Preparation of miniaturized hydrophilic affinity monoliths: Towards a reduction of non-specific interactions and an increased target protein density

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Keywords

Frontal affinity chromatography, hydrophilic organic monolith, non-specific interactions, fragment screening

Abstract

In affinity chromatography, non-specific interactions between the ligands and the affinity column may affect the results, leading to misinterpretations during the investigation of protein-ligand interactions (detection of false positives in ligand screening, lack of specificity in purification). Such non-specific interactions may arise both from the underlying support or from the target protein itself. If the second ones are protein-dependent (and cannot be studied in a general framework), the first ones occur in the same way regardless of the immobilized target. We propose a methodology to identify the origin of such non-specific interactions with the underlying material of the affinity column. This methodology relies on the systematic investigation of the retention behavior of a set of 41 low-molecular weight compounds covering a wide chemical space (net charge, log D, functionality). We first demonstrate that the main source of non-specific interactions on the most commonly used GMA-co-EDMA monolith comes from hydrophobic effects. To reduce such non-specific interactions, we developed a new hydrophilic glycidyl methacrylate-based monolith by replacing the EDMA crosslinker by the more hydrophilic N-N' Methylenebisacrylamide (MBA). Optimization of the synthesis parameters (monomer content, initiation type, temperature) has focused on the reduction of non-specific interaction with the monolithic support while maximizing the amount of protein that can be grafted onto the monolith at the issue of its synthesis. The retention data of the 41 test solutes on the new poly(GMA-co-MBA) monolith shows a drastic reduction of non-specific interactions except for cationic compounds. The particular behavior of cationic compounds is due to their electrostatic interactions with carboxylic groups resulting from the partial acidic hydrolysis of amide groups of MBA during the epoxide ring opening step. So, the ring opening step in acidic media was replaced by a hot water treatment to avoid side reaction on MBA. The new monolith poly(GMA-co-MBA) not only has improved hydrophilic surface properties but also a higher protein density (16 ± 0.8 pmol cm^{-1} instead of 8 ± 0.3 pmol cm^{-1}). To highlight the benefits of this new hydrophilic monolith for affinity chromatographic studies, frontal affinity chromatography experiments were conducted on these monoliths grafted with con A.

1- Introduction

Affinity monolith chromatography (AMC) is a type of liquid chromatography that uses monolithic supports functionalized with biological targets such as proteins, receptors, antibodies or enzymes [1–3]. AMC is a powerful method for clean-up and preconcentration purposes in sample treatment [4,5], chiral separation [6] and ligand or fragment screening in Drug discovery [7,8]. Applications also include the study of biological interactions to get information on the stoichiometry, thermodynamics and kinetics of the interaction between the immobilized biological target and ligands in solution.

Miniaturized affinity monolith chromatography µAMC, with columns in the few tens µm range covers all the application fields of AMC with the tremendous advantage of reducing the quantity of biological material to be immobilized.

Several reasons have driven the development of miniaturized affinity chromatography with monolithic supports. First, these supports are synthesized *in-situ* as continuous bed supports avoiding the cumbersome filling of columns with particles. Their external porosity can be tuned to increase their permeability and decrease back pressures thus allowing higher flow rates (useful for increasing the throughput in screening campaigns, for decreasing the percolation time in purification or clean up applications). Whatever the application field, the underlying monolithic material supporting the biomolecules must satisfy two main criteria: the quantity of protein per unit volume must be as high as possible and its intrinsic capacity to limit non-specific interactions. The quest for a stealthy underlying support in affinity chromatography is a complex and difficult task [9,10].

Organic monoliths are usually preferred to their silica counterparts for their easier preparation (thermically or photochemically initiated polymerization). Moreover the great diversity of commercially available monomers allows tuning their surface properties and adjusting available functional moieties for their subsequent grafting with a biological target [11]. They also usually offer a satisfactory mechanical resistance and a tunable permeability allowing their *in-line* coupling with another separation dimension (liquid chromatography or capillary electrophoresis) [12–14]. A great diversity of organic monoliths has been synthesized. Among those, GMA-co-EDMA monoliths have found applications in many areas (for the preparation of IMERS dedicated to *on-line* solid-supported enzymatic digestion, immunoaffinity preconcentration, separation…)[12,15–17]. Moreover, their synthesis is well-documented, they present a satisfactory chemical and thermal stability, and their surface epoxy groups open multiple grafting-ways to prepare a wide range of stationary phases, from ion-exchange to affinity columns through biomolecules immobilization (*e.g.,* aptamers and proteins).

