SELECTIVITY OF AFFINITY MEMBRANES FOR IMMUNOGLOBULIN CAPTURE

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Abstract

Affinity membranes have been prepared with a synthetic ligand that exhibits affinity for the Fc portion of immunoglobulins. The immobilization of the ligand has been optimised with respect to time and temperature of reaction and ligand density has been determined.

These new affinity membranes have been characterized and tested for adsorption with pure polyclonal human IgG, murine IgG and human IgM obtaining equilibrium and kinetic parameters. The membranes have been tested with human serum in order to determine the selectivity towards immunoglobulins and to evaluate immunoglobulin recovery.

Materials and methods

Membranes

Ultrabind® U450 membranes have been used as affinity supports for ligand immobilization. These polyethersulphone matrices are pre-activated with aldehyde surface chemistry; they have an average pore size of 0.45 μm, thickness of 155 μm and 48.4 % void fraction. The membranes have been kindly provided by Pall Corporation.

Ligand

D-PAM (Protein A Mimetic) is a synthetic ligand obtained through screening of combinatorial libraries that exhibit affinity for immunoglobulins [1]. Lyophilized D-PAM, kindly provided by Xeptagen SpA (Italy), has been used in this work.

Proteins, chemicals and solutions

Murine IgG, human IgM, human serum and all other chemicals, unless otherwise stated, have been purchased from Sigma-Aldrich.
Human polyclonal IgG, Gammanorm, has been purchased from Octapharma, Sweden.
BCA protein assay has been purchased from Pierce. Protein markers and all electrophoresis reagents have been purchased from Bio-Rad Laboratories.

Equipment

The concentration of pure protein solutions has been measured with absorbance readings at 280 nm by using a UV-Visible spectrophotometer, Shimadzu UV-1601.
SDS–PAGE analysis of the human serum was performed with Criterion electrophoresis system from Bio-Rad Laboratories, using precast gels.

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Membrane modification

The membranes have been modified following a two stage protocol. First the circular membranes (25 mm diameter) were immersed in a solution prepared with 2 g/L of D-PAM, and 3.2 g/L of $NaCNBH_3$ as catalyst in coupling buffer (0.1 M NaCl, 0.115 M NaH_2PO_4, pH 7). The solution was gently agitated overnight at room temperature. A second stage, to block the remaining aldehyde active groups, was performed by immersing the membranes in a solution of 0.42 g/L monoethanolamine and 1.26 g/L of $NaCNBH_3$ in coupling buffer for a period of 4 hours at room temperature. After the cross-linking stage the affinity membranes have been extensively washed with deionised water and coupling buffer.

Since D-PAM has a protein like structure, the BCA protein assay has been profitably utilised to measure the amount of ligand immobilized on the membrane surface [2].

Experimental and results

Affinity membranes were tested in batch experiments in order to determine kinetic parameters and measure the static binding capacity. The circular membranes (diameter of 25 mm) were immersed in a beaker containing protein solution and gently agitated in an orbital shaker. Then the membranes were washed twice with PBS buffer pH 7.4 to remove proteins and other molecules non-specifically adsorbed. Finally, the adsorbed protein was eluted with 0.1 M glycine HCl pH 2.8.

During the kinetic tests, the decrease of absorbance due to the protein uptake has been monitored with time up to a stable absorbance value. These experiments have been repeated with solutions of different initial concentration in order to determine the time needed to reach equilibrium. Results of experiments performed with murine IgG are reported in fig.1. It can be noticed that, for the concentration range inspected, the effect of the feed concentration on the time needed to reach equilibrium is almost negligible.

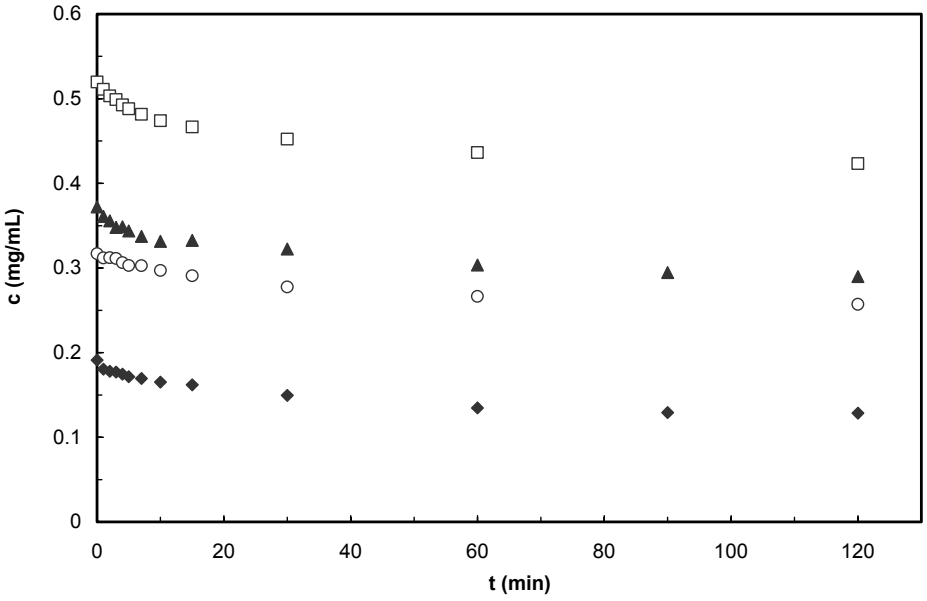

Fig. 1. Adsorption kinetics of murine IgG solutions on D-PAM-U450 affinity membranes.

