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# Mercaptoheterocyclic ligands grafted on a poly(ethylene vinyl alcohol) membrane for the purification of immunoglobulin G in a salt independent thiophilic chromatography

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**Abstract**

In this study, we attempted a limited combinatorial approach for designing affinity ligands based on mercaptoheterocyclic components. The template, divinyl sulfone structure (DVS), which was grafted on poly(ethylene vinyl alcohol) (PEVA) hollow fiber membrane, has served for the tethering of different heterocyclic compounds as pyridine, imidazole, purine and pyrimidine rings. Their ability to adsorb specifically IgG in a salt independent manner out of pure IgG solution, mixture of IgG/albumin and human plasma was demonstrated. Mercapto methyl imidazole (MMI) has shown the best adsorption of IgG in terms of binding capacity. No subclass discrimination was observed on all tested ligands except for mercapto methyl pyrimidine where the major IgG subclass adsorbed was IgG3. MMI gave an IgG binding capacity of 100 $\mu g/cm^2$ of hollow fiber membrane surface area.


*Keywords:* Salt-independent thiophilic chromatography; Mercaptoheterocyclic ligands; Poly(ethylene vinyl alcohol); Immunoglobulin G

## 1. Introduction

The recovery of immunoglobulin G (IgG) from natural sources such as human plasma and from in vitro hybridoma cell cultures is gaining more importance. Furthermore, the application of affinity adsorbents for extracorporeal removal of autoimmunue IgG is being reported widely. Conventional ligands such as protein A/G have been extensively studied and used for the IgG recovery [1,2]. However, these conventional bioaffinity ligands have some drawbacks. The harsh elution conditions used (low pH), due to their high binding strength to the IgG, can compromise target stability and affect further processing [3]. The drastic "clean in place" (CIP) and sanitation treatments could not be used due to the protein nature of ligand. Contamination, leakage of ligand and the decrease of ligand and hence the decrease of performance could occur after a few purification cycles. All these drawbacks led researchers to find alternatives to these bioaffinity ligands. Pseudobiospecific affinity ligands such as metal chelates [4], immobilized histidine [5] and thiophilic ligands [6] have been proposed as alternatives. These ligands are robust, not expensive and compatible with CIP treatment. These pseudobioaffinity ligands have good binding capacities and specificity towards IgG. However, thiophilic, L-histidine and IMA chromatography have some limitations. In fact, these kinds of chromatography need sample adjustments such as dilution of human plasma in case of L-histidine, the need of high concentration of water structuring salt (lyotropic salt) for thiophilic ligand, and the presence of 1.0 M of NaCl for IMAC.

Hoping to minimize or eliminate these requirements, several ligands have been recently developed for the purification and separation of IgG from plasma or sera without sample preparation. These ligands are composed of sulfur atoms and heterocyclic rings. They have shown their capability to adsorb specifically IgG in a salt independent manner [7]. 3-(2-Mercaptoethyl)quinazoline-2,4-(1H, 3H)dione (MECH), grafted on soft gel, allowed the adsorption of antibodies without addition of lyotropic salt. In this case, desorption was achieved by injection of 20 mM NaOH. However, some traces of IgM and IgA were present in the eluate [8].

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A new chromatographic method has been developed by Burton and Harding [9], the "hydrophobic charge induced chromatography" (HCIC). HCIC was based on the hydrophobic binding of the target protein to the ligand and the desorption was based on ionic repulsion between the ligand, when the pH is changed in an appropriate direction. One of the commercially available sorbents for HCIC is named "MEP HyperCel" from BioSepra [10]. This sorbent carries a ligand, the mercapto ethyl pyridine, where the head is a pyridine ring, associated with a sulfur atom separated from the ring by two carbons [11]. This head is attached to a cellulose solid matrix by a hydrophobic chain. This resin gave a binding capacity of 30 mg of IgG/ml of wet resin from culture cell supernatant [12]. An aromatic ring in the ligand structure such as pyridyl, thiazolyl seemed to be important for salt independent chromatography of IgG. Another ligand, L-histidine, has shown good binding capacities and specificity towards IgG at neutral physiological conditions. This amino acid residue have an imidazole ring in its structure and allowed mixed mode interactions such as electrostatic, hydrophobic, ionic [13].