Despite their frequent use, there is very little information on the non-specific interactions generated by these GMA-co-EDMA monolithic based stationary phases. During a previous ligand/protein interaction study by AMC [7], we have noticed that non-specific interactions can be non negligeable when these GMA-co-EDMA monoliths are used as underlying material for affinity columns. Indeed, GMA-co-EDMA monoliths are used in experimental conditions close to physiological conditions (purely aqueous medium at pH 7.4, with high salt concentration) favoring secondary interactions by hydrophobic effect. We have shown that such non-specific interactions occur regardless of the mode of grafting but are of less importance after grafting according to the Schiff base method *i.e.,* when residual reduced aldehyde groups remain at the monolith surface instead of epoxy ones even after

end-capping. The existence of such interactions can lead to (i) a lack of specificity/recovery if they are used for purification/preconcentration, (ii) a lack of recovery of the products resulting from the enzymatic digestion (for IMERS), (iii) the detection of false positives in the case of ligand screening and to (iv) a misestimation of thermodynamics parameters such as affinity constant determination. Because non-specific interactions are a concern in bioaffinity chromatography, their characterization seems a prerequisite to any stationary phase optimization. To our knowledge, sources of non-specific interactions with the underlying support on affinity monoliths have not been thoroughly investigated. For instance, no dedicated method was developed and accepted to characterize the level and origin of non-specific interactions of small molecules on monolithic phases used as chromatographic underlying support for affinity columns.

Our study stems on improving the conditions for detecting low affinities in affinity chromatography by reducing unwanted interactions with the chromatographic support. In a preliminary investigative study, we report a methodology aiming at characterizing non-specific interactions due to the GMA-co-EDMA underlying material widely used for the preparation of affinity columns. To achieve this task, we selected a set of 41 molecules from a library of fragment-like molecules covering the whole range of physico-chemical properties in terms of net charge, logD, H-bond donor and acceptor. The physical-chemical properties and chemical structures of the set of fragments are detailed in Table S1. Based on the outcomes of the preliminary study (non-specific interactions mainly due to hydrophobic effects), we propose the synthesis of a new glycidyl based monolith, by using MBA crosslinker (instead of EDMA) to increase the hydrophilicity of the resulting monolith. Both the grafting capacity and the surface properties regarding the non-specific interactions of the newly prepared monolith are optimized and characterized. Non-specific interactions are characterized using the developed methodology based on the library of small chemical compounds. The grafting capacity is evaluated by determining the amount of biological active sites present at the monolith surface after the grafting of a biological target onto the monolith (amount of sites able to bind a ligand of known activity). Streptavidin is used as model target protein and HABA as a test ligand. Finally, to evaluate the improvement of the new monolith in affinity chromatography, low affinity ligands are used on a Concanavalin A grafted monolith. These experiments highlight the benefits associated with reduced non-specific interactions and an increased target protein density. All these experiments are carried out by frontal affinity experiments (with a dedicated in-house developed instrumentation [18]) as described in the Material and Methods Section.

2- Material and methods

2.1- Reagents and Buffers.

Streptavidin (from *Streptomyces avidinii*, affinity purified, ≥13 U mg^{-1} of protein), Concanavalin A (Con A) from Canavalia *ensiformis*, (3-methacryloxypropyl)-trimethoxysilane (γ-MAPS), ethylene dimethacrylate (EDMA), glycidyl methacrylate (GMA), acrylamide, N,N'-Methylenebis(acrylamide) (MBA), Acrylamide (AA), 1- propanol, 1,4-butanediol, dimethyl sulfoxide (DMSO), sodium periodate, lithium hydroxide, dipotassium hydrogen phosphate (K_2HPO_4), o-phosphoric acid, sulfuric acid, sodium cyanoborohydride, triethylamine (TEA), azobis(isobutyronitrile) (AIBN), 4'-Hydroxyazobenzene-2-carboxylic acid (HABA), p-nitrophenyl-α-D-mannopyranoside (αPNM), p-nitrophenyl-α-D-glucopyranoside (αPNG) and p-nitrophenyl-α-D-galactopyranoside (αPNGal) and ligands (Table S1) were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesne, France). All aqueous solutions were prepared using >18 MΩ deionized water. Phosphate buffer was prepared by dissolving 1.17 g of K_2HPO_4 in 100 mL of ultrapure water and the pH was adjusted to 7.4 with phosphoric acid.

2.2- Monolithic capillary column synthesis

Fused-silica capillaries with UV transparent coating (TSH, 75-µm i. d.) or polyimide coating (TSP, 75-µm i.d.) were purchased from Polymicro Technologies (Molex). In all cases, capillaries were pre-treated by flushing capillaries with a 5% (v/v) solution of γ-MAPS in methanol/water (95/5, v/v) with 2.5% TEA for 1 h at 7 bar. Next, they were rinsed with methanol for 15 min at 7 bar and dried at room temperature under nitrogen stream.

2.2.1 GMA-co-EDMA Monolith synthesis:

Poly(GMA-co-EDMA) monoliths were synthesized as described in a previous work [7]. The polymerization mixture was prepared by mixing 0.9 mL GMA, 0.3 mL EDMA, 1.05 mL 1-propanol, 0.6 mL 1,4-butanediol, 0.15 mL ultra-pure water and 12 mg of AIBN initiator. The pre-treated capillary (TSH Capillary) was then filled with the polymerization mixture under 1 bar N_2 pressure. The photopolymerization reaction was performed in a Bio-link UV cross-linker (VWR International, France) under 365 nm UV light for a total energy of 6 J cm^{-2}. To localize the monolith inside the silica capillary, a PEEK tubing (380 µm i. d.) was used as a mask to cover non-irradiated areas. After polymerization, the capillary was rinsed with methanol for 1 h.

