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Comparison of Ligand Density and Protein Adsorption on Dye-Affinity Membranes Using Different Spacer Arms

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ABSTRACT

The use of 1,6-diaminohexane, 1,10-diaminodecane, 1,6-diaminohexane + ethylene glycol diglycidyl ether (EGDGE), and 1,10-diaminodecane + EGDGE as spacer arms for the immobilization of Cibacron Blue 3GA onto Immobilon AV membranes was investigated in this work. The dye ligand density was optimized by the employment of 1,6-diaminohexane, followed by 1,10-diaminodecane, 1,10-diaminodecane + EGDGE, and 1,6-diaminohexane + EGDGE. The adsorption properties of lysozyme onto the dye-affinity membranes were also studied. The results show that the use of 1,10-diaminodecane as a spacer molecule appears to offer the best adsorption capacity and hence results in the highest percentage of ligand utilization. Consequently, a 12-atom straight molecule such as 1,10-diaminodecane is a good choice for dye-affinity membrane separations.

INTRODUCTION

Recently, the use of membrane materials as solid supports has demonstrated its potential in overcoming the severe mass-transfer limitations of traditional column techniques (1–4). Low transmembrane pressure drops and high flow rates reduce the required time for a separation cycle. As a result of the reduced cycle time, the possible denaturation of ligand and biomolecules can be minimized, thus raising the economic efficiency. Accordingly, more and more

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studies have been made in an attempt to improve the application of adsorptive membranes (5–17).

Different types of membrane have been exploited for the adsorption process, including membrane disk, hollow fiber, spiral-wound membrane, polymer rod, etc. (3, 4). Among them, the membrane disk and hollow fiber are most frequently utilized and studied in the literature. Their adsorption performance and efficiency on a laboratory scale have been extensively investigated in recent studies (5–17). The major advantages of using membrane disks come from the fact that their commercial products are of various sizes and materials, which can provide more choices for the customers to meet their requirements. Moreover, the design of a disk holder is usually simpler and the cost for both the holder and membrane itself is lower as compared to other types of membranes. The practical applications of membrane disks, however, are still restricted by several problems. The crude solutions may not be directly applied due to the fouling of membranes. In addition, flow maldistribution and leaking from the disk edge may become severe in a large-scale design (3). On the other hand, hollow fibers are selected as the solid supports specifically due to their high specific surface area, which increases the relative adsorption capacity. Besides, since the traditional hollow fiber holder is frequently designed in a crossflow manner to facilitate an efficient filtration process, it is possible to induce an effective separation of the crude solutions. Combining the advantages of both the flat-sheet membrane disk and hollow fiber design, a crossflow design of a flat-sheet membrane, that is, a plate-and-frame adsorptive filter, may be suggested for more effective adsorptive membrane separation.

Because of its rectangular shape and design for affinity purposes, the Immobilon AV (IAV) membrane of Millipore Company (Bedford, MA, USA) can be a good choice as the solid support for a plate-and-frame adsorptive filter. The base material of the IAV membrane is hydrophilic PVDF (polyvinylidene difluoride) that provides low levels of nonspecific protein adsorption. The membrane surface has been chemically activated for the purpose of immobilizing ligands with amine groups (18, 19). In the case where the ligand lacks an active amine group, a suitable spacer arm can be used to connect both the membrane and the ligand molecule. In addition, it is also suggested that utilization of a spacer arm increases ligand accessibility when poor adsorption performance is exhibited for low molecular weight ligands due to their low steric availability (4, 20). To further improve its application, this study is aimed at finding a better length of spacer arm for the immobilization of dye ligand onto the IAV membrane. The results could be eventually applied to the practical application of the IAV membrane in the plate-and-frame filter.

Cibacron Blue 3GA, a popular triazine dye for group-specific affinity separation, was used as the affinity ligand in this work. Due to the absence of ac-

tive amine groups on Cibacron Blue 3GA, diamine was employed in this study as a spacer arm for linkage binding. The diamines tested were 1,6-diaminohexane and 1,10-diaminodecane. Furthermore, considering that the epoxide group is more reactive than the amino group but can not be directly bound with the IAV membrane, ethylene glycol diglycidyl ether (EGDGE) with two epoxide terminals was added after the diamine conjugation. The addition of EGDGE was expected to have two functions: lengthening the spacer arm and increasing the immobilized ligand amount by use of the more reactive epoxide group. The results of ligand density using different spacer arms were compared. The properties of lysozyme adsorption onto the dye-affinity membranes were also investigated.

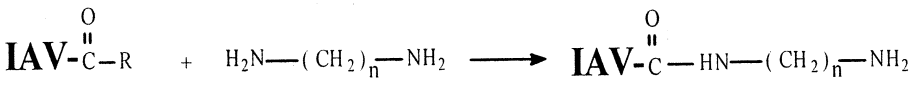
EXPERIMENTAL

Materials

The IAV membrane, with an average pore size of 0.65 μm and a thickness of 140 μm, was purchased from Millipore Company (Bedford, MA, USA). Cibacron Blue 3GA (C9534, MW 774.2) and chicken egg white lysozyme (L6876, MW 14300) were obtained from Sigma Chemical (St. Louis, MO, USA). The reagents 1,6-diaminohexane, 1,10-diaminodecane, and EGDGE were from TCI Company (Tokyo, Japan)