Similar experiments have been performed using regenerated membranes to estimate the different performances; a comparison between the kinetic behaviour of new and regenerated membranes is shown in fig.2.

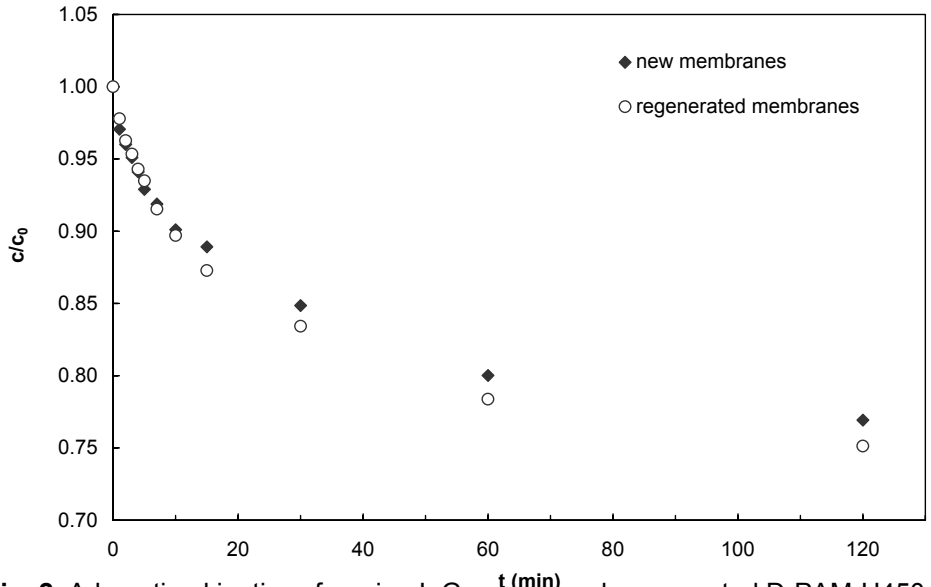

Fig. 2 Adsorption kinetics of murine IgG on new and regenerated D-PAM-U450 affinity membranes.

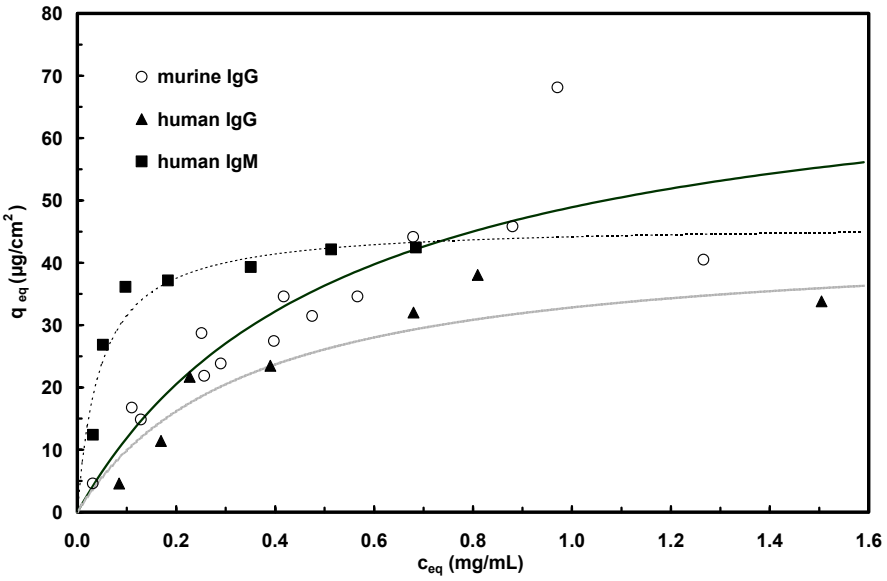

Fig.3: Adsorption isotherms of murine IgG, human IgG and human IgM onto D-PAM-U450 affinity membranes.