2.2.2 GMA-co-MBA monolith synthesis

Poly(GMA-co-MBA) monoliths were prepared as follows, adapting protocols from [19]. First, a mixture of solvents was prepared by mixing 3330 mg of DMSO, 1480 mg of 1,4-butanediol and 1850 mg of

dodecanol. Then, 320 mg of MBA were added and sonicated 1 h at room temperature. After the dissolution of MBA, 480 mg of GMA were added and sonicated 1 h at room temperature. 8 mg of AIBN were added and the final mixture was sonicated 15 min at room temperature. The pre-treated capillary (TSP Capillary) was then filled with the polymerization mixture under 1 bar N_2 pressure and the ends of the capillary sealed. The polymerization reaction was performed in water bath at 57°C for 18 hours. After polymerization monoliths were rinsed with methanol for 1 h.

2.3 Protein (streptavidin) immobilization

Streptavidin was grafted using the Schiff base method as previously described [7]. First, the epoxy groups of the GMA-co-EDMA monoliths were hydrolyzed into diols flowing 1 M sulfuric acid for 2 h at 7 bar. After water rinsing, the diol-monolith was oxidized to aldehyde using a 0.12 M $NaIO_4$ solution at pH 5.5. Then, a 1 mg mL^{-1} Streptavidin and 4 mg mL^{-1} $NaBH_3CN$ solution in 67 mM phosphate buffer (pH 6) was percolated through the column for 18 h at 7 bar and room temperature. After immobilization, the column was flushed with sodium borohydride (2.5 mg mL^{-1}, phosphate buffer 67 mM pH 8) (2 h, 7 bar) to reduce residual aldehydes. The Streptavidin columns were then rinsed with phosphate buffer and stored at 4 °C.

2.4 Nano-FAC experiments

Nano-FAC Setup. Frontal Weak Affinity Chromatography experiments were carried out with a 7100 capillary electrophoresis Agilent system (Agilent Technologies, Waldbronn, Germany) equipped with an external nitrogen tank to reach pressures up to 12bar. System control and data acquisition were carried out using the Chemstation software (Agilent). All experiments were carried out in “short-end” injection mode, with the inlet of the capillary immersed in the solution to be infused, so that by applying external pressure at 12bar the liquid is forced to flow inside the capillary column. The detection was achieved *in-situ* on an empty section of the column, right after the end of the monolith, thanks to a diode array detector operated in multi-wavelength mode. [18] The revelation window on the capillary was obtained by burning away the external coating of the capillary. The analyses were all carried out under controlled room temperature at 25°C.

2.4.1 FAC for the evaluation of non-specific interactions

Non-specific interactions were evaluated by infusing compounds individually (100 µM solutions prepared in phosphate buffer, 67 mM, pH=7,4) on the reduced aldehyde monolithic support until the breakthrough time was reached. The columns were rinsed for 30 min in-between each infusion. The breakthrough time is measured for each compound. In the case of non-specific interactions, this breakthrough time $t_{breakthrough}$ is independent of the solute concentration [L] provided this

concentration is low enough to work is the linear range of the adsorption isotherm. If no non-specific interactions take place, the breakthrough time occurs at the dead time (the reduced breakthrough time is equal to 1). If non-specific interactions occur, the solute is captured by the support until the equilibrium is reached, and the breakthrough time $t_{breakthrough}$ is delayed. The higher the non-specific interactions, the higher the quantity of solute captured and the higher the breakthrough time.

$$q_{capt} = \left(t_{breakthrough} - t_0\right) * F * [L] = \frac{\left(t_{breakthrough} - t_0\right)}{t_0} * V_0 * [L] = k_{ns} * V_O * [L]$$

Where F is the flow rate, t_o the dead time, V_0 the dead volume of the column and k_{ns} the retention factor of the solute due to non-specific interactions.

2.4.2 Quantification of the number of protein active sites

For the determination of the amount of streptavidin active sites available after grafting, HABA (Kd = 100 µM) was infused as test solute with increasing concentrations (5, 10, 50, 100 and 200 µM solutions prepared in phosphate buffer, 67 mM, pH=7,4), without any rinsing step in-between the percolation of different concentrations (staircase experiments). The amount of ligand captured at each step is determined and the cumulated amount of ligand captured is calculated by summing the captured quantity at all the steps. The double reciprocal plot showing the amount of ligand captured vs the ligand concentration allows for the simultaneous determination of both the Kd value of the target–ligand interaction and the number of active sites available (Bact) (see S1 for the rationale of the staircase approach). Rationality of the results was ensured by comparing the obtained Kd value (range 90-120 µM) with literature values (100 µM).