In this study, we attempted a limited combinatorial approach to find suitable ligands for IgG isolation. The divinyl-sulfone (DVS) activation has been described as an efficient way to obtain reactive sites and create a stable covalent link in a wide pH range. DVS activated matrices can react with primary amino-containing ligand or mercapto ligands. Moreover, the sulfone group seemed to be essential for the IgG specific adsorption [14]. Scholz et al. [7] have also shown that the sulfone group, introduced by DVS, was reported to play a role for salt independent chromatography. Consequently, DVS was used as template and several mercaptoheterocyclic compounds have been grafted on these DVS–poly(ethylene vinyl alcohol) (PEVA) hollow fiber membranes. Their ability to specifically adsorb IgG in a salt independent manner under physiological conditions was investigated.

## 2. Experimental

### 2.1. Materials

Divinyl-sulfone (DVS), (3-[*N*-morpholino]propane sulfonic acid) (MOPS), (tris[hydroxymethyl]aminomethane) (Tris), phosphate, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane sulfonic acid]) (Hepes), acrylamide, bis-acrylamide, sodium dodecyl sulfate, ammonium persulfate (APS), silver nitrate, 6-mercapto-purine, 2-mercapto-1-methyl-imidazole, 2-mercapto-4-methyl-pyrimidine, 2-mercapto-nicotinic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). High molecular weight standard markers from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade. Ultrapure milliQ$^{(r)}$ water was obtained from the Millipore milli RO/milliQ plus system (Millipore, Bedford, MA, USA). The hollow fibers membranes a copolymer of ethylene and vinyl alcohol (PEVA, Kuraray, Japan), had an internal diameter of 200 μm, a wall thickness of 20 μm and a nominal molecular mass cut-off of $600 \times 10^3$. The ethylene content was 30 mol% [15].

### 2.2. Hollow fiber PEVA–DVS activation

Wet hollow fibers PEVA cut in small pieces (1 mm of length) have been washed with 0.6 M NaOH then carbonate buffer 0.5 M pH 11. Fibers have been degassed during 1 h. Ten milliliters of DVS over period of 15 min was added under stirring and stirred during 10 h at room temperature, then fibers were rinsed intensely with ultrapure water.

### 2.3. Ligand grafting

An amount of 1.5 g of wet PEVA–DVS were rinsed with carbonate buffer. Ligands (Table 1) were dissolved in 30 ml of 0.5 M NaOH and 10 ml of carbonate buffer, 1.25 mM, final concentration.

The mixture was shaken for 24 h at room temperature, then fibers were rinsed with water and stored at 4 °C.

### 2.4. Preparation of human plasma

Human citrated blood samples were collected from healthy persons. The samples were centrifuged for 5 min at $200 \times g$ at 4 °C. Human plasma thus obtained was used for adsorption experiments.

### 2.5. Chromatographic procedure

All chromatographic experiments were carried out in static mode, e.g. 31 mg of dry mass of liganded PEVA

Table 1
Recovery of IgG and albumin (in percentage), from different mercaptoheterocyclic ligands, in the eluted fractions from a mixture of IgG/albumin (1:4) and 10 times diluted plasma injections

| Ligand | Mixture of IgG/albumin | | Diluted human plasma (1:10) | |
|---|---|---|---|---|
| | IgG recovery (%) | Albumin recovery (%) | IgG recovery (%) | Albumin recovery (%) |
| Mercapto methyl pyrimidine (MMPy) | 15 | BT | 12 | BT |
| Mercapto nicotinic acid (MNi) | 31.7 | BT | 12.9 | BT |
| Mercapto purine (MPu) | 32.2 | BT | 13 | BT |
| Mercapto methyl imidazole (MMI) | 51.0 | BT | 20 | BT |