Spacer Arm Conjugation

The IAV membrane was cut into small pieces of 1 cm by 1 cm for the following experiments. An excess amount of diamine was dissolved in 5 mL of 0.5 M phosphate buffer (pH 7.2) and put into a container to be reacted with one piece of the cut, dry IAV membrane. The amount of diamine used was 1 g for 1,6-diaminohexane and 1.5 g for 1,10-diaminodecane, both about 0.008 mole. The reaction was conducted at room temperature for 12 hours. After reaction, the membrane was thoroughly rinsed to remove the unreacted diamine. It may be worthwhile to note that the pH value of the diamine solution was raised to 11 due to the use of a high diamine concentration; this value was beyond the suggested pH range for the membrane, 3 to 10. To check the membrane stability, a piece of blank membrane was incubated in an aqueous solution of pH 11 for 12 hours. The result certified the stability of the membrane under this pH value. The diamine reaction is



As to the addition of EGDGE, 3 g (about 0.005 mole) was dissolved in 5 mL of deionized water and mixed with a piece of membrane with bound di-

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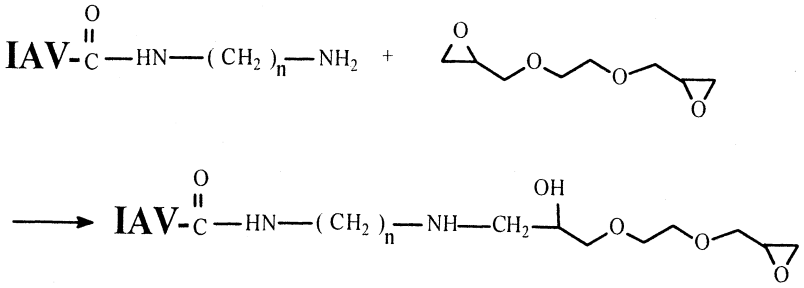
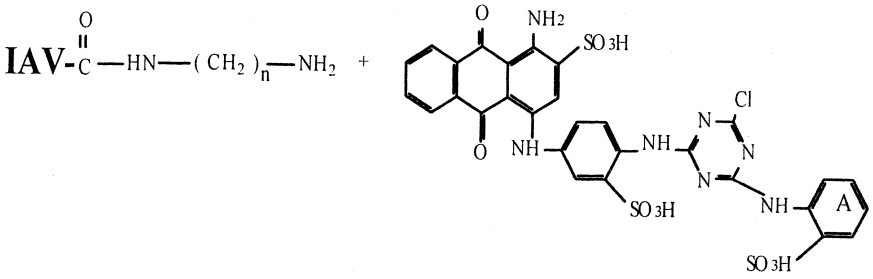
amine at room temperature for 12 hours. The membrane was then washed with deionized water to remove the unreacted reagent. The EGDGE reaction is

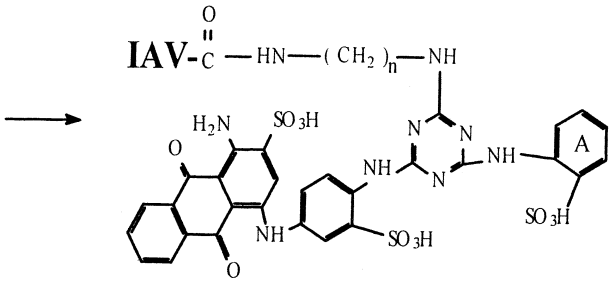


Ligand Immobilization

The immobilization procedure is described as follows: A piece of spacer-bound membrane was incubated with 10 mL of 3% (w/v) dye solution under 60°C for 30 minutes. Ten milliliters of 6% (w/v) NaCl solution was then added into the mixture and the reaction was continuously conducted at the same temperature for 1 hour. Ten milliliters of 2% (w/v) Na_2CO_3 solution was finally added and the temperature was raised to 80°C for another 1 hour of incubation. The membrane was thoroughly rinsed until no blue dye was eluted.

The immobilization of Cibacron Blue 3GA onto diamine-activated IAV membrane is based on the binding of the triazine group of Cibacron Blue 3GA with the free amine group of the spacer molecule. The reaction is





On the other hand, the coupling site of Cibacron Blue 3GA with the amino-EGDGE-activated membrane is on the primary amine on the anthraquinone

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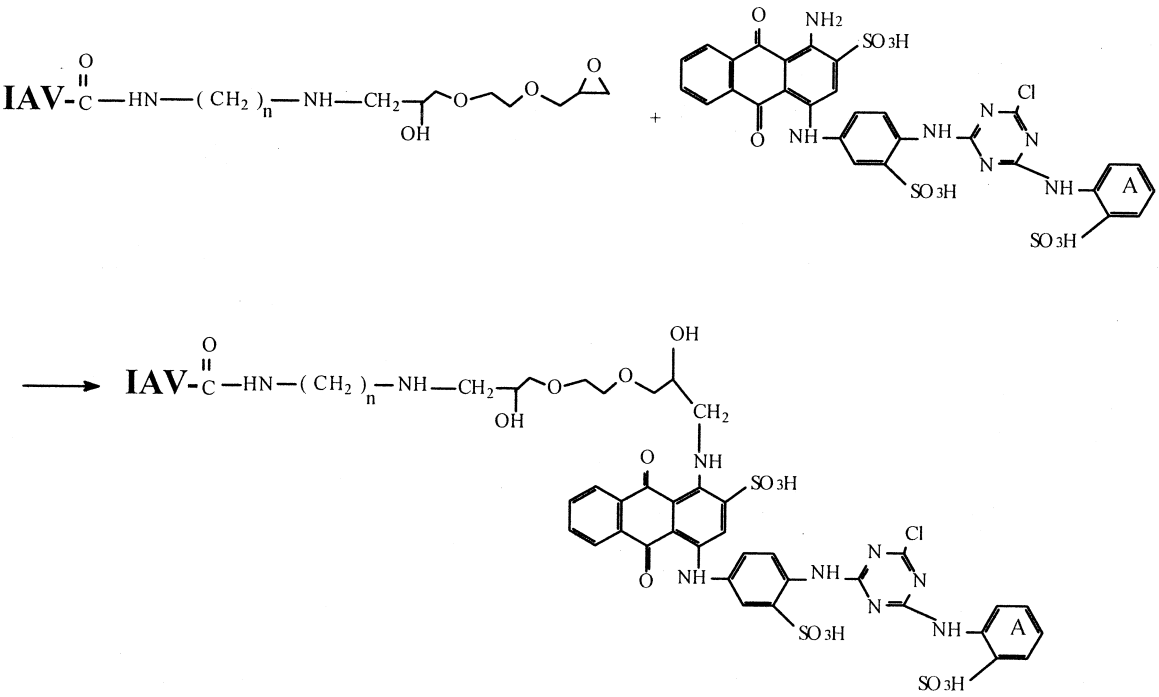
ring of the dye. The reaction is