3- Results and discussion

3.1 Investigation of non-specific interactions on GMA-co-EDMA monoliths

The starting monolith uses glycidyl methacrylate (GMA) as functional monomer and ethylene dimethacrylate (EDMA) as crosslinker. Preparation of (bio)affinity monoliths requires their subsequent functionalization with target (bio)molecules such as proteins. The preferred functionalization pathway implements the hydrolysis of epoxides, followed by the periodate-mediated oxidation of the resulting diols into aldehyde reactive groups. The protein is then covalently grafted onto the monolith via Schiff base method. The remaining aldehyde groups after the grafting step are reduced to form alcohol groups (Figure S2). This biofunctionalization method gives more hydrophilic stationary phases compared to the one obtained by direct grafting of proteins onto the GMA-based monolith [7]. In addition, the reaction is faster and results in improved grafting protein densities compared to the

epoxy method [20,21]. Using a 75-µm inner diameter capillary, up to 8 ± 0.3 pmol of active streptavidin binding sites per cm of monolith are reached. The investigation of non-specific interactions on GMA-co-EDMA based monoliths (interactions with the underlying material and not the whole affinity column) was carried out on GMA-co-EDMA monoliths subjected to the entire functionalization process except for the protein grafting step, *viz* on reduced aldehyde GMA-co-EDMA monoliths. Figure 1 presents the breakthrough curves obtained by nano-FAC experiments for three different molecules of the library solubilized in a 67 mM phosphate buffer at pH=7,4. For f412, non-specific interactions are not observed, and the breakthrough time is equal to the dead time (k=0). The presence of non-specific interactions between the ligands and the stationary phase is observed by a shift of the breakthrough curve from the dead time. The higher the non-specific interactions the higher the breakthrough times as for f771 (a moderately retained compound, k=3.9) and f366 (a highly retained compound k=14.7) (Figure 1).

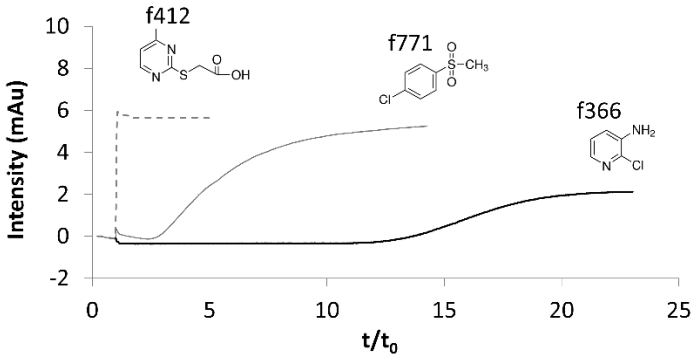
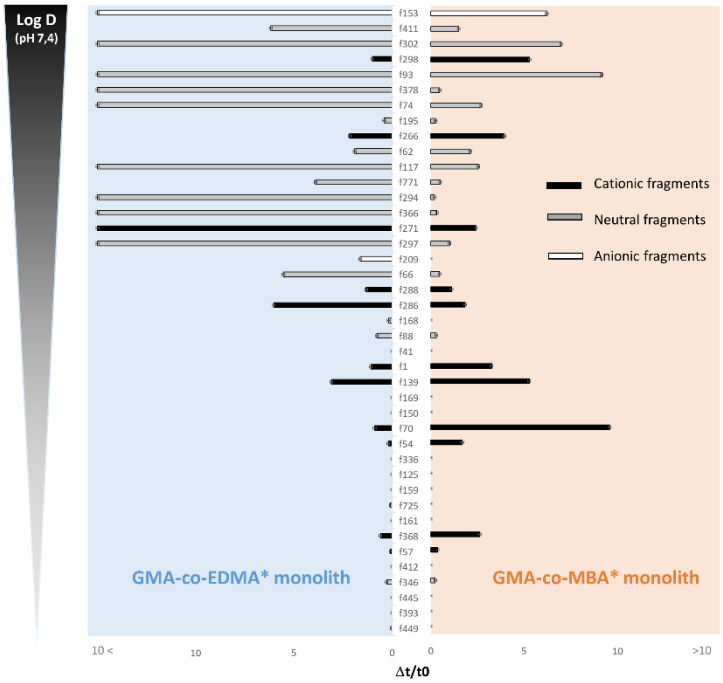


Figure 1. Nano-FAC-UV chromatograms of three molecules exhibiting different retention behavior on the reduced aldehyde GMA-co-EDMA monolith. Ligands were infused in pH 7.4 phosphate buffer solution at 67 mM and detected at 230, 254 and 280 nm, respectively for f771, f412 and f366.

The retention factors (reduced breakthrough times) measured for the 41 molecules are ranked in ascending order of their logarithm value of the distribution coefficient (log D). All the experiments were carried out in purely aqueous mobile phase at pH 7.4, i.e., in the experimental conditions classically used for affinity studies. Results are summarized in Figure 2 (left).



237

238 Figure 2. Bar graphs of the reduced breakthrough times for the 41 molecules on reduced aldehyde
239 GMA-co-EDMA* monolith (left) and reduced aldehyde GMA-co-MBA* monolith (right).