Eluted fractions were assayed for IgG and albumin by Nephelometry. BT: below threshold.

fibers were placed in 1.5 ml tube volume. Protein solution (pure IgG, mixture of IgG/albumin or human plasma solution) were loaded onto affinity hollow fiber membranes. To promote diffusion of the protein into the fibres and pores, solution was slowly shaken during 60 min. Then, fibers were rinsed several times with binding buffer and adsorbed protein was eluted by addition of 0.4 M of NaCl to the binding buffer. The chromatographic supports were regenerated by passing 20 mM NaOH through the fibers.

### 2.6. Binding capacity of MMI–PEVA

Binding capacity of mercapto methyl imidazole MMI–PEVA hollow fiber membranes were tested in batch mode using different concentrations of IgG (0.7–5.7 mg/ml). An amount of 1.5 ml of IgG solution in MOPS, 25 mM, buffer has been adsorbed into fiber during 1 h under stirring at room temperature. IgG desorption was performed by adding 0.4 M of NaCl to the starting buffer.

### 2.7. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The chromatographic fractions were analyzed by SDS–PAGE (10%) under non-reducing conditions according to Laemmli [16] methods using the BioRad system miniprotean II (BioRad Labs., CA, USA). The presence of IgG and albumin was identified by comparison with standard markers: myosin (212,000 relative molecular mass), α2-macroglobulin 170,000 relative molecular mass, β-galactosidase (116,000 relative molecular mass), transferrin (76,000 relative molecular mass) and glutamic dehydrogenase (53,000 relative molecular mass).

### 2.8. Nephelometric quantification of IgG and albumin

In fractions obtained after adsorption of human plasma proteins, concentrations of IgG and albumin were determined by nephelometry, using a Beckman Array Protein System with the Beckman reagents for IgG and albumin (Beckman Instrument, Fullerton, CA, USA (see www.http://beckman.com-IMMAGE systems)).

### 2.9. Identification of adsorbed IgG subclasses

ELISA plate (Nunc from Poly labo, Strasbourg, France) was coated with 100 μl of anti-human IgG (1 mg/ml) (P.A.R.I.S., Compiegne, France) in coating buffer (15 mM $Na_2CO_3$–35 mM $NaHCO_3$, pH 9.5) overnight at 4 °C. Then, three washing steps with PBS Tween 0.1% and 200 μl of BSA solution (1% w/v) in PBS Tween 0.1% were added onto the wells during 1 h at 37 °C. The wells were then washed three times with PBS 0.1% Tween 20 and 100 μl of each elution fraction, corresponding to the different ligands, were deposited on the wells for 1 h at room temperature. The 96-wells plate was rinsed five times with PBS 0.1% Tween 20 and the biotinylated anti-IgG-subclasses, e.g. anti-IgG1, anti-IgG2 and anti-IgG3 (Sigma, St. Louis, MO, USA) was added to the wells with a dilution of 1:4000 in PBS Tween 0.1% and incubated for 1 h at 37 °C. The plate was again washed five times with PBS Tween 0.1% and a solution of avidin–peroxidase (Sigma) (1:4000) in 10 mM of phosphate buffer (pH 7.4)–0.15 M NaCl–0.1% Tween 20 was incubated for 1 h at 37 °C. The plates were washed with PBS containing 0.1% Tween 20. Finally, 100 μl per well of substrate solution for peroxidase (3 mM *O*-phenylenediamine dihydrochloride, 50 mM citric acid, 100 mM $Na_2HPO_4$, 16 μl $H_2O_2$ 9%, pH 5) was added and the reaction was stopped after 30 min incubation at room temperature in the dark with 100 μl of HCl (2 M). The adsorption was measured at 410 nm.

