]. RB-10 has an acidic sulfonate and carboxyl groups, a chelated Cr(III) ion and a bulk hydrophobic group. RG-5 molecule has seven acidic sulfonate, three secondary amino groups and Cu(II) ion containing phtahalocyanine group (Fig. 2). As seen from the figure, the binding of lysozyme on the dye-ligands immobilised membranes should be a combination of ion-exchange and hydrophobic interactions [1,5].



Procion Brown MX 5BR



Ptcy = Phthalocyanine moiety

Procion Green H-4G

Fig. 2. Chemical structure of Procion Brown MX 5BR and Procion Green H-4G.

3.2.2. Effect of temperature

The adsorption rates for the binding of lysozyme to the RB-10 and RG-5 immobilised affinity membranes were obtained at different temperatures. The adsorption rate is exemplified in Fig. 3 for RG-5 immobilised membrane. The equilibrium adsorption of lysozyme onto both affinity membranes significantly increased with increasing temperature. At higher temperature, the contact area between the

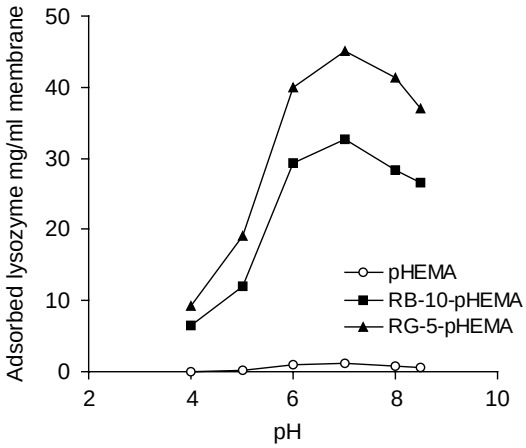


Fig. 1. Effects of pH on lysozyme adsorption on the RB-10 and RG-5 immobilised affinity membranes; initial concentration of lysozyme: 1.0 mg ml^{-1}; temperature: 25 °C.

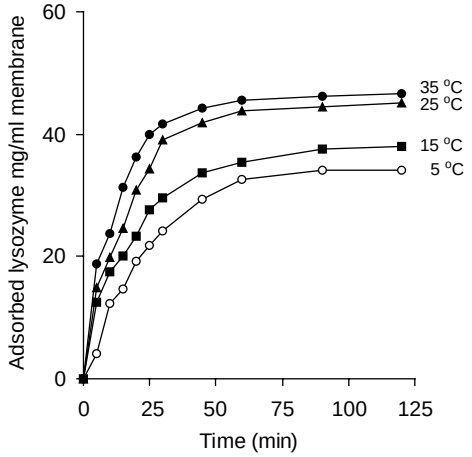


Fig. 3. Effect of temperature on the lysozyme adsorption capacity on the RG-5 immobilised affinity membranes; initial concentration of lysozyme: 1.0 mg ml^{-1}; pH: 7.0.

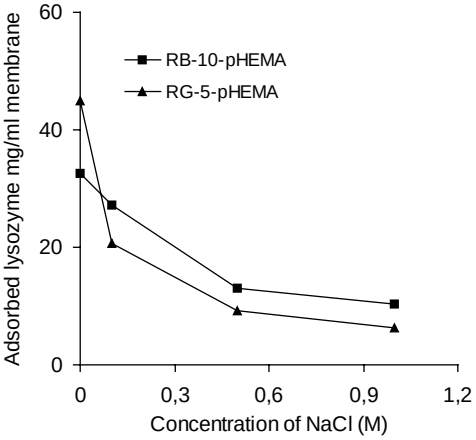


Fig. 4. Effects of ionic strength on lysozyme adsorption on the RB-10 and RG-5 immobilised affinity membranes; initial concentration of lysozyme: 1.0 mg ml^{-1}; temperature: 25 °C, pH: 7.0.

protein and the dye-ligand on the matrix should increase, resulting in increase in the affinity of proteins for the adsorbents [10]. From 5 to 35 °C, the adsorption capacity of the RB-10 and RG-5 immobilised affinity pHEMA membranes for lysozyme increased about 30, and 27%, respectively. These could be attributed to the chemical interaction between the dye-ligand and the lysozyme molecules as the temperature increased.

