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Miniaturized Antithrombin III affinity monolithic columns coupled to TOF-MS for the selective capture and release of fondaparinux a high affinity antithrombin III ligand

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Abstract

This work explores the capability of antithrombin III-functionalized capillary monolithic columns (in-line coupled with MS detection) to selectively capture, release and detect high affinity binders of antithrombin III (AT III) from oligosaccharides mixtures. The *in-situ* characterization of home-made AT III affinity columns was done by frontal affinity chromatography coupled to MS detection using fondaparinux as model ligand. Three different preparation methods of miniaturized antithrombin III monolithic affinity columns were optimized and compared. Immobilization of antithrombin III onto Concanavalin A functionalized column is the simplest method but leads to lowest protein density. The two other methods, direct grafting on aldehyde preactivated monoliths and immobilization of biotinylated antithrombin III to streptavidin-functionalized columns, require the presence of fondaparinux to protect the heparin binding site during the grafting process. Up to $1.3 \pm 0.3 \text{ pmol cm}^{-1}$ of antithrombin III were immobilized with both methods. The direct coupling of such miniaturized affinity columns to MS-detection was made possible by optimization of the elution step. Ammonia (0.1 M) was chosen as an efficient and MS compatible solvent to elute fondaparinux. Finally, the complete analytical workflow (capture/elution/detection) was demonstrated to allow the selective capture and elution of fondaparinux initially contained in a complex oligosaccharide mixture with a limit of detection of 1 pmol.

INTRODUCTION

Glycosaminoglycans or GAGs are key polysaccharides in the organism due to the broad range of relevant biological roles supported by this class of biomolecules. Besides the biomechanical properties displayed by hyaluronan and chondroitin sulfate in some tissues, GAGs interact with various protein families (Extracellular matrix proteins, membrane proteins, lipoproteins, enzymes, cytokines, pathogens) to mediate various biological activities. The biological functions depend on the GAGs location (organ and cell compartment) [1]. The key to a better understanding of the role of GAGs is the elucidation of the structure-function relationship, which is still poorly understood [2–4]. The first challenge is to identify the interacting sequence inside these long, linear polysaccharides and then to sequence the identified binding pattern (nature and sugar positions, sulphation and N-acetylation patterns). This ambitious objective requires a demanding methodological development effort. In this context, the development of an analytical method on a miniaturized scale is of major interest to ultimately make it possible to target a particular cell compartment. Different approaches have recently been proposed : "filter-entrapment enrichment pull-down" methods [2] which are not easily amenable to a fully integrated and automated solution and solid-phase supported methods (e.g. microarray, SPR)[4,5] which require immobilization of GAGs and suffer from lack of sensitivity due to the low quantity of immobilized molecules on flat solid support.

The main objective of this study is to implement a miniaturized and in-line analytical strategy for the selective capture/elution and detection of small GAG binding motifs in a mixture of oligosaccharides. The proposed strategy relies on the coupling of nano-affinity chromatography (with the target protein immobilized on the chromatographic support) with mass spectrometry leveraging several key points. First, the porous stationary phase used in chromatography improves the surface to volume ratio compared to 2D-flat solid support and hence increases the amount of captured GAG binding motif which is beneficial for detection purpose. Then, the use of column at the capillary scale is useful to drastically reduce the protein consumption and allows to handle minute quantity of sample solution. Lastly, the in-line coupling represents the ultimate degree of integration as the loading/elution/separation steps are performed sequentially in a fully automated way. Moreover, this in-line coupling strategy is more advantageous than off-line strategy where dilution impairs the overall analytical performance (detectability) [6].

Our goal is to provide a proof of concept at the nano-scale that affinity columns can selectively capture high affinity binding motifs before their elution and detection by mass spectrometry. The well-studied antithrombin III / heparin complex was used as GAG/protein

interaction model. The development of home-made miniaturized Antithrombin-III (AT III) affinity capillary columns was investigated based on previous studies reporting immobilization of Antithrombin-III (AT III) on Sepharose supports for the preparation of affinity resins [7–9]. Such resins, composed of about 100 µm diameter beads (45-165 µm bead diameter Sepharose 4B), are not adapted to fill capillary column (75 µm i.d). So, we chose to focus our development on the use of *in-situ* synthesized glycidyl-functionalized monolithic (poly(GMA-co-EDMA) supports [10–12]. Although organic poly(GMA-co-EDMA) monoliths are widely-used stationary phases for (bio-) functionalization purposes [13,14], no report dealing with AT III immobilization on such stationary phases is available. Three immobilization strategies have been investigated and compared in this work (Figure 1).