## 3. Results and discussion

Thiophilic mercaptoheterocyclic ligands like 2-mercaptopyridine and 2-mercaptopyrimidine have been already used for the purification of IgG in a salt-promoted chromatography [14,17,18]. More recently, other mercaptoheterocyclic ligands have been developed for salt independent chromatography for purification of IgG such as MECH (3-(2-mercaptoethyl)quinazoline-2,4-(1H, 3H)dione) [8] and mercapto ethyl pyridine (MEP) [10]. Here, a screening of four mercaptoheterocyclic ligands (Fig. 1) coupled onto hollow fiber membranes in PEVA via DVS activation have been tested for the purification of IgG in a salt-independent chromatography.

### 3.1. IgG adsorption

An amount of 1.5 ml of IgG solution (1 mg/ml) was injected in tubes. Different buffer systems such as Tris–HCl 25 mM, pH 7.4; PBS pH 7.4 and MOPS 25 mM, pH 6.5

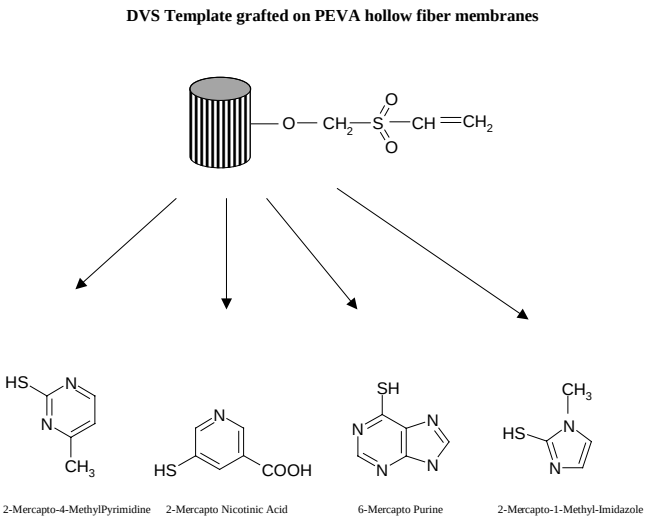


Fig. 1. Different mercaptoheterocyclic ligands grafted on DVS-activated PEVA hollow fiber membranes used as template.

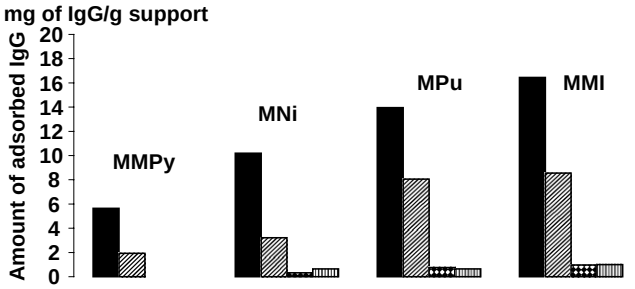


Fig. 2. Determination of adsorbed IgG after chromatography on a thiophilic mercaptoheterocyclic ligand as function of buffer systems. From left to right, IgG in MOPS buffer pH 6.5; MOPS buffer pH 7.4; Tris–HCl pH 7.4; and PBS pH 8.