3.2.3. Effect of salt concentration

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 lysozyme and the immobilized dye-molecules. The adsorption capacities of RB-10 and RG-5 immobilised affinity membranes to lysozyme were reduced by 3.1 and 7.2 folds with increasing presence of salt concentration from zero to 1.0 M (Fig. 4). The decrease in lysozyme adsorption capacities of both affinity membranes with increasing ionic strength should be resulted from decrease in the electrostatic interactions between lysozyme and the dye-ligand molecules. RB-10 and RG-5 contain both hydrophobic and charged groups. It is to be noted that the combination of these two forces should be involved during the adsorption process between dye-ligands and lysozyme molecules. As the ionic strength increased in the adsorption medium, the electrical double layer around the molecules, given by the Debye–Hünkel length, decreased. Rusremier and Killmann reported that with increasing electrolyte concentration in the medium the surface charge are screened [28,29]. These effects cause the electrostatic force between molecules to decrease. Consequently, the total electrostatic energy between the molecules decreases and the system becomes aggregating.

3.2.4. Effect of initial lysozyme concentration

The adsorption capacities of the affinity membranes were determined by changing the initial concentration of lysozyme between 0.05 and 3.0 mg ml^{-1}. An increase the lysozyme concentration in adsorption medium led to a linear increase in the amount of adsorbed lysozyme onto both affinity membranes up to 2.0 mg lysozyme ml^{-1} in the adsorption medium. It should be noted that there was a small amount of lysozyme adsorption on the pHEMA membrane (1.9 mg ml^{-1}). The attachment of RB-10 and RG-5 on the pHEMA membrane significantly increased the lysozyme adsorption capacity (about 32.4 and 42.9 fold) of the pHEMA membrane up to 60.6 and 80.2 mg ml^{-1}, respectively. It is clear that this increase is due to ternary complex formation between pHEMA membrane, dye-ligand and lysozyme molecules.

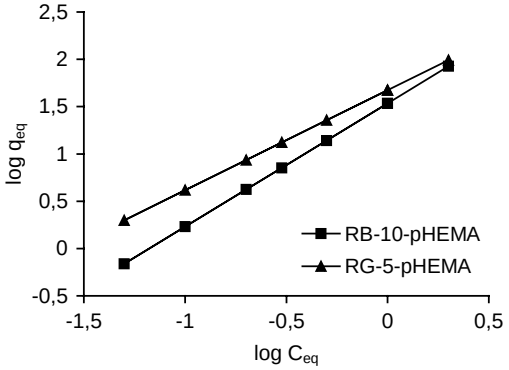


Fig. 5. The Freundlich isotherm plots for the RB-10 and RG-5 pHEMA membranes.

3.3. Adsorption isotherms

Three theoretical isotherm models were used to fit the experimental data: Langmuir, Freundlich and Temkin model. The Langmuir model is based on assumption homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. Since the Langmuir model is formulated for homogenous adsorption. The Temkin and the Freundlich isotherm models are usually adopted for heterogeneous adsorption.

The Freundlich isotherm is frequently used to describe the adsorption. It relates the adsorbed concentration as the power function of solute concentration. One limitation of the Freundlich model is that the amount of adsorbed solute increases indefinitely with the concentration of solute in the solution. This empirical equation takes the form:

$$q_{eq} = K_F (C_{eq})^{1/n} \quad (6)$$

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 (Fig. 5).

The Temkin isotherm model describes the behaviour of many adsorption systems on heterogeneous surface and it is based on the following equation:

Table 1
Freundlich constants for dye-ligands immobilised affinity membranes

Types of membranes	Freundlich parameters		
	K_F (mg ml^{-1})	n	R^2
pHEMA-RB-10	34.1	1.3	0.984
pHEMA-RG-5	47.2	1.1	0.989

$$q = q_T \ln(1 + K_T c) \quad (7)$$

where K_T (ml mg^{-1}) is the equilibrium binding constant corresponding to the maximum binding energy ($K_T = \exp(-\Delta G_{max}/RT)$, c (mg ml^{-1}) is the concentration of protein in the solution at equilibrium, q (mg protein ml^{-1} membrane) is the amount of protein adsorbed on the affinity membrane surface, and q_T (mg protein ml^{-1} membrane) is the differential surface capacity for protein adsorption per unit binding energy. In the case of Temkin-type fit the experimental data, the semi logarithmic plot of $\ln C_{eq}$ versus q_{eq} was employed to generate the intercept value of $\ln K_T$ and the slope of q_T.