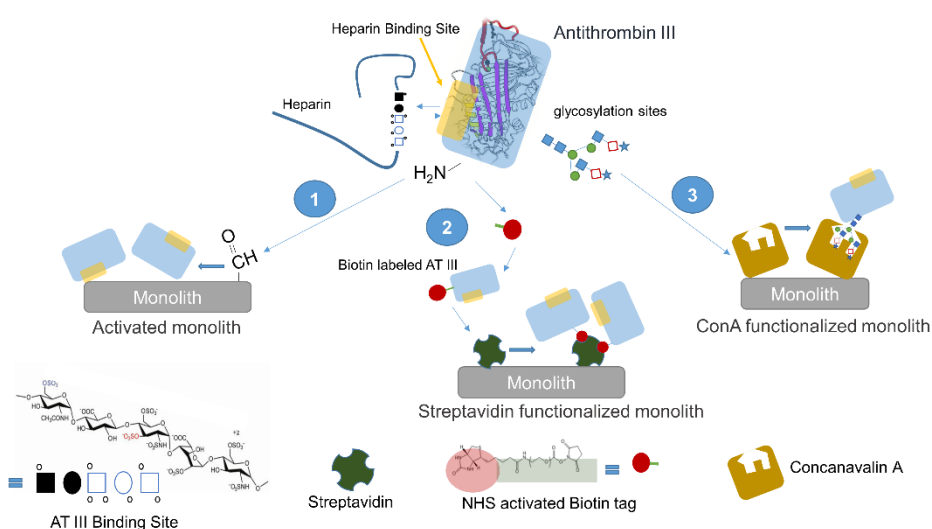


Figure 1. Schematic illustration of the strategies of immobilization of Antithrombin III on monolith capillary columns. Direct grafting of antithrombin III on aldehyde activated monolith (1), biotinylation of Antithrombin III and immobilization of the biotin labeled protein on streptavidin functionalized column (2) and immobilization of Antithrombin III on Concanavalin A functionalized column (3).

The first one is the direct covalent grafting of AT III on aldehyde-activated monoliths (Schiff base method). The second one, is an indirect non-covalent method that requires the labelling of AT III with a biotin hetero cross-linker and pre-functionalization of the monolith with streptavidin. A last method, based on lectin (Concanavalin A) interactions with glycosylated proteins (AT III has 3 to 4 glycosylation sites), was also investigated.

The column characterization was done by nano-frontal affinity chromatography - Mass spectrometry (nano-FAC-MS) experiments. Fondaparinux (FDPX), a highly specific ligand of AT III, was used as model ligand. This pentasaccharide was used to determine the binding capacity of the columns (which has to be as high as possible) and to compare the three grafting methods. The elution conditions of FDPX were optimized to be MS-compatible.

Finally, we present the selective capture of FDPX in a complex mixture of oligosaccharides, its elution and in-line MS detection.

Material et methods

- *Chemicals*

Ethylene dimethacrylate (EDMA, 97%), glycidyl methacrylate (GMA, 98%), 1-propanol, 1,4-butanediol, (3-methacryloxypropyl)-trimethoxysilane (γ -MAPS), methanol (HPLC grade), triethylamine (TEA), pentylamine (PTA), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), sodium periodate, lithium hydroxide, dipotassium hydrogen phosphate (K_2HPO_4), o-phosphoric acid, acetic acid, sodium cyanoborohydride. 4-nitrophenyl- α -D-mannopyranoside, Concanavalin A, Biotinylated Concanavalin A were purchased from Sigma-aldrich (Saint-Quentin Fallavier, France), Ammonia solution (28-30 %) was purchased from Merck (Darmstadt, Germany), sulfuric acid (18 M) from VWR (Fontenay-sous-bois, France). Low Molecular Weight Heparin (LMWH, molecular range 1800 – 7500 dalton) was purchased from Iduron (Manchester, United Kingdom). All aqueous solutions were prepared using >18 M Ω deionized water. Preparation of the phosphate buffer was done by dissolving 1.17 g of K_2HPO_4 in 100 mL of ultrapure water and pH was adjusted to 7.4 with o-phosphoric acid.