Batch Adsorption Experiment

The binding of lysozyme with Cibacron Blue 3GA is mainly due to both ionic and hydrophobic interactions (21). At pH values far below 11, the lysozyme molecule would be cationic based on its isoelectric point of 11, and this allows a cationic interaction with Cibacron Blue 3GA. Under this condition, the addition of ionic strength in the adsorption buffer would lead to an adsorption competition between other ions and the lysozyme molecule. Therefore, low ionic strength should be used in the adsorption process in order to enhance lysozyme adsorption onto the Cibacron Blue 3GA-immobilized membrane. In our work a buffer with a low salt concentration and a pH value under 9 was used as the loading buffer, which was 50 mM Tris-HCl, pH 7, with 0.005% NaN_3. In addition, a loading buffer with a higher salt concentration (1 M KCl in Tris-HCl) was used as the desorption agent, i.e., the elution buffer. Both buffers were filtered through 0.2 μm nylon membranes (Lida Manufacturing, Kenosha, WI, USA). A lysozyme solution was prepared with the loading buffer and filtered through 0.45 μm filters (Millex-HV, Millipore, Bedford, MA, USA).

Batch adsorption experiment was conducted at room temperature as follows. Two milliliters of lysozyme solution with a given concentration and a piece of dry membrane with immobilized Cibacron Blue 3GA were incubated for 12 hours in order to reach equilibrium. The lysozyme concentration was

determined by using a UV-Vis spectrophotomer (UV-1601, Shimadzu, Auburn, Australia) at 280 nm. The extinction coefficient of lysozyme $E^{280\ nm}_{1\ mg/mL}$ is 2.65 (21). After each experiment the membrane was washed using elution buffer to completely elute the bound protein out of the membrane.

Ligand Density Measurement

After batch adsorption experiments, the Cibacron Blue 3GA-immobilized membrane was dissolved in 5 N HCl at 120°C for 12 hours. Any remaining membrane backbone was discarded. The solution of dissolved dye was neutralized to pH 7 using 5 N NaOH and then concentrated using a rotary evaporator under reduced pressure. The dye concentration in the concentrated solution was detected by UV-Vis at 620 nm. The extinction coefficient for Cibacron Blue 3GA at 620 nm is 8682 L/mol/cm.

RESULTS AND DISCUSSION

Cibacron Blue 3GA Immobilization and Density on the Membrane

In this work, diamines and EGDGE were used as spacer molecules for the immobilization of Cibacron Blue 3GA onto the IAV membranes. The blue color of the dye displayed on the outer membrane surface indicated the success of using 1,6-diaminohexane, 1,10-diaminodecane, 1,6-diaminohexane + EGDGE, and 1,10-diaminodecane + EGDGE as spacer arms. For comparison, blank IAV membranes (without spacer arm) and using only EGDGE as the spacer arm were tested for the immobilization procedure. It was found that the blue dye could not be bound onto the membrane surface and was completely rinsed out in both these tests. Therefore, it was concluded that at least an active amino group is required for Cibacron Blue 3GA immobilization onto the IAV affinity membrane.

The color intensity obtained with different spacer arms is also visually appreciated. The blue color on both membranes conjugated with diamine + EGDGE was paler than those only bound with diamine. This phenomenon suggests that the employment of EGDGE lessens the ligand immobilization. These results are in contradiction with the expectation that the high reactivity of the epoxide group may assist the immobilization reaction and raise the amount of immobilized ligand. This contradiction may be explained by two reasons. One is that the steric encumbrance of the NH_2 group on the anthraquinone ring of the dye hinders its reaction with the free epoxide group of the amino-EGDGE-bound membranes. The other is that the high reactivity of the epoxide group may lead to the possibility that both end groups of the EGDGE molecule simultaneously reacted with the free amine groups on the

TABLE 1
Ligand Density and % Utilization for Adsorption

Spacer arm	Cibacron Blue 3GA density	Lysozyme capacity	% Utilization
1,6-Diaminohexane	0.110 μmol/cm^2	0.026 μmol/cm^2	24
1,6-Diaminohexane + EGDGE	0.032 μmol/cm^2	0.011 μmol/cm^2	34
1,10-Diaminodecane	0.093 μmol/cm^2	0.045 μmol/cm^2	48
1,10-Diaminodecane + EGDGE	0.053 μmol/cm^2	0.015 μmol/cm^2	28

diamine-bound membrane, which resulted in a decrease of free reactive groups on the membrane surface, and thus lessened immobilization.