The corresponding semi-reciprocal plots and Scatchard plots gave a non-linear plot for the RB-10 and RG-5 immobilised affinity membranes. In other words, a non-linear Scatchard plot indicates the adsorption heterogeneity [30,31]. Since the adsorption of lysozyme onto the affinity membrane cannot be described in terms of the Langmuir model. The magnitude of K_F and n values of Freundlich model showed easy uptake of lysozyme from aqueous medium with a high adsorption capacity of the dye-ligands immobilised pHEMA membranes. Values of $n > 1$ for both affinity membranes indicates positive cooperativity in binding and a heterogeneous nature of adsorption (Table 1).

In the case of the Temkin model, the corresponding semi-logarithmic plots gave rise to linear plot for the binding of lysozyme, to RB-10 and RG-5 immobilised pHEMA membranes and the correlation coefficient of the semi-logarithmic plots (R^2) was above 0.989 for the affinity membranes, indicating the Temkin model best fitted the experimental data. The fitted curves and the fitted parameter values for Temkin model are presented in Fig. 6 and Table 2, respectively. For the adsorption process of a protein, the possible binding sites can be non-specific (such as ionic, hydrophobic, etc.) or specific (affinity). All these interactions between lysozyme and RB-10 and RG-5 immobilised pHEMA membranes should result in uniform binding energies, up to some maximum binding energy (ΔG_{max}). In these cases, ΔG_{max} values were decreased from −6.71 to −7.57 and from −7.01 to −8.60 kcal mol^{-1} for the RB-10 and RG-5 immobilised pHEMA membranes as the adsorbed lysozyme increase on the adsorbents surface, respectively. These results indicate that there is a relationship between the surface interactions groups of lysozyme with the presented affinity pHEMA membranes. From these observations, the range and distribution of binding energies should depend on the density, and type of functional groups, both on the protein and the adsorbent surface. As indicated in the Temkin model, the binding energy decreased with increasing the amounts of adsorbed protein on the adsorbents surface (Table 2).

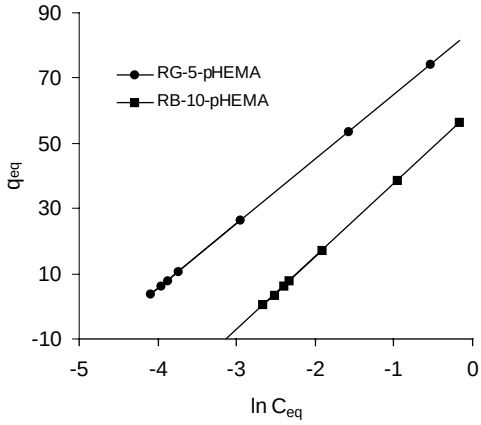


Fig. 6. Semi-logarithmic plots for the Temkin isotherm for RB-10 and RG-5 immobilised affinity membranes. RB-10 content: 0.312 μmol ml^{-1}; RG-5 content: 0.254 μmol ml^{-1}; pH: 7.0; temperature: 25 °C.

3.4. Thermodynamic parameters

The dependency of the equilibrium binding constant (K_T) versus $1/T$ for the binding of lysozyme on the RB-10 and RG-5 immobilized membranes was analysed in terms of Van't Hoff plots. The ΔG values for lysozyme adsorbed on the dye-ligands immobilized membrane were calculated for each temperature and tabulated in Table 2. The negative

Table 2
The Temkin isotherms parameters for dye-ligands immobilised affinity membranes