and 7.4, were tested as binding buffer (Fig. 2). IgG adsorption varied with the type of buffer systems used. For all thiophilic mercaptoheterocyclic ligands, best IgG adsorption was observed in 25 mM MOPS buffer, pH 6.5. Here, the thiophilic mercaptoheterocyclic ligands were greatly influenced by buffer systems when the IgG adsorption was achieved in a salt independent chromatography. This influence was already shown for L-histidine ligand which had a maximal IgG adsorption in MOPS buffer [19,20]. It was shown that pseudo-biospecific affinity chromatography with L-histidine–PEVA membranes under zwitterionic buffers systems, like HEPES and MOPS, yielded much higher adsorption capacities in comparison with other buffers like Tris–HCl and PBS. Indeed, zwitterionic buffers did not interfere via charge–charge interactions with protein binding sites for the ligand, in contrast to "mono-charged" buffers. Here, the mercaptoheterocyclic ligands seemed to interact by charge–charge interactions and MOPS buffer have been chosen for IgG adsorption. However, when IgG were applied onto T-PEVA (2S) membranes in the presence of lyotropic salt, IgG adsorption was not influenced by the buffer system [21]. We supposed that thiophilic mercaptoheterocyclic ligands seem to adsorb IgG by a different mechanism under salt independent chromatography where electrostatic interactions are mainly involved. The amounts of adsorbed IgG, were 16 and 14.9 mg of IgG/g of support for mercapto methyl imidazole (MMI) and mercapto purine (MPu), respectively, whereas mercapto nicotinic acid (Mni) gave 10.3 mg/g of adsorbed IgG. Mercapto methyl pyrimidine (MMPy) had the lowest IgG adsorption (4.9 mg/g) in comparison with other ligands. MMPy grafted on a gel via DVS activation adsorbed specifically IgG in presence of lyotropic salt. However, in a salt independent chromatography, no significant amount of adsorbed IgG was detected. Only a small amount of IgG were recovered with 20 mM NaOH [7,8]. The pyrimidine ring seemed to be not suitable for IgG adsorption in a salt independent manner in contrast to MMI, MPu and MNi rings showed significant the immunoglobulin G adsorption. The pyridine ring was known to interact with IgG by a π-electron system and hydrophobic interactions [14], whereas the imidazole ring was known to have a mixed mode interaction mechanism by hydrophobic interaction, Van der Waals forces, electrostatic interactions and hydrogen bonds [13]. The addition of sodium chloride resulted in IgG desorption, which proved the weak interaction of the studied ligands with IgG. Moreover, in Fig. 2 a significant decrease of binding capacity for all ligands was observed when MOPS buffer at pH 7.4 was used as starting buffer. At this pH, IgG molecules were neutral which confirms the presence charge–charge interactions between ligands and IgG.

### 3.2. Selectivity of adsorption

#### 3.2.1. Mixture of IgG/albumin

The selectivity of adsorption was tested following the protocol described in Section 2. A mixture of IgG/albumin (ratio 1:4) was prepared in MOPS buffer and 1.5 ml of this mixture was loaded during 1 h onto each support with the different ligands grafted on membranes, followed by washing steps in the same buffer and elution by the addition of 0.4 M NaCl to this buffer. Table 1 shows the selectivity of IgG adsorption on different ligands tested. All of them presented good selectivity towards IgG and no albumin adsorption was detected. Though the sulfur atom introduced by the activation of DVS, can contribute to the selectivity of IgG adsorption in all the cases as already described by Oscarsson and Porath [14], the best IgG adsorption was obtained for MMI ligand with 51% of IgG recovery. MPu and MNi showed about the same adsorption e.g. 32% of injected IgG. The heterocyclic ring had a cooperative effect in terms of selectivity of adsorption of IgG but also in the capacity to bind IgG via different interactions mostly electrostatic and π-electron system.

Albumin could interact with ligands by hydrophobic interactions and hamper the IgG adsorption onto the liganded matrices. However, binding buffer could sweep albumin away from the matrices as no traces of albumin could be detected in the eluted fractions. This phenomena could explain the range of IgG recovery of 30 and 50% according to the ligands (15% for MMPy) when mixture of IgG/albumin was injected. Nevertheless, the adsorption of IgG seemed to be more efficient when imidazole core was present in the ligand structure.

#### 3.2.2. Human plasma

The selectivity of IgG binding for each ligand was tested using 10 times diluted human plasma. As in the case of mixture IgG/albumin no albumin adsorption has been detected for all different ligands (Table 1). Twenty percent of injected IgG was bound to the MMI fibers while 12–13% of IgG for the other ligands. SDS–PAGE Fig. 3A and B) indicated the purity of the eluted fraction for each ligands after contact with human plasma. The combination of sulphone group and heteroaromatic resulted in specific adsorption.