To investigate quantitatively the degree of immobilization, the ligand densities immobilized on the membrane using different spacer arms were compared. The experimental results of Cibacron Blue 3GA density are listed in Table 1. It was found that 1,6-diaminohexane has the highest ligand density, followed by 1,10-diaminodecane, 1,10-diaminodecane + EGDGE, and 1,6-diaminohexane + EGDGE. The density using only diamines is two to three times as much as that using diamine + EGDGE. This difference is clearly reflected by the color intensity on the outer membrane surface.

Adsorption Isotherms and Scatchard Plots

The experimental results of lysozyme adsorption to Cibacron Blue 3GA-immobilized affinity membranes using different spacer molecules are illustrated in Fig. 1. To distinguish the effects of specific and nonspecific binding, a blank IAV membrane and membranes with only spacer arms bound but without Cibacron Blue attached were tested for lysozyme adsorption at room temperature. The adsorption capacities for those membranes were found to be near zero, which indicates negligible nonspecific binding. In other words, the existence of free acyl, amine, or epoxide groups on the blank or spacer-bound membranes would not contribute to the binding with lysozyme.

A higher adsorption capacity is observed in Fig. 1 for the results employing only diamine as the spacer arm, which agrees with the ligand density investigation discussed in the previous section. More specifically, the adsorption capacity using 1,10-diaminodecane is greater than that using 1,6-diaminohexane. Related studies in the literature offer a few clues to explain this phenomenon. Using a cellulose and acrylic composite matrix, Hou et al. (5) indicated that a spacer arm length of 11 atoms was sufficient for ligand extension from the solid surface when Protein A was employed as the ligand. They also pointed out that the possibility of arm folding with longer spacer arms

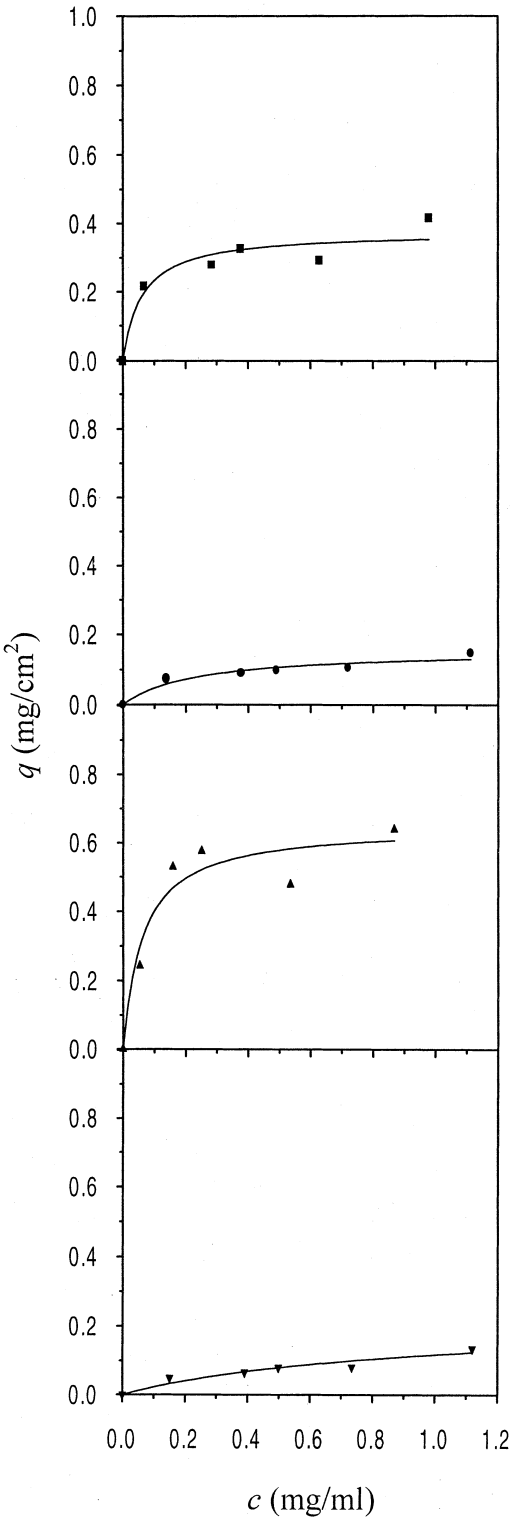


FIG. 1 Isotherms for lysozyme adsorption onto Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Fitting model: Langmuir.

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may cause a difficulty for the protein to touch the ligand on the free end of an arm and thus lead to a sharp reduction in adsorption capacity. Other research (12) on the separation of IgG by L-histidine-immobilized hollow fibers also showed that a spacer arm of 10 carbon atoms rendered the ligand more accessible for adsorption in the separation process than a 3-carbon-atom spacer. In this study, 1,6-diaminohexane is a spacer arm of 8 atoms, 1,10-diaminodecane is of 12 atoms, and the use of EGDGE contributes to an extra length of 10 atoms. The results of adsorption capacity research indicate that the 12-atom straight molecule more effectively eliminates steric hindrance to ligand accessibility than does the 8-atom spacer, which is similar to the results in the studies mentioned above. As to the low protein capacity accompanying the use of diamine + EGDGE, one possible cause is the corresponding low ligand density as obtained in the earlier section, and the other may be arm folding for these long spacer arms, as explained by Hou et al. (5).