Types of membranes	Temperature (K)	q_m (mg ml^{-1})	q_t (mg ml^{-1})	K_T (M^{-1}) × 10^5	ΔG_{max} (kcal mol^{-1})	ΔS (kcal mol^{-1} K^{-1})
pHEMA-RB-10	278	51.66	17.59	1.87	−6.71	29.41
	288	56.64	18.26	1.99	−6.98	29.31
	298	60.56	22.39	2.16	−7.27	29.27
	310	68.23	26.51	2.35	−7.57	29.29
pHEMA-RG-5	278	60.82	18.4	3.21	−7.01	54.38
	288	67.72	18.42	5.24	−7.54	54.24
	298	80.21	19.87	8.14	−8.06	54.16
	310	84.24	20.97	12.77	−8.61	53.84

Table 3
The first-order and second-order kinetics constants for dye-ligands immobilised affinity membranes

Types of membranes	Experimental q_{eq} (mg ml^{-1})	First-order kinetic			Second-order kinetic		
		$k_1 \times 10^2$ (min^{-1})	q_{eq} (mg ml^{-1})	R^2	$k_2 \times 10^2$ g mg^{-1} min^{-1}	q_{eq} (mg ml^{-1})	R^2
pHEMA-RB-10	60.6	3.85	50.2	0.993	1.04	64.9	0.998
pHEMA-RG-5	80.2	5.11	70.0	0.959	9.01	83.7	0.997

ΔG values for each temperature indicated that adsorption of lysozyme on the both dye-ligand immobilized membrane was a favourable process and those were ranged between −6.71 and −7.57 kcal mol^{-1} for RB-10 and −7.01 and 8.61 kcal mol^{-1} for RG-5. These values are of similar magnitude to those obtained by Finette et al. [10] in their study of the binding of lysozyme onto IDA-Cu(II) immobilized onto Fractosil 1000, where the ΔG values were ranged between −5.54 and −5.80 kcal mol^{-1} at different temperatures.

The ΔS values for the adsorption of lysozyme to both dye immobilized affinity membranes are presented in Table 2. At lower temperatures, due to lower kinetic motion of the dye-ligands, the value of the phase ratio will be different to that at higher temperatures. Positive values for the ΔS were obtained for the adsorption of lysozyme onto dye-ligand immobilized membrane, indicating an increase in the total disorder of the system during adsorption [32]. The calculated ΔH values of the system for the interaction for the lysozyme with the RB-10 and RG-5 affinity membranes were 1.46 and 8.08 kcal mol^{-1}, respectively.

3.5. Kinetics modelling

In order to analyse the adsorption kinetics of lysozyme, the first-order and the second-order kinetics models were applied to the experimental data. The second-order equation fitted well with the experimental data. The comparison of experimental adsorption capacities and the theoretical values estimated from the above two equations are presented in Table 3. The theoretical q_{eq} values estimated from the first-order kinetic model gave significantly different values compared to experimental values, and the correlation coefficients were also found to be slightly lower. These results showed that the first-order kinetic model did not well describe these affinity membrane systems.

The correlation coefficients for the linear plots of $1/qt$ against $1/t$ for the second order equation are greater than 0.99 for all the adsorbents for contact times of 120 min (Fig. 7). The theoretical q_{eq} values for all the tested adsorbent systems were very close to the experimental q_{eq} values in the case of second order kinetics. The second order kinetics best described the data.

In addition, Arrhenius plots in the temperature range from 5 to 35 °C obtained from $1/T$ versus $\ln k_2$ (k_2; second order rate constant) appear linear; activation energies were found to be 18.22 and 5.84 kJ mol^{-1} for RB-10 and RG-5 immobilised membranes, respectively. The lower activation energy calculated for the RG-5 ligand indicates that the adsorption of lysozyme on the RG-5 ligand is more favourable than the RB-10 ligand. The binding of lysozyme on the RB-10 dye-ligand immobilised membrane may require a large conformational deformation, thereby resulting in higher activation energy for the molecule to reorganise and attain the proper conformation for binding to the immobilised dye-ligand. This result also supports that the second-order kinetic model well fit the experimental data.