- *Instrumentation*

Capillary columns were prepared using a pressure gradient microfluidic flow controller MFCS-EZ100 system (Fluigent, Villejuif, France) to handle liquids inside the capillary. Inlet pressure values are reported to specify the liquid flow conditions (outlet pressure was always set up at atmospheric pressure). An LC pump L-6000 (Merck, Darmstadt, Germany) was used to rinse the freshly prepared monolithic capillary columns.

Nano-FAC-UV experiments (frontal weak affinity chromatography experiments) with in-situ UV detection were carried out with a capillary electrophoresis Agilent 7100 system (Agilent Technologies, Waldbronn, Germany) equipped with an external pressure nitrogen supply allowing to work up to 1.2 MPa (up to $0.1 \text{ cm}\cdot\text{s}^{-1}$ with aqueous solutions for a 8.5 cm monolith). The affinity experiments were carried out with the CE system in the short-end injection mode. The system was exclusively operated in the pressurization mode by applying external pressure on the outlet vial (no voltage applied). The detection was achieved “on-column” (in an empty section of the 10-cm capillary located just after the monolith), with a diode array detector operated in a multi-wavelength mode. The effective length of the affinity column was 8.5 cm. System control and data acquisition were carried out using the Chemstation software (Agilent). The carousel and cassette temperatures were set at 25°C. After each analysis, the capillary column was rinsed with 20 column volumes of phosphate buffered solution.

Nano-FAC-TOF-MS analyses were carried out with a Nano-LC (from Eksigent) connected to an Electro-Nano-Spray Ionization source (from Agilent) with a TOF analyzer (Agilent 6230 TOF LC/MS). Optimization of the coupling set-up and MS parameters for the in-line coupling is presented in supplementary material (S1). An additional make-up flow (300 nL min^{-1}) made of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 50 mM) in mixture with pentylamine (PTA, 30 mM) in methanol was added with the loading pump of the nano-LC device and mixed with the chromatographic flow in a zero-dead volume union (UH-750 from IDEX (Cluzeau info labo, France)). Nanospray stainless steel emitters with a uniform $30 \text{ }\mu\text{m}$ -inner diameter were purchased from Pepsep (Marslev, Denmark).

- *Antithrombin III biotinylation*

Antithrombin III was biotinylated with a long chain (29 \AA), water soluble biotinylating reagent using the Thermo Scientific EZ-Link™ NHS-PEG4 Biotinylation Kit, following the manufacturer's instructions. The biotinylation molar excess was 20:1 to control the biotinylation ratio. The biotin incorporation ratio was estimated thanks to the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) Assay, executed with the reagents included in the Thermo Scientific EZ-Link™ NHS-PEG4 Biotinylation Kit.

Two biotinylated AT III batches were prepared. The first batch was prepared in absence of fondaparinux ((-) FDPX) and the second one in presence of fondaparinux during the labelling step ((+) FDPX). Considering the high affinity between Antithrombin III and Fondaparinux, a 1:10 molar ratio was used to completely complex Antithrombin III with Fondaparinux. A volume of $205 \text{ }\mu\text{L}$ of a $1000 \text{ }\mu\text{M}$ Fondaparinux is added to $900 \text{ }\mu\text{L}$ of a $22.7 \text{ }\mu\text{M}$ Antithrombin III solution in PBS buffer before the biotinylation step. The reaction mixture is mixed and incubated at room temperature during 15 minutes before used. Then this solution is biotinylated according to manufacturer's instructions.

- *Preparation of the AT III functionalized monolithic capillary columns*

Synthesis of poly-(GMA-co-EDMA) monoliths

Fused-silica capillaries with UV transparent coating (TSH, $75\text{-}\mu\text{m}$ i.d.) were purchased from Polymicro Technologies (Molex, France). Poly(GMA-co-EDMA) monoliths were synthesized as described in a previous work [11,15]. Briefly, capillaries were pre-treated (at 0.7 MPa) with a 5 % (v/v) solution of γ -MAPS in methanol/water (95/5, v/v) 2.5 % TEA for 1 h. Next, they were rinsed with methanol for 15 min at 0.7 MPa and dried at room temperature under nitrogen stream. A 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 γ -MAPS pre-treated capillary was then filled with the polymerization mixture under 0.1 MPa 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 8.5-cm monolithic segment of the 15-cm capillary was rinsed with methanol for 1 h with the chromatographic pump (30 bars).