Three single-solute isotherm models with two parameters only were used to fit the experimental data:

Langmuir model:

$$q = \frac{cq_{\mathrm{m}}}{K_{\mathrm{d}} + c} \tag{1}$$

Freundlich model:

$$q = kc^{n} \tag{2}$$

Temkin model (22):

$$q = q_{\mathrm{T}} \ln(1 + K_{\mathrm{T}}c) \tag{3}$$

The Langmuir model is based on assumptions of adsorption homogeneity such as all equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. The other two isotherm models are usually adopted for heterogeneous adsorption. The fitted curves are plotted with solid lines in Figs. 1 to 3 and the fitted parameter values are listed in Table 2. From the results in Table 2, the Freundlich model shows a slightly better fitting than the other two models, except for the case of 1,10-diaminodecane where the Langmuir model is best suited.

As to the affinity strength, the reciprocal of the K_{d} values for the Langmuir model and the K_{T} values for the Temkin model can be examined for comparison. The K_{d} and K_{T} values are almost the same for the diamine spacers, which implies that the length of the diamine spacer may not affect the affinity strength of lysozyme with Cibacron Blue 3GA. Thus, the use of a 12-atom diamine spacer provided a better arm length for the lysozyme molecule to touch the immobilized dye than did the 8-atom diamine, and the

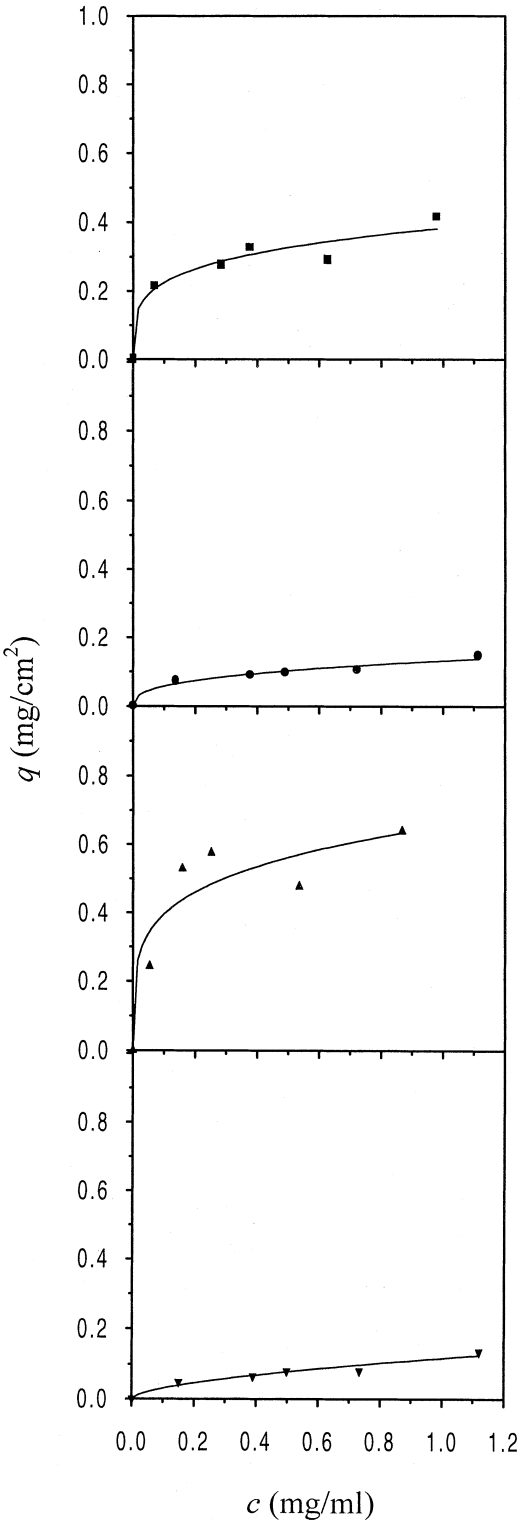

FIG. 2 Isotherms for lysozyme adsorption onto Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Fitting model: Freundlich.

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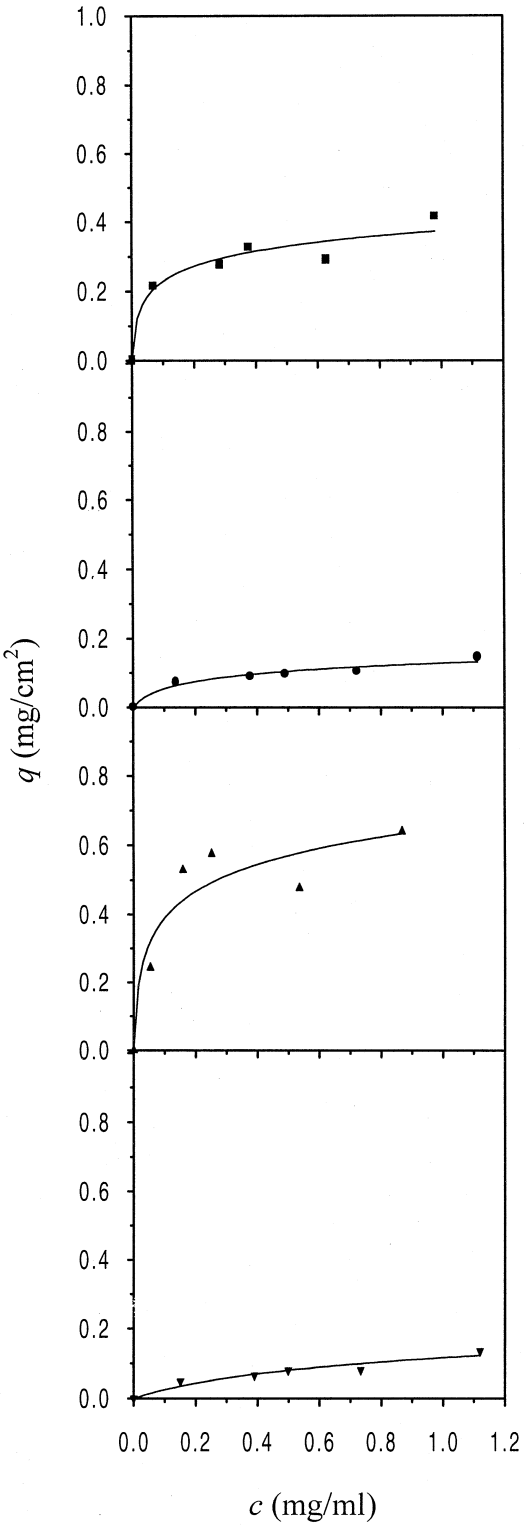