240

241 The ranking of the fragments according to their log D value (at pH 7.4) allows to feature the correlation
242 between the retention and the hydrophobic character of test compounds, whatever the net charge of
243 the molecules (Figure 2 left). Classification of the ligands according to other molecular or
244 physicochemical properties (the whole molecular descriptors used are listed in table S1) did not reveal
245 any other correlation. So, despite efforts to limit hydrophobicity of this monolithic stationary phase,
246 non-specific interactions seem to be mainly governed by hydrophobic effects. Reducing these
247 undesirable interactions involves increasing the hydrophilicity of the monolithic support. Two
248 strategies have thus been considered: replacing the EDMA with a more hydrophilic crosslinker (MBA)
249 and/or substituting part of the GMA functional monomer with a more hydrophilic monomer such as
250 acrylamide [19] at the risk of reducing the amount of active epoxy groups *i.e.* the amount of protein
251 that can be immobilized on the monolith.

252

253 3.2 Synthesis and characterization of GMA-co-MBA monoliths

254 Synthesis of GMA-co-MBA monoliths was carried out on the basis on the work of Zhu et al [19]. In this
255 work, hydrophilic GMA-based monoliths were prepared using N, N' methylenebis(acrylamide) (MBA,
256 log P -1.44) as crosslinker and acrylamide (AA, log P -0.77) as co- functional monomer with GMA (log P

1.39). Although monolithic structures were easily obtained in our laboratory using the recipe proposed by Zhu [19], the quantity of active proteins measured after grafting (less than 1 pmol cm^{-1}) was significantly lower than on our GMA-co-EDMA monolith (to 8 ± 0.3 pmol cm^{-1}). The optimization of the monolith synthesis parameters (e.g., polymerization mixture composition, temperature and polymerization time) was undergone to increase the density of active protein that can be grafted on the monolithic stationary phase. Assuming the limiting factor is the density of glycidyl reactive groups on the monolithic surface, the GMA/AA/MBA monomer ratio was modified, the porogen to monomer ratio being kept constant (Table 1). GMA content was increased by decreasing acrylamide and MBA content. The optimization criteria of the GMA-co-MBA monolith synthesis was based on the ability to still obtain a monolith structure with a satisfactory permeability and on the quantity of active protein that could be grafted. Best conditions of the thermically initiated polymerization (18 hours at 57 °C) gives rise to a quantity of immobilized active protein of 13 ± 0.8 pmol per cm of monolith. This is a big improvement over the initial monolith described by Zhu [19] and the GMA-co-EDMA monolith (8 ± 0.3 pmol cm^{-1}).

Table 1. Comparison of the monomer composition of the GMA-co-MBA polymerization mixture of the initial recipe and after optimization in this work to increase the active protein density.

% monomer (w/w)	***GMA-co-MBA*** [19]	***GMA-co-MBA This work***
GMA	24.8	60
Acrylamide	21.9	0
N,N'-methylenebis(acrylamide)	53.3	40

Although the protein content is a key parameter, our first goal was to increase the hydrophilicity of the monolith. This was evaluated by following the retention behavior of two tests solutes (thiourea a polar test solute with a log P value of -1.08 and octylbenzene a highly hydrophobic test solute with a log P value of 6.6 in an acetonitrile /water (70:30, v:v) mobile phase. The two chromatograms are illustrated Figure 3.

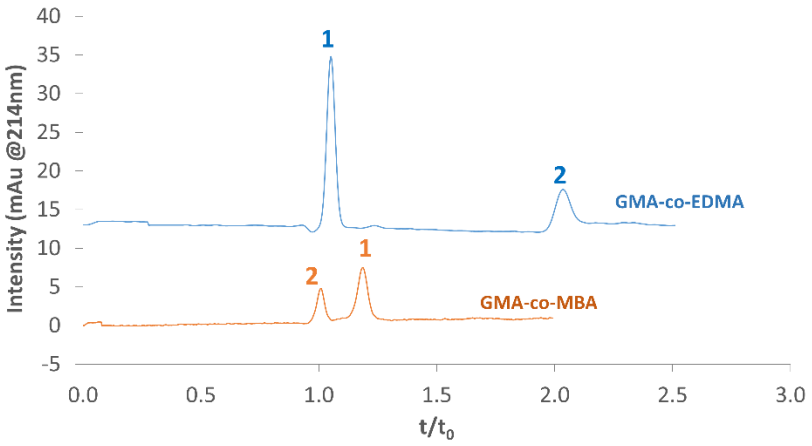


Figure 3. Chromatograms of two test solutes on GMA-co-EDMA and GMA-co-MBA monoliths. Solutes : Injection of thiourea (1) and octylbenzene (2) in acetonitrile/water (70/30, v :v) mobile phase. UV detection at 254 nm. Applied pressure 12 bars.

In these mobile phase conditions (acetonitrile /water, 70:30, v:v), the elution order of the two test solutes is reversed on the two monolithic columns. On the GMA-co-EDMA monolith, thiourea elutes at the hold-up time, while octylbenzene, which is more hydrophobic, elutes with a retention factor of approximately 1. This separation takes place according to a reversed-phase mechanism. On the GMA-co-MBA monolith and with the same mobile phase, the elution order is reversed, *viz* octylbenzene elutes first, indicative of a HILIC separation mechanism. Such a modification of the retention mechanism clearly reflects the highly hydrophilic character of this new GMA-co-MBA monolith stationary phase.