Covalent coupling of protein through Schiff base method

First, the epoxy groups of the GMA-co-EDMA monoliths were hydrolyzed into diols flowing 1 M sulfuric acid for 2 h at 0.7 MPa. After rinsing, the diol moieties were oxidized into aldehyde ones using a 0.12 M NaIO₄ solution at pH 5.5. Then, a 1 mg ml⁻¹ protein solution (AT III, Streptavidin or Concanavalin A) and 8 mg mL⁻¹ NaBH₃CN solution in 67 mM pH 6 phosphate buffer was percolated through the column for 12 h at 0.7 MPa (room temperature). After immobilization, the column was flushed (2 h, 0.7 MPa) with sodium borohydride (NaBH₄ at 2.5 mg mL⁻¹ in phosphate buffer 67 mM pH 8) to reduce residual aldehydes. The affinity columns were then rinsed with phosphate buffer and stored at 4°C.

For AT III immobilization, the protocol was performed either without fondaparinux ((-) FDPX) or with fondaparinux ((+) FDPX) added at 100 µM to the protein reaction mixture to completely saturate the Heparin binding sites before the grafting. The columns grafted in presence of fondaparinux ((+) FDPX), were thoroughly rinsed with a high saline buffered solution (1 M NaCl, for 2 h) to remove FDPX from AT III before any use.

Streptavidin-biotin immobilization pathway

Streptavidin was grafted on the organic monolith following the Schiff base method described above using a 1 mg mL⁻¹ streptavidin solution. Streptavidin columns can be prepared and stored for several months at + 4°C. Finally, biotinylated-AT III either without fondaparinux ((-) FDPX) or with fondaparinux ((+) FDPX) solution was percolated through the streptavidin column using the Agilent CE system at 1.2 MPa pressure, monitoring UV absorbance at 280 nm. The percolation was stopped when saturation of the support was reached i.e., when the biotin-AT III grafting solution reaches the detector (observation of a plateau at 280 nm). The breakthrough volume was used to estimate the quantity of captured protein. The columns grafted in presence of fondaparinux ((+) FDPX), were thoroughly rinsed with a high saline buffered solution (1 M NaCl, for 2 h) to remove FDPX from biotinylated-AT III before any use.

Concanavalin (Con A) immobilization method

Con A columns were first prepared by grafting Con A onto monolithic columns by the Schiff base method. Due to the dependence of Con A activity on the presence of calcium ions, phosphate buffer 67 mM with Ca²⁺ (67 mM K₂HPO₄, pH 7.4, 100 µM Ca²⁺) was percolated through the column for 40 minutes before AT III immobilization. Immobilization of AT III was carried out using a 5-µM AT III solution in phosphate buffered solution (67mM K₂HPO₄ pH

7.4). The grafting experiments were conducted using the CE system in the short-end injection mode. The detection was achieved “on-column” (in an empty section of the 10-cm capillary located just after the monolith), with a diode array detector operated in a multi-wavelength mode. The effective length was 8.5 cm. Percolation of AT III solution on Con A column was carried out overnight at 12 bars pressure.

- *Column characterization*

Column characterization was assessed by nano-FAC with UV or MS detection. The accurate determination of the breakthrough times, t_{plateau} , was achieved using the 1st derivative of the UV or MS signal.

Characterization of the ATIII columns by nano-FAC-MS

The determination of the number of ATIII active sites ($B_{\text{act,ATIII}}$) was assessed by nano-FAC-TOF-MS experiments using a 5 μM FDPX solution in ammonium acetate solution (20 mM, pH 7.4). Considering the high affinity between FDPX and ATIII (K_d in the nM range) and the high FDPX concentration (in the μM range) with regard to the K_d value, one may consider that the column is saturated at such concentration. Thus, the number of active sites can be estimated in a single experiment according to equation 1 :

$$B_{\text{act,ATIII}} = (t_{\text{plateau}} - t_0) \times F \times [\text{ligand}] \quad (\text{eq 1})$$

with F being the flow rate and t_0 the column hold-up time, t_{plateau} the breakthrough time of FDPX as ligand.