FIG. 3 Isotherms for lysozyme adsorption onto Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Fitting model: Temkin.

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TABLE 2
Fitted Parameter Values for Different Isotherm Models

Spacer arm	Isotherm model	Parameter values		Correlation coefficient (R)
1,6-Diaminohexane	Langmuir	q_m	0.38 ± 0.04	0.96
		K_d	0.06 ± 0.04	
	Freundlich	k	0.38 ± 0.03	0.98
		n	0.23 ± 0.07	
	Temkin	q_T	0.06 ± 0.02	0.97
		K_T	340 ± 520	
1,6-Diaminohexane + EGDGE	Langmuir	q_m	0.16 ± 0.08	0.96
		K_d	0.2 ± 0.4	
	Freundlich	k	0.13 ± 0.01	0.98
		n	0.36 ± 0.07	
	Temkin	q_T	0.04 ± 0.02	0.98
		K_T	35 ± 34	
1,10-Diaminodecane	Langmuir	q_m	0.65 ± 0.04	0.96
		K_d	0.06 ± 0.02	
	Freundlich	k	0.65 ± 0.08	0.94
		n	0.2 ± 0.1	
	Temkin	q_T	0.11 ± 0.05	0.95
		K_T	300 ± 530	
1,10-Diaminodecane + EGDGE	Langmuir	q_m	0.2 ± 0.3	0.96
		K_d	0.9 ± 2.0	
	Freundlich	k	0.12 ± 0.01	0.98
		n	0.6 ± 0.1	
	Temkin	q_T	0.07 ± 0.03	0.96
		K_T	4 ± 4	

binding strength between lysozyme and the dye was basically not influenced. On the other hand, the introduction of EGDGE raised the K_d values and decreased the K_T values. In other words, the affinity strength between lysozyme and Cibacron Blue 3GA was reduced. It is worthwhile to recall that the coupling site of the epoxide end of the amino-EGDGE-bound membrane with Cibacron Blue 3GA during the immobilization process is on the primary amine of the anthraquinone ring of the dye, which is also a possible adsorption site between lysozyme with Cibacron Blue 3GA. Consequently, the employment of EGDGE as part of the spacer arm may have encumbered lysozyme binding and therefore decreased both the adsorption capacity and the affinity strength.

To further analyze the heterogeneity of lysozyme adsorption onto dye-affinity membranes, Scatchard plots of the experimental data are displayed in Figs. 4 to 6, together with the corresponding plots for the three isotherm

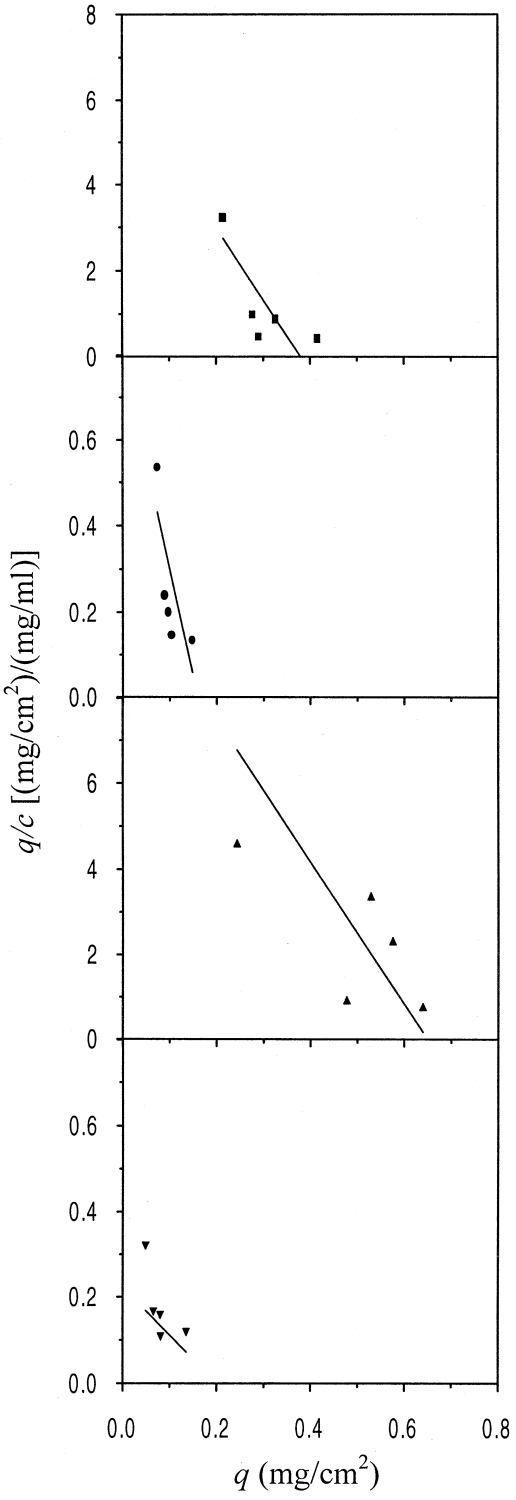

FIG. 4 Scatchard plots for adsorption isotherms of lysozyme to Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Solid lines: Langmuir fitting results from isotherms.