3.6. Evaluation of lysozyme adsorption behaviour of affinity membranes

The variation of the wetting force is extremely sensitive to the surface characteristics since it reflects the effect of functional groups in a surface layer thick (less 10 Å) and in direct contact with the liquid phase [26,33]. The contact angle values of two polar liquids (i.e. water and glycerol) and an apolar liquid (i.e. diiodomethane) for the investigated membrane samples are presented in Table 4. The pHEMA and both dye-ligand immobilised and their lysozyme-adsorbed counterpart membranes gave quite different surface characteristics depending on the presence of functional groups. The variation of the contact angle values after immobilisation of dye-ligands show that the hydrophobicity of the surfaces is increased with respect to the pHEMA membrane. As seen from Table 4, the RG-5 immobilised pHEMA membrane surface is less hydrophobic than that of the RB-10-pHEMA as shown by contact angle measurements whereas has a higher lysozyme adsorption capacity (80.2 mg ml^{-1}). It was 60.6 mg ml^{-1} for RB-10 immobilized membrane. The

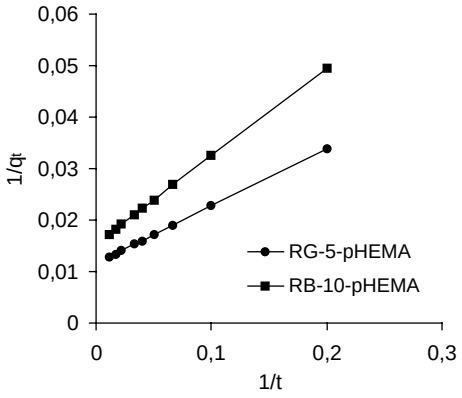


Fig. 7. Second-order-kinetics plot of the experimental data for RB-10 and RG-5 immobilised affinity membranes.

Table 4
Contact angles of various test liquids on the pHEMA and RG and RB dye-ligand immobilised affinity membranes

Types of membranes	Test liquid		
	Water θ (°)	Glycerol θ (°)	Diiodomethane θ (°)
pHEMA	55.6 ± 1.4	53.6 ± 4.8	39.3 ± 1.0
pHEMA-RG-5	63.9 ± 1.7	59.2 ± 2.5	33.0 ± 0.1
pHEMA-RG-5-lysozyme	71.1 ± 1.2	60.1 ± 1.1	46.5 ± 1.2
pHEMA-RB-10	78.0 ± 1.2	64.9 ± 3.0	28.3 ± 0.2
pHEMA-RB-10-lysozyme	63.7 ± 3.7	58.5 ± 1.8	34.6 ± 1.3

lysozyme adsorption modifies the surface characteristics of both affinity membranes as is presented by the changing contact angle values of membranes (Table 4). These results indicated that lysozyme adsorptions strongly depend on the structure and distribution of functional groups (i.e. ionic and hydrophobic groups) on the surface of the different dye-ligand immobilised membranes.

Lysozyme with a p*I* value of 11.2 contains a large number of –OH and $–NH_2$ groups and it is positively charged at pH 7.0 whereas RG-5 dye-ligand molecule has seven net negative charges. From this point of view, the interactions between lysozyme and immobilised RG-5 should be governed by the charge-charge interactions. For RB-10 immobilised membrane more hydrophobic and adsorbed amount of lysozyme lower than the RG-5 immobilised membrane. In this case, hydrophobic interaction should be predominant and, as observed in the purification studied, the obtained purity of lysozyme with RB-10 is lower than the RG-5 dye-ligand immobilised membrane. This may be resulted from the non-specific hydrophobic interactions of RB-10 dye-ligand with other proteins present in the egg white solution. The others egg proteins (i.e. ovalbumin, conalbumin, ovomucoid, ovomucin, G2 globulin, G3 globulin, and ovoinhibitor) have p*I* values between 4.1 and 6.1 [22]. Only, the lysozyme positively charged at pH 7.0 and all other egg white proteins negatively charged. Therefore, we expected that the charge–charge interaction could predominantly take place between the RG-5 immobilised membrane and lysozyme. From these observations, the lysozyme molecules should interact from different patches with each immobilised dye-ligand. After first lysozyme layer formation on each dye-ligand immobilised membrane surfaces, the adsorbed lysozyme molecules should re-organise and create another energetically favourable layers according to interaction side of the immobilised ligand molecules. By this way, as stated in the Temkin isotherm model, the adsorbed lysozyme layers on the affinity membrane surfaces should increase until the formation of energetically unfavourable layers.