Characterization of Con A columns by nano-FAC-UV

The determination of the number of Con A active sites ($B_{\text{act,ConA}}$) was assessed by nano-FAC-UV, with 4 nitrophenyl- β -D-mannopyranoside (a weak affinity ligand with a K_d value of few tens μM) as test solute. The solute was infused at different concentrations $[L_i]$ ranging from 5 μM to 40 μM , and the amount of ligand captured at each concentration (q_i) calculated according to equation 1. The total number of active sites on the affinity column, $B_{\text{act,ConA}}$, and the dissociation constant, K_d , of the Con A-ligand complex were determined according to eq. 1 after plotting $1/q$ versus $1/[L]$:

$$\frac{1}{q_i} = \frac{K_d}{B_{\text{act,ConA}}} \times \frac{1}{[L_i]} + \frac{1}{B_{\text{act,ConA}}} \quad (\text{Eq 2})$$

Results

- *Biofunctionalization of capillary monolithic columns: Antithrombin-III immobilization strategies*

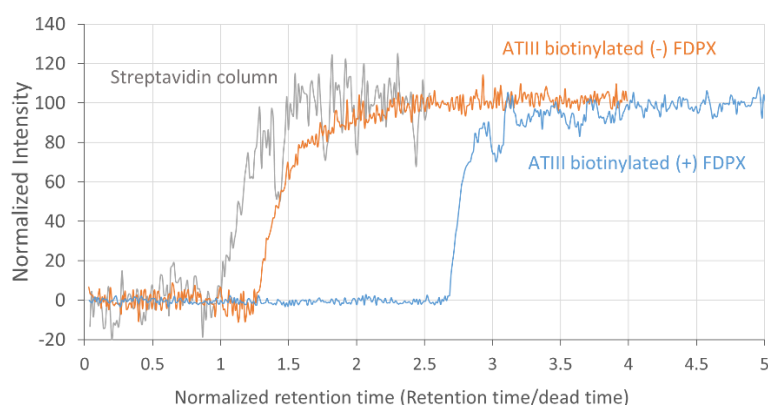
The key parameter for affinity column development is the binding capacity per volume unit. Such binding capacity is not related to the total amount of immobilized protein that can be evaluated by colorimetric assay (BCA, ADECA) but only depends on the number of accessible active sites (B_{act}) [16]. The in-capillary determination of B_{act} can be performed by nanoFAC-MS experiments (see material and methods section). This determination is based on the measurement of the breakthrough time of a high affinity ligand continuously perfused through the stationary phase bearing the immobilized protein. FDPX was used as a high affinity ligand of AT III (K_d value of few tens nM depending the ionic strength among others [17,18]). Using a high FDPX concentration (5 μ M) with regard to the K_d value, the number of active sites can be estimated in a single experiment and is equal to the amount of ligand captured. For a given ligand-protein complex, all other conditions being equal, the longer the ligand breakthrough time, the greater the amount of ligand captured and the greater the number of active sites on the stationary phase. The B_{act} value was used to evaluate the three different grafting methods.

Streptavidin-biotin immobilization pathway: This approach takes advantage of the instantaneous capture of a biotinylated-target protein onto streptavidin-modified supports. The strong streptavidin-biotin interaction ($K_d = 10^{-15}$ M) allows dynamic affinity studies under mild conditions [19]. In this approach, the biotinylated protein is infused through the streptavidin-functionalized monolith. The capture of AT III is quantitative thus limiting the amount of protein required to the strict minimum. Nevertheless, this approach requires the biotinylation of the target protein. Bio-engineered biotinylation (using Avi-tagTM technology) is suitable for oriented biotinylation (direct integration of a biotin tag at one end of the protein). However, it requires development of recombinant proteins that is beyond the scope of our work. So, we chose the alternative chemical derivatization of the protein using the PEG-NHS biotinylation reagents that target the amino groups of the protein. However, such a derivatization through chemical reactions targeting cationic amine groups of AT III is likely to modify the Heparin Binding Site (HBS) involved in the AT III-heparin interaction. Indeed, interactions of AT III with heparin is mainly mediated by electrostatic interactions [3,20] between the cationic heparin binding site (HBS) of AT III (lysine, arginine groups) [21] and the sulphate groups of heparin. To prevent such side-effect, the protection of the HBS with its ligand during the derivatization was considered as it has been reported to largely increase the number of remaining active sites after grafting [22,23]. The effect of such protection during the chemical biotinylation of AT III was also investigated herein. Two batches of

biotinylated ATIII were prepared with biotinylation ratios of 2,4 and 2,7 for the biotinylation in absence ((-) FDPX) and in presence ((+) FDPX) of fondaparinux respectively, which is well-adapted to the preparation of functionalized dense protein monolayer [24]. These two batches were used to prepare AT III affinity columns by percolation of the biotinylated AT III on streptavidin pre-functionalized monoliths.