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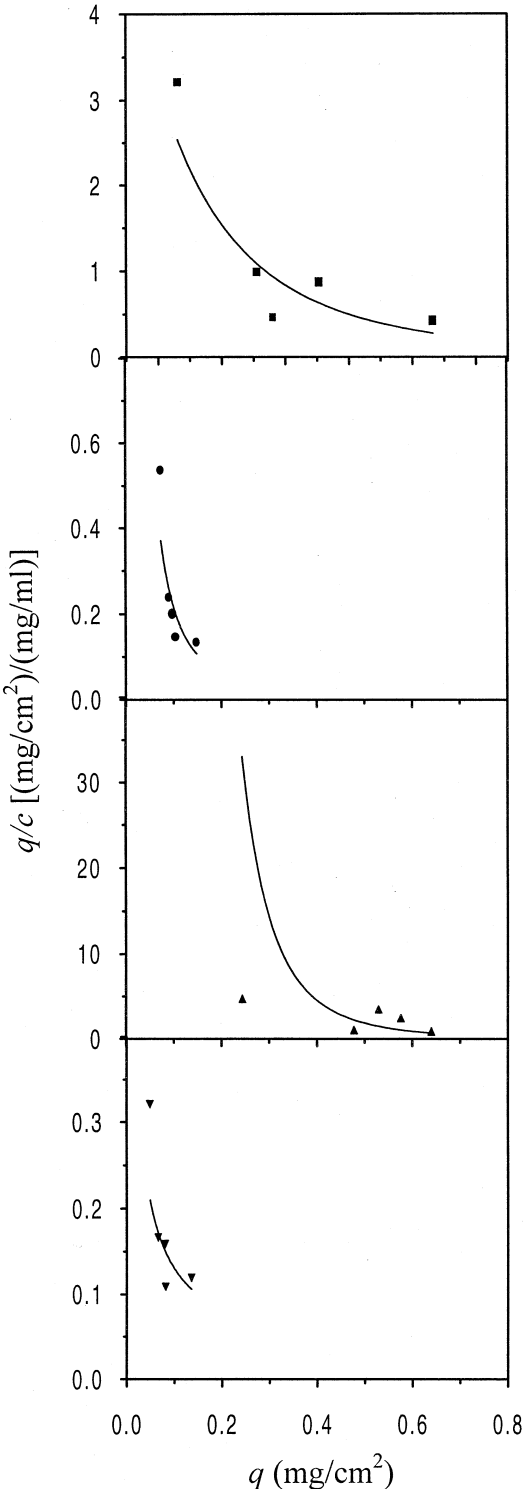

FIG. 5 Scatchard plots for adsorption isotherms of lysozyme to Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Solid lines: Freundlich fitting results from isotherms.

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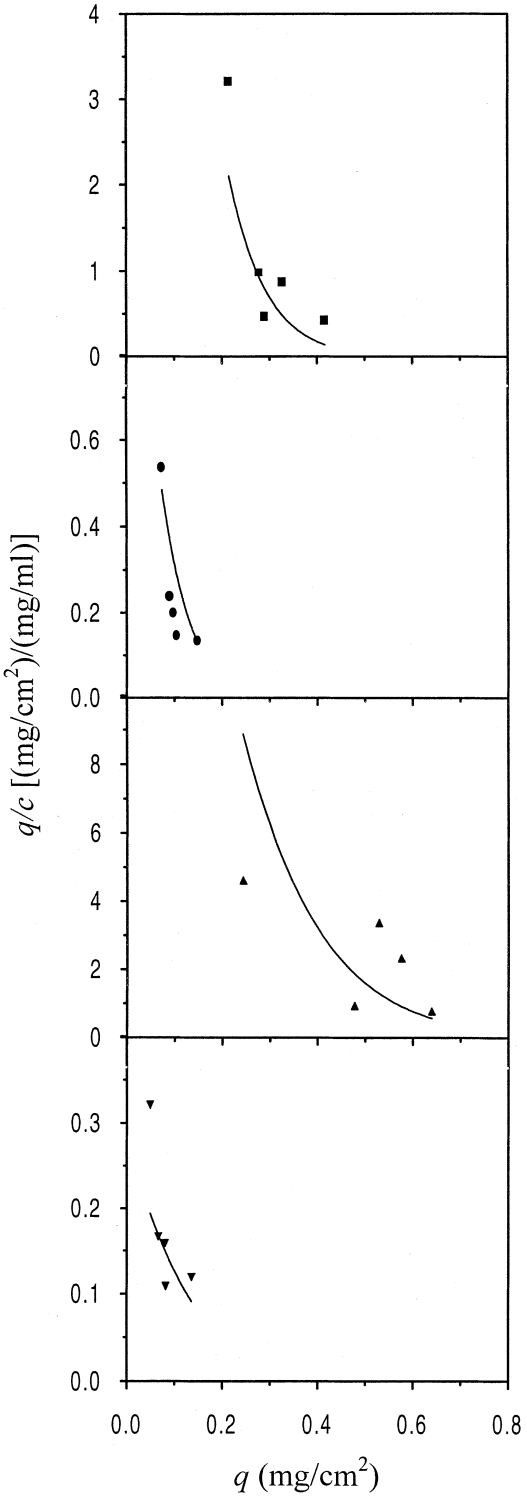

FIG. 6 Scatchard plots for adsorption isotherms of lysozyme to Cibacron Blue 3GA-immobilized IAV membranes. Spacer arms used: (■) 1,6-diaminohexane; (●) 1,6-diaminohexane + EGDGE; (▲) 1,10-diaminodecane; (▼) 1,10-diaminodecane + EGDGE. Solid lines: Temkin fitting results from isotherms.