The static binding capacity at equilibrium of the D-PAM-U450 affinity membranes has been measured in batch experiments for three different proteins: murine IgG, human IgG and human IgM. In all cases the experimental data show a Langmuir behaviour, with different kinetics and different binding capacities. Clearly, the membranes have different affinity for

different immunoglobulins [3] and show the highest binding capacity towards murine IgG, while exhibit the lowest adsorption with human IgG. The results obtained are reported in fig. 3 in which the experimental data and the adsorption isotherms are presented for all three different proteins. The Langmuir parameters, q_m and K_d, have been calculated and are explicitly reported in table 1.

Table 1. Langmuir parameters for immunoglobulins onto D-PAM-U450 affinity membranes.

protein	q_m ($\mu g/cm^2$)	K_d (mg/mL)
murine IgG	83.84	0.624
human IgG	44.03	0.347
human IgM	46.22	0.047

The affinity membranes were tested for selectivity towards immunoglobulins in batch experiments with human serum. Human serum has been diluted 1:20 and pre-filtered with 0.22 µm syringe filters. The procedure adopted for the adsorption, washing and elution steps is the same used for the previous experiments with pure proteins. The membrane behavior has also been analyzed with SDS-PAGE gel electrophoresis; the results of a duplicate experiment are reported in fig. 4.

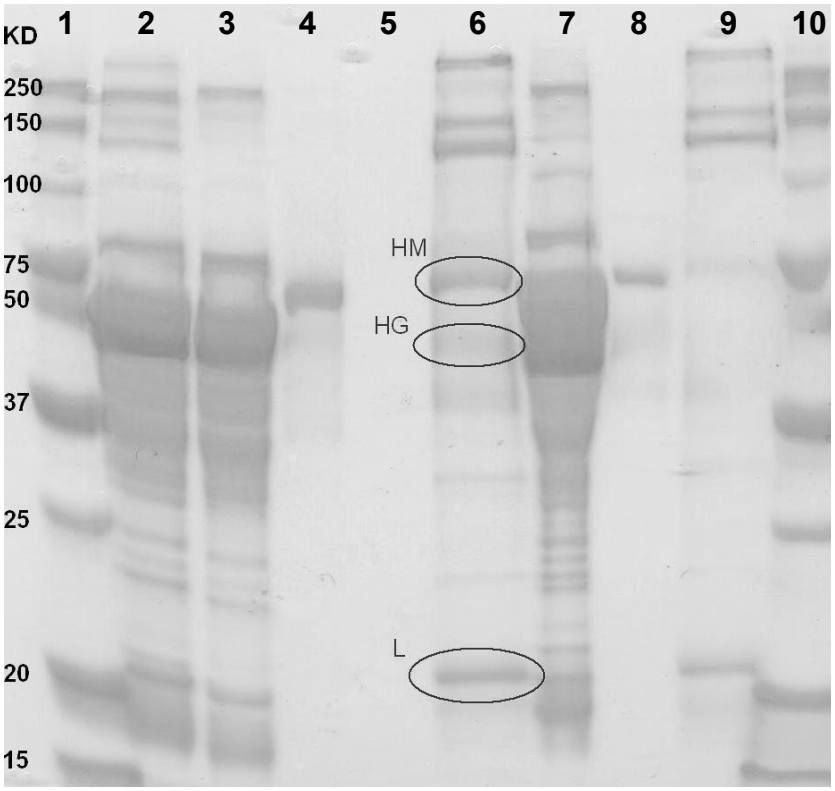

Fig. 2. Gel electrophoresis results of batch adsorption of human serum on D-PAM-U450. Lanes 1 and 10 MW markers; lane 2 human serum initial solution; lanes 3 and 7 human serum solution after the adsorption step; lanes 4 and 8 first wash, lane 5 second wash, lanes 6 and 9 concentrated eluted fractions.

In lane 6 the bands of heavy and light chains of IgG and IgM are clearly visible showing a good selectivity of the D-PAM-U450 for immunoglobulins. However, other bands do indicate the presence of other proteins that should be further removed in order to obtain a pure product.

Conclusions

D-PAM has been successfully immobilised onto Ultrabind-U450 membranes. The affinity membranes obtained have a good static binding capacity for immunoglobulins and in particular for murine IgG and human IgM.

The affinity membranes were tested with human serum in order to verify their selectivity towards immunoglobulins. Interestingly, the results obtained point out a marked selectivity versus IgM with respect to IgG. This result suggests promising applications for the purification of IgM , for which Protein A is not selective. Future work will investigate further this possibility with the aim to find the operating conditions more suitable for that separation.

Binding kinetics has a significant effect on selectivity indicating that the affinity membrane process maybe rate limited. However, this could be turned to an advantage of membranes with respect to chromatography, since different proteins can be isolated on the basis of different sorption kinetics and not only on the basis of different binding capacity.

Acknowledgements

MIUR (Ministry of Education University and Research) for financial support (PRIN 2004).
Pall Corporation for supplying Ultrabind® U450 membranes and Xeptagen SpA for supplying D-PAM are gratefully acknowledged.

References

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