To investigate the benefits of this new monolith on non-specific interactions, the retention factors of the 41 fragment-like compounds were measured by nano-FAC on reduced aldehyde GMA-co-MBA monolith (GMA-co-MBA*) and compared with those obtained on reduced aldehyde GMA-co-EDMA monolith (GMA-co-EDMA*) (figure 2, right). As for the first study on GMA-co-EDMA monoliths all this set of experiments was carried out in a purely aqueous mobile phase buffered at pH=7.4. For most compounds, the non-specific interactions are drastically reduced. The higher the hydrophobic character of the solute, the higher the reduction of non-specific interactions. Figure 4 illustrates the impact of the reduction of the non-specific interaction for the ligand F366.

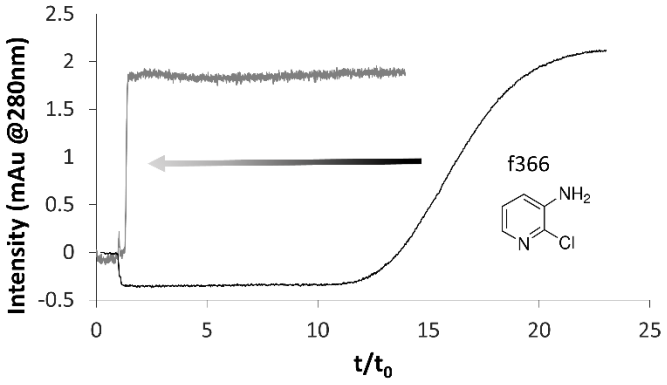


Figure 4. Nano-Frontal affinity chromatograms of the molecule F366 on the GMA-co-EDMA* and GMA-co-MBA* monolith. UV detection at 280 nm, mobile phase sodium acetate 20mM pH 7.4.

However, this effect observed for hydrophobic neutral compounds must be qualified by the increase in retention for some cationic compounds. This unexpected side effect on cationic compounds led us to seek its origin. A systematic investigation of the non-specific interactions observed for cationic compounds was performed at the different stages of functionalization. This non-specific interactions with cationic compounds appear from the first acid hydrolysis step. One hypothesis is the potential degradation of the acrylamide functions into carboxylic acids [22]. An alternative hydrolysis route to acid hydrolysis has therefore been evaluated to avoid this side-effect. A hot water hydrolysis step was therefore studied [23,24].

3.3 Optimization of the GMA-co-MBA monolith preparation: hot water hydrolysis

Following the reported procedures, GMA-co-MBA monolithic capillary columns were subjected to thermal treatment in hot water at 80°C for 18 h before undergoing the subsequent streptavidin grafting step. The efficiency of this hydrolysis of the epoxide groups method was evaluated through the determination of the quantity of grafted protein after the overall process. An amount of streptavidin binding sites of 16 ± 0.8 pmol cm^{-1} was obtained. While longer than acidic hydrolysis, this milder method using hot water is also effective to prepare affinity columns. To determine the impact of such modification on the physico-chemical properties of the resulting monolith, the retention behavior of the 41 ligands on reduced aldehyde GMA-co-MBA* monolith prepared by hot water hydrolysis was compared to those obtained by acidic hydrolysis. Figure 5 compares the reduced breakthrough times measured on the two GMA-co-MBA* monoliths. For clarity purpose, the ligands were classified by charge state (anionic, neutral and cationic ligands).