Adsorption of IgG subclasses were investigated on different coupled ligands. Eluted fractions were assayed using

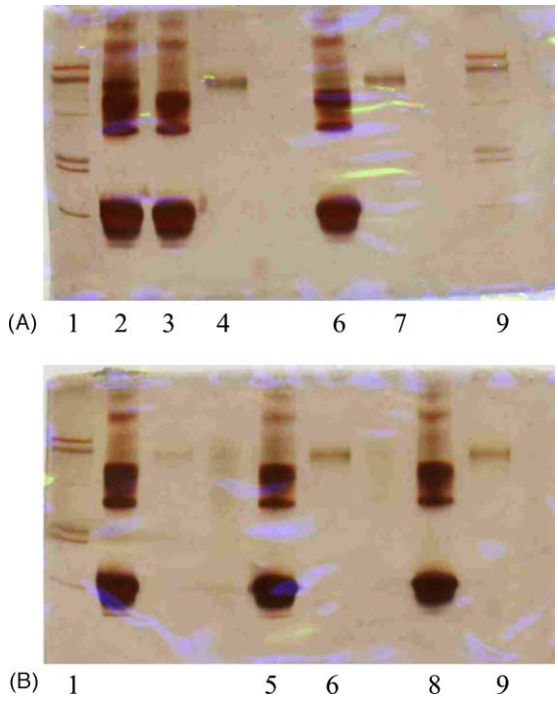


Fig. 3. SDS–PAGE under non-reducing conditions of the fractions from chromatography on mercaptoheterocyclic ligand grafted on PEVA hollow fiber membranes. (A) (Lane 1) molecular mass markers; (Lane 2) human plasma; (Lane 3) non-retained fraction from MMI; (Lane 4) adsorbed and eluted fraction from MMI; (Lane 6) non-retained fraction from MPu; (Lane 7) adsorbed and eluted fraction from MPu; (Lane 9) molecular mass markers. (B) (Lane 1) molecular mass markers; (Lane 5) non-retained fraction from MNi; (Lane 6) adsorbed and eluted fraction from MNi; (Lane 8) non-retained fraction from MMPy; (Lane 9) adsorbed and eluted fraction from MMPy.

ELISA test as described in Material and Methods section. As we can see in Table 2, no significant differences appeared in the IgG subclasses adsorption onto MMI, MPu and MNi ligands. Nevertheless, for mercapto methyl pyrimidine (MMPy), the major IgG subclass adsorbed was IgG3. This subclass was less represented in human plasma in comparison with IgG1 and IgG2. This finding could explain the small amount of adsorbed IgG on MMPy in comparison with the other ligands. In spite of a poorly adsorbed fraction, an enrichment in IgG3 was found.

### 3.2.3. MMI–PEVA membranes

We decided to study in more detail MMI fibers for the adsorption of IgG out of human plasma without any dilution

Table 2
Distribution of IgG subclasses adsorbed on mercaptoheterocyclic ligands

| IgG subclasses ligand type | IgG1 | IgG2 | IgG3 |
|---|---|---|---|
| Mercapto methyl pyrimidine (MMPy) | + | + | ++++[a] |
| Mercapto nicotinic acid (Mni) | +++ | ++ | +++ |
| Mercapto purine (MPu) | ++++ | ++ | +++ |
| Mercapto methy imidazole (MMI) | ++++ | +++ | ++++ |

[a] Maximum OD at 490 nm.