3.7. Purification of lysozyme from egg white

The content of lysozyme in chicken egg white is about 3.4%. The classical lysozyme purification method required several steps, such as precipitation, centrifugation and adsorption. The purity of the lysozyme eluted from both dye-ligand immobilised affinity membrane was determined by HPLC (Fig. 8). The purities of the eluted lysozyme, as determined by HPLC, were 76 and 92% with recovery 63 and 77% for RB-10 and RG-5 membranes, respectively. For separation and purification of lysozyme the RG-5 immobilised membrane provided the best results. The RG-5 immobilised pHEMA membrane provided an efficient single step method to purify lysozyme from diluted egg white, showing high binding capacity and high selectivity for lysozyme. On the other hand, the RB-10 immobilised pHEMA membrane produced a low recovery yield and purification factor for lysozyme than that of the RG-5 immobilised pHEMA membrane. The specific activity of the lysozyme purified with RG-5 affinity membrane was 41 400 U mg^{-1}. This was for RB-10 immobilised membrane 34.200 U mg^{-1}.

3.8. Elution of adsorbed lysozyme and regeneration of affinity membranes

The lysozyme loaded affinity membranes was placed within the elution medium containing 1.0 M NaCl at pH 8.0. Both affinity membranes more than 98% of the adsorbed lysozyme were eluted when 1.0 NaCl was used as an elution agent. The elution results showed that NaCl is a suitable desorption agent for lysozyme from both affinity membranes.

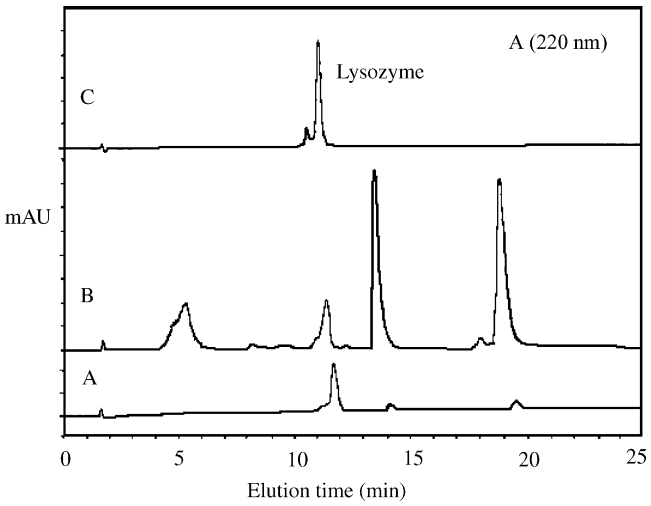


Fig. 8. HPLC chromatogram showing: (A) commercial lysozyme; (B) whole egg white; (C) purified lysozyme with RG-5 immobilised affinity membrane.

In order to show the reusability of the both affinity membrane, adsorption–desorption cycle of lysozyme was repeated five times by using the same affinity membrane and the adsorption capacity of the affinity pHEMA membranes did not changed during the repeated adsorption–desorption operations. After washing the affinity membranes with 0.1 M NaOH and 1.0 M NaCl for 4 and 2 h, respectively, the affinity membranes recovered their original capacities.

4. Conclusion

The immobilization of two different dye-ligands on the membrane changed the contact angles values and the polarity of the membrane surfaces. Both dye-ligands immobilised and their lysozyme adsorbed counterpart membranes gave quite different surface characteristics depending on the presence of functional groups. The pH of the medium has an important effect on the adsorption equilibrium of lysozyme, and there is a preferential interaction between lysozyme and both dye-ligands immobilised pHEMA membrane at pH 7.0. The equilibrium adsorption of lysozyme onto all the tested adsorbents significantly increased with increasing temperature. The lysozyme adsorption capacities of both affinity membranes were decreased with increasing ionic strength. As indicated in the Temkin model, the binding energy decreased with increasing the amount of adsorbed lysozyme on the affinity membrane surface. The theoretical q_{eq} values for all the tested adsorbent systems were very close to the experimental q_{eq} values in the ease of second order kinetics. The second order kinetics best described the data. The purities of the eluted lysozyme, as determined by HPLC, were 76 and 92% with recovery 63 and 77% for RB-10 and RG-5 membranes, respectively.

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