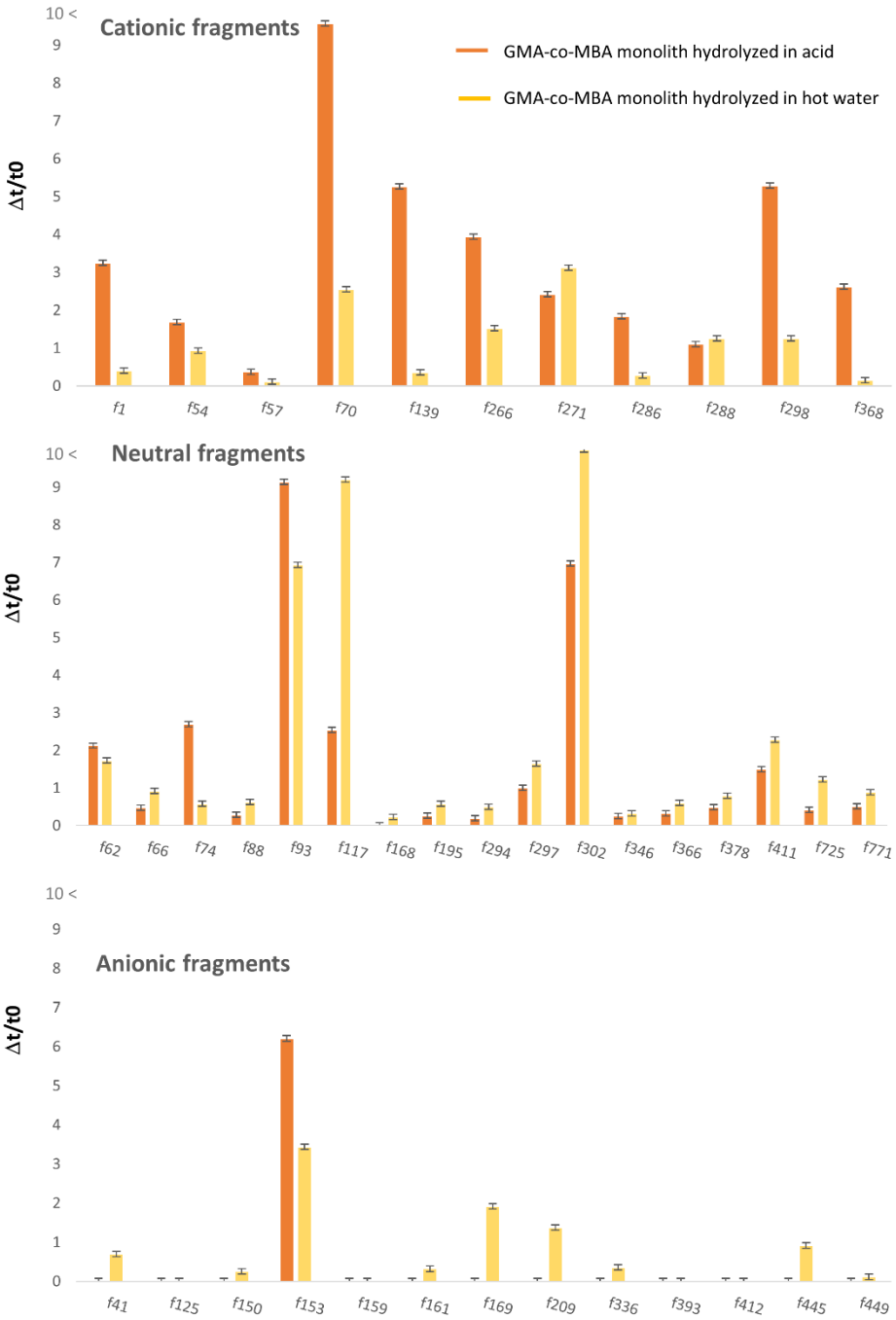


Figure 5. Bar graphs of the breakthrough times for the 41 molecules (classified according to their global charge: anionic, cationic or neutral fragments) on reduced aldehyde GMA-co-MBA* monolith hydrolyzed in acid (orange) and reduced aldehyde GMA-co-MBA* monolith hydrolyzed in hot water (yellow).

Regarding cationic fragments, except for compound f221 that is not affected, the hot water hydrolysis drastically reduced the level of non-specific interactions observed by nano-FAC experiments. Meanwhile, this new preparation method has not significantly modified the retention factor for neutral ligands, except for f117 and f302 for unknown reason. On the contrary, the retention of anionic ligands slightly increased. The very low level of retention of anionic ligands on the GMA-co-MBA* monolith prepared by acid hydrolysis seems to be stressed by repulsive ionic interactions between species of the same charge. Hydrolysis in hot water is intended to suppress the presence of anionic charge on the support. In return, the level of retention of anionic ligands slightly increases. The modification of the physico-chemical properties of the monolith generated by the modification of the mode of hydrolysis seems to confirm the initial hypothesis according to which the high level of retention for the cationic ligands came from the presence of negatively charged groups created during the acidic hydrolysis step. In addition to graft as much as protein than with the previous one, this new hydrolysis method drastically reduced the level of non-specific interactions for the cationic ligands without affecting the non-specific interactions for the neutral and anionic ligands. The stability of this GMA-co-MBA monolith hydrolyzed in hot water was evaluated. The retention factors remained constant for the duration of use (one week of continuous use) and even after 3 additional months of storage (the column was store in aqueous solution at room temperature).

3.4 Application of the new GMA-co-MBA monolithic affinity columns for the measurement of protein (Con A)-ligand interactions

Use of FAC for ligand screening generally relies on the comparison of breakthrough curves of a set of compounds using a protein immobilized on the stationary phase. Working at a fixed ligand concentration, the longer the breakthrough time, the higher the affinity. This straightforward relationship turns out to be more complex when non-specific interactions are not negligeable. It is therefore necessary to find methodological strategies to avoid misinterpretation. For example, it is possible to use control columns without protein to assess the retention level due to non-specific interactions on the stationary phase itself. Minimizing the non-specific interactions is necessary both for reducing the duration of experiments and limiting false positives. To illustrate the impact of non-specific interactions during screening experiments and show the improvement in the use of a stationary phase that limits non-specific contributions, nano-FAC experiments were performed on GMA-co-EDMA and GMA-co-MBA monoliths. Concanavalin A, a lectin that binds mannose-containing ligands with high affinity, or other carbohydrates with lower affinities [25–27], was used as protein model. Four ligands were used to illustrate the impact of the non-specific interactions when screening ligand by nano-FAC. P-nitro-α-D-mannopyranoside (αPNM) and P-nitro-α-D-glucopyranoside (αPNG)

were used as low affinity ligands (Kd values of 50 µM and > 200 µM, respectively [27]) and P-nitro-α-D-galactopyranoside (αPNGal) and f366 were used as controls (non-ligands).