Table 3
Human plasma, non-diluted and diluted four times in 25 mM MOPS, pH 6.5 (A), injected on MMI–PEVA hollow fiber membranes

| MMI–PEVA | IgG adsorbed (μg) | Albumin adsorbed (μg) | Capacity (mg/g) |
|---|---|---|---|
| 1:4 dilution | 225 | BT | 7.25 |
| A + 0.15 M NaCl | BT | BT | BT |
| Non-diluted | 195 | 15 | 6.29 |

BT: below threshold.

and with a dilution factor of 1:4. An amount of 7.25 mg of IgG/g of support was bound to the MMI fibers for the 1:4 dilution and 6.3 mg of IgG/g of support out of non diluted plasma. Traces of albumin were detected in the eluted fractions, only when non-diluted plasma was injected (Table 3). The traces of albumin adsorbed with injection of non diluted plasma were acceptable in terms of biomedical applications. MMI could adsorb specifically IgG from human plasma, without sample pre-treatment such as dilution or addition of lyotropic salt. When non-diluted human plasma was directly injected onto MMI fibers, IgG were desorbed by just the addition of NaCl. The interactions between IgG and this ligand seemed to be weak and easily reversible. The weakness of the interaction was confirmed by addition of 0.15 M NaCl in the equilibration buffer, before injection of plasma (Table 3).

### 3.2.4. IgG binding capacity on MMI–PEVA fibers

A maximum IgG binding capacity of MMI–PEVA was achieved by loading IgG solutions at different concentration (0.7–5.7 mg/ml). An amount of 1.5 ml of IgG solution in MOPS 25 mM, buffer was loaded onto the adsorbent in a batch mode by stirring the mixture during 1 h, at room temperature. IgG was desorbed by adding 0.4 M of NaCl to the starting buffer. Eluted fractions were assayed by the Bradford method [22]. Fig. 4 shows an adsorption isotherm of MMI–PEVA and gave a maximum binding capacity of

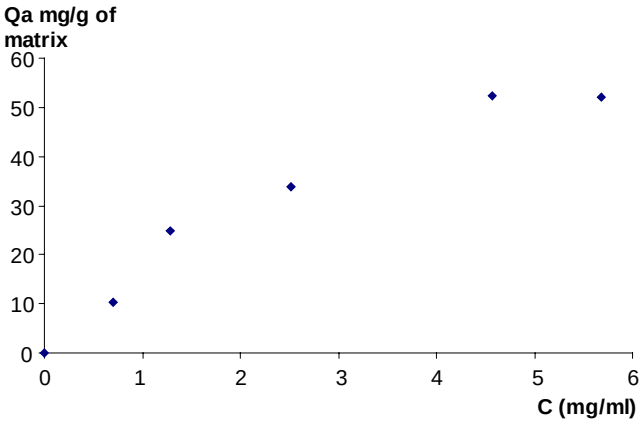


Fig. 4. Adsorption isotherm in batch of IgG from Cohn fraction II on MMI–PEVA hollow fiber membranes. MOPS buffer pH: 6.5, elution with 0.4 M NaCl.

52.5 mg of IgG/g of support corresponding to 100 μg/cm$^2$. This result was in order of magnitude with the binding capacity found for L-histidine–PEVA membrane and T-PEVA [19,21].

## 4. Conclusion

In this paper, we attempted a limited combinatorial approach for ligand designing for IgG adsorption using DVS as template onto which different heterocyclic compounds have been grafted. These heterocyclic structures were based on pyridine, imidazole, purine and pyrimidine rings. Matrix used was hollow fiber membranes in PEVA. The cooperative effect the thiol group and the heterocyclic ring of each compound were important for the selectivity of adsorption in a salt independent manner at neutral pH. Their ability to adsorb specifically IgG in a salt independent manner from pure IgG solution, a mixture of IgG/albumin and human plasma was demonstrated. IgG adsorption seemed to be influenced by the buffer type and no albumin adsorption was observed when mixture of IgG/albumin and diluted plasma were injected in all different supports. Although the same template has been used, these ligands have shown differences in their binding capacities. This funding proved the importance of the heterocyclic ring type for the selectivity of adsorption but also for the capacity to bind IgG. The most efficient ligand was MMI (imidazole ring) followed by MPu (pyrimidine and imidazole rings) and MNi (pyridine ring). All of them, except MMPy have shown their great potential to adsorb IgG in a salt independent manner. A more detailed study of MMI has shown than the imidazole ring was able to bind selectively IgG from non-diluted human plasma and could be eluted under gentle elution conditions with only small traces of albumin present as contaminant. The maximum binding capacity found was 100 μg of IgG/cm$^2$ of hollow fibers membranes surface area.