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models governed by the following equations and using the parameters in Table 2.

Langmuir model:

$$\frac{q}{c} = \frac{q_{\mathrm{m}}}{K_{\mathrm{d}}} - \frac{1}{K_{\mathrm{d}}} q \tag{4}$$

Freundlich model:

$$\frac{q}{c} = k^{1/n} q^{1-(1/n)} \tag{5}$$

Temkin model:

$$\frac{q}{c} = \frac{K_{\mathrm{T}} q}{e^{q/q_{\mathrm{T}}} - 1} \tag{6}$$

Since the Langmuir model is formulated for homogeneous adsorption, the negative-slope linearity shown on the Scatchard plot can be considered as an index for adsorption homogeneity. In other words, the data that fail to be reasonably fitted by negative-slope straight lines imply heterogeneous adsorption. On the other hand, both the Freundlich ($n < 1$) and Temkin models construct concave-up Scatchard curves which indicate either the presence of multiple independent heterogeneous binding sites or negative cooperativity in the binding process (23).

Most Scatchard curves from experimental data resemble concave-up curves, as shown in Figs. 4 to 6. This explains why the Freundlich and Temkin models fit the isotherms better than the Langmuir model as presented in Table 2. We conclude from the Scatchard results that lysozyme adsorption onto Cibacron Blue 3GA-immobilized IAV membranes was heterogeneous, no matter what spacer arms were used.

Utilization Percentage

From the above analyses on adsorption isotherms, the Freundlich model seems to be a better choice for describing lysozyme adsorption onto dye-affinity membranes. However, it should be noted that no limit is set for saturation capacity in applying both the Freundlich and Temkin models, which causes a problem for calculation of the ligand utilization percentage. Therefore, only the saturation capacity data from the Langmuir model was used to evaluate the utilization percentage of immobilized dye for lysozyme adsorption. The results are listed in Table 1. The use of 1,10-diaminodecane as the spacer arm provided the highest utilization percentage, followed by 1,6-diaminohexane + EGDGE, 1,10-diaminodecane + EGDGE, and 1,6-diaminohexane. Recalling that the spacer arm of 1,6-diaminohexane resulted in the best ligand immobilization, its lowest utilization percentage means that a large portion of the

ligand sites of the 1,6-diaminohexane-conjugated membrane were not utilized and were still empty during the adsorption process. This phenomenon may be because the 1,6-diaminohexane spacer was not long enough to make it easy for the lysozyme molecule to surpass the resistance of liquid–film mass transfer and to touch the immobilized ligand. Lengthening the spacer arm is an option to reduce this steric hindrance, as shown by the use of 1,10-diaminodecane. In addition, a combination of two molecules as a spacer arm, such as diamine + EGDGE, should be avoided as reflected in the results.

CONCLUSIONS

In this work, 1,6-diaminohexane, 1,10-diaminodecane, 1,6-diaminohexane + EGDGE, and 1,10-diaminodecane + EGDGE were used as spacer arms for the immobilization of Cibacron Blue 3GA on IAV membranes. The blue color of the dye displayed on the outer membrane surface indicated success in the use of spacer arms. The ligand densities using different spacer arms were also measured and compared. It was found that 1,6-diaminohexane has the highest density, followed by 1,10-diaminodecane, 1,10-diaminodecane + EGDGE, and 1,6-diaminohexane + EGDGE. The adsorption isotherms of lysozyme to dye-affinity membranes were also investigated. The results show that the use of 1,10-diaminodecane as the spacer arm provided the highest percentage of ligand utilization, followed by 1,6-diaminohexane + EGDGE, 1,10-diaminodecane + EGDGE, and 1,6-diaminohexane. Consequently, the correct selection of a suitable spacer molecule is important for affinity membrane adsorption. A 12-atom straight molecule such as 1,10-diaminodecane is a good choice, according to this investigation. Spacer arms with a longer molecule or a combination of two molecules may reduce both the ligand immobilization density and the protein adsorption capacity, which should be avoided.

The ligand density of the 1,10-diaminodecane-conjugated membrane is 22,000 μM, based on the solid membrane volume (0.7 porosity). In comparison with the ligand density of the commercial product Blue Sepharose CL-6B (Pharmacia AB, Sweden), 1700–2300 μM based on the drained gel, the dye-affinity membrane produced in this work is obviously superior. Even with only 50% utilization for lysozyme adsorption, this membrane can still provide a better adsorption capacity than traditional gel beads. Accordingly, membrane adsorbents which demonstrate better mass transfer characteristics further prove their potential for practical application by their greater ligand density.

SYMBOLS

c	concentration in solution (mg/mL)
k	parameter of the Freundlich model

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K_d equilibrium dissociation constant (mg/mL); parameter of the Langmuir model

K_T equilibrium binding constant corresponding to the maximum binding energy (mL/mg); parameter of the Temkin model

n parameter of the Freundlich model

q adsorbed concentration based on the membrane front surface (mg/cm^2)

q_m saturation capacity based on the membrane front surface (mg/cm^2); parameter of the Langmuir model

q_T differential adsorption capacity per unit binding energy (mg/cm^2); parameter of the Temkin model

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