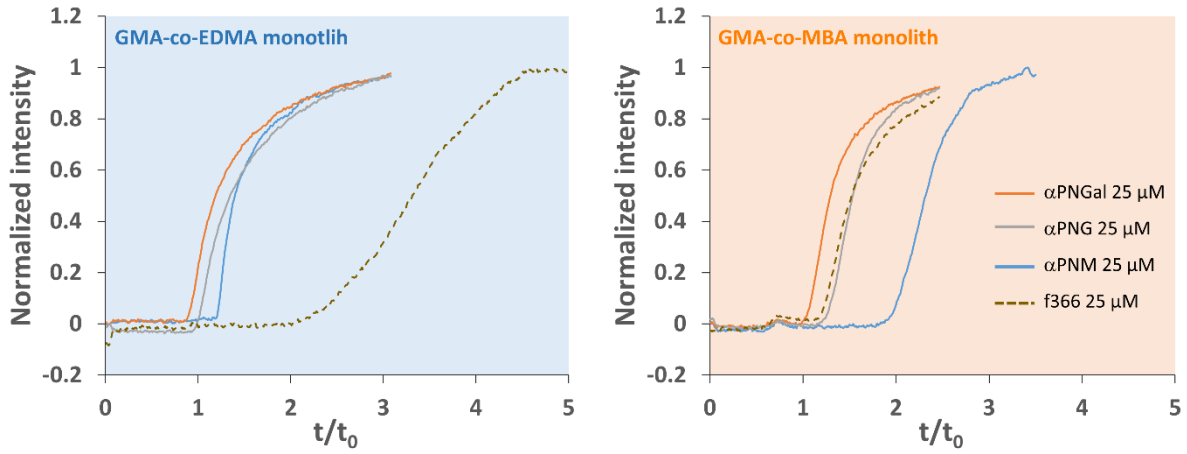


Figure 6. Nano-Frontal affinity chromatograms of αPNM, αPNG, αPNGal and the molecule f366 on the GMA-co-EDMA and GMA-co-MBA monolith. UV detection at 240 nm for the molecule f366 and 300 nm for αPNM, αPNG and αPNGal. Mobile phase sodium acetate 20mM pH 7.4.

Figure 6 shows the frontal affinity chromatograms obtained for the 4 tested ligands on both stationary phases. Regarding the glycosides residues, the galactose residue (αPNGal) as a non-ligand of ConA elutes early followed by αPNG and αPNM) on the two columns. These stereoisomers of monoglycoside share comparable physico-chemical properties and are highly polar compounds. This supports the observed behavior of αPNGal that elutes at the hold-up time of the column (t/t_0=1). The order of elution follows the affinity of the three ligands. The situation is more complex if we look to the more hydrophobic ligand f366. On the GMA-co-EDMA column, this ligand elutes in last position while it elutes with αPNGal on the MBA-co-EDMA column. F366 is a neutral and moderately nonpolar compound (log D = 0.97 at pH 7.4). Interpretation of the raw breakthrough curves on the GMA-co-EDMA monolith would lead to a wrong conclusion about the affinity of the ligands for f366. Conversely, the use of the highly polar GMA-co-MBA column allows to conserve the order of elution logical with respect to the ligand affinity. Moreover, the higher amount of active ConA grafted on the monolith allows a better discrimination between solutes with different Kd values.

4- Conclusions

In affinity chromatography, non-specific interactions between the ligands and the chromatographic support may affect the results, leading to misinterpretations during the investigation of protein-ligand interactions (detection of false positives in ligand screening, lack of specificity in purification). Optimizing the underlying support of the affinity column to reduce nonspecific interactions is an ongoing goal. A new methodology was first proposed for the characterization of non-specific interactions. This methodology relies on the determination of the retention behavior of a set of 41 selected small molecules. Such methodology applied to GMA-co-EDMA monolithic capillary columns has shown that non-specific interactions are mainly due to hydrophobic effects and must be taken into account. This led us to propose the modification of the monolith synthesis by using MBA crosslinker to increase the hydrophilicity of the resulting monolith. A large reduction of non-specific interactions was observed for all the ligands excepted the cationic ones. This behavior of cationic solutes has been attributed to electrostatic interactions with the monolith after the acidic hydrolysis step (side reaction onto the MBA crosslinker). Acidic hydrolysis was successfully replaced by a hot water treatment. The new poly(GMA-co-MBA) monolith not only exhibits improved hydrophilic surface properties, but also higher protein density (16$\pm$0.8 pmol cm^{-1} instead of 8$\pm$0.3 pmol cm^{-1} for the GMA-co-EDMA monolith). The new highly hydrophilic monolith should find applications in various fields of affinity chromatography (e.g., sample purification, preconcentration, chiral separation, IMERs …).

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