MMI could be of great interest in the great challenge, which is the adsorption of IgG from complex fluid as human plasma without any sample pre-treatment (e.g. dilution, salt addition, . . . ) in biomedical application (extracorporeal removal of pathogenic immunoglobulin G) and in biotechnology applications.

This study could serve as basic work and help the ligand designer to make new sorbents for IgG purification. On the imidazole ring, more charges could be added, the ligand density should be increase to improve the binding capacity and the experimental conditions fine-tuned.

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## References

[1] P.L. Ey, S.J. Prowse, C.R. Jenkin, Immunochemistry 15 (1978) 429.
[2] S. Duffy, B.J. Moellering, G.M. Prior, K.R. Doyle, C.P., Prior, Bio-Pharm. June (1989) 35.
[3] J. Buchner, M. Renner, H. Lilie, H.J. Hinz, R. Jaenicke, T. Kiefhabel, R. Rudolph, Biochemistry 30 (28) (1991) 6922.
[4] V. Boden, J.J. Winzerling, M.A. Vijayalakshmi, J. Porath, J. Immunol. Methods 181 (1995) 225.
[5] A. El-Kak, S. Mandjiny, M.A. Vijayalakshmi, J. Chromatogr. 604 (1992) 29.
[6] J. Porath, F. Maisano, M. Belew, FEBS 185 (1985) 306.
[7] G.H. Scholz, P. Wippich, S. Leistner, K. Huse, J. Chromatogr. Biomed. Appl. 709 (1998a) 189.
[8] G.H. Scholz, S. Vieweg, S. Leistner, J. Seissler, W.A. Scherbaum, K. Huse, J. Immunol. Methods 219 (1998) 109.
[9] S.C. Burton, D.R.K. Harding, J. Chromatogr. A 814 (1998) 71.
[10] BioSepra® Application Note No. 417, 1999.
[11] E. Boschetti, D. Judd, E. Warren, W. Schwartz, P. Tunon, Genetic Engineering, 20 (2000).
[12] L. Guerrier, P. Girot, W. Schwartz, E. Boschetti, Bioseparation 9 (2000) 211.
[13] O. Pitiot, M.A. Vijayalaksmhi, in: M.A. Vijayalakshmi (Ed.), Taylor & Francis, Biochromatography—Theory and Practice, London, 2002, p. 496.
[14] S. Oscarsson, J. Porath, J. Chromatogr. 499 (1990) 235.
[15] Y. Sakurada, A. Sueoka, I. Kawahashi, Polym. J. 19 (1987) 501.
[16] U.K. Laemmli, Nature 227 (1970) 680.
[17] A. Schwarz, F. Kohen, M. Wilchek, J. Chromatogr. Biomed. Appl. 664 (1995) 83–88.
[18] P.P. Berna, N. Berna, J. Porath, S. Oscarsson, J. Chromatogr. A 800 (1998) 151.
[19] S.M.A. Bueno, K. Haupt, M.A. Vijayalakshmi, J. Chromatogr. B 667 (1995) 57.
[20] K. Haupt, S.M.A. Bueno, M.A. Vijayalakshmi, J. Chromatogr. B 674 (1995) 13.
[21] Y. Coffinier, C. Legallais, M.A. Vijayalakshmi, J. Membr. Sci. 208 (2002) 13.
[22] M. Bradford, Anal. Biochem. 72 (1976) 248.