Figure 2 presents an example of the breakthrough curves obtained for FDPX on AT III-monolithic capillary columns prepared by the streptavidin-biotin immobilization pathway, using AT III biotinylated in presence or absence of fondaparinux. The FDPX breakthrough time obtained for the column prepared with AT III biotinylated in presence of FDPX ((+) FDPX) is higher than the one measured on the column prepared with AT III biotinylated in absence of FDPX ((-) FDPX). So, $B_{act,ATIII}$ is higher for the former column ((+) FDPX) than for the latter one ((-) FDPX) (respectively $1.1 \pm 0.1 \text{ pmol cm}^{-1}$ (n=3) and $0.20 \pm 0.02 \text{ pmol cm}^{-1}$ (n=3)). In addition, the total quantity of AT III immobilized (determined by the UV monitoring during the AT III immobilization step) is quite similar for both columns ($B_{tot,FDPX}$ about $3.8 \pm 0.4 \text{ pmol cm}^{-1}$ (n=6)). It shows that the presence of FDPX during the biotinylation step makes it possible to increase the fraction active protein ($B_{act,ATIII}/B_{tot,ATIII}$) from 5 to ~ 30%. The higher $B_{act,ATIII}/B_{tot,ATIII}$ for the column prepared with AT III biotinylated in presence of FDPX ((+) FDPX) highlights the importance of protecting the heparin binding site (HBS) during the biotinylation step.

The comparison with a control column (streptavidin functionalized column with no AT III) for which the breakthrough of FDPX occurs at the dead time, shows that the capture is due to specific interactions with AT III and not to non-specific interactions with the streptavidin monolith. Moreover, this comparison also confirms that AT III-monolithic columns prepared



with AT III biotinylated in absence of fondaparinux exhibit only a limited activity.

Figure 2. Breakthrough curves of fondaparinux $1 \mu\text{M}$ in ammonium acetate buffer (pH 7.4, 20 mM) obtained by percolation on streptavidin-column (grey line), streptavidin-column after immobilization of biotinylated antithrombin

III in presence (+) (blue line) and absence (-) (orange line) of fondaparinux during the biotinylation step. Intensity was measured using the 1233 Th extracted ion signal.

These results show that the biotinylation of AT III must be done in presence of FDPX to preserve the HBS and hence to ensure high activity of the resulting AT III column.

The streptavidin-biotin immobilization pathway is rapid and limits the total amount of protein consumed (about 30 pmol for one column). While this method requires biotinylated proteins, which involves additional protein manipulations, the streptavidin/biotin immobilization is a “generic” method adapted to a large diversity of biomolecules. This method implies the use of streptavidin functionalized column which can be a potential source of non-specific interactions. Interestingly, the UV-monitored AT III immobilization allows the *in-capillary* determination of the total amount of immobilized protein without any additional experiments, which is very interesting for quality control purposes [19].

The same methodology was applied to evaluate the AT III monolithic capillary columns prepared with the two other immobilization methods.

AT III direct grafting (Schiff base method): The direct grafting of AT III is carried out after hydrolysis of the surface glycidyl groups into diols, followed by an oxidation step yielding amine-reactive aldehyde groups at the monolith surface. This way is preferred to the direct grafting of proteins onto glycidyl-functionalized monolith to reduce non-specific interactions (residual epoxy groups leads to a more hydrophobic surface). Although this method is widely used, adaptation to AT III immobilization had to be specifically considered. Indeed, the random reaction of the amine groups at the protein surface with the aldehyde groups of the monolith can lead to hinder the access or block the active binding site of the protein. Considering the results obtained for the streptavidin-biotin immobilization pathway, the direct grafting of AT III on aldehyde pre-functionalized column (Schiff base method) was implemented in presence or absence of FDPX during the grafting step. Results, expressed as number of active sites, are given figure 3. As already observed for the streptavidin-biotin method, the number of active sites was greatly improved by shielding the HBS, thus confirming that protection of the binding site during the immobilization (by adding FDPX to the grafting mixture) step is critical. A $B_{act,ATIII}$ up to $1.3 \pm 0.2 \text{ pmol cm}^{-1}$ (n=3) was obtained for this direct grafting method in presence of FDPX.

This grafting method is direct *viz* does not require specific preparation of the protein. The covalent coupling ensures a high reusability of the affinity column but is time consuming (12 h) and requires high protein concentrations (in the mg mL^{-1} range) [25,26]. However, considering the low column volume (about 330 nL for a 8.5-cm capillary length) and the low

flow rate used during the grafting step, the protein consumption is limited to about 50 $\mu\text{g}/\text{column}$ i.e. 860 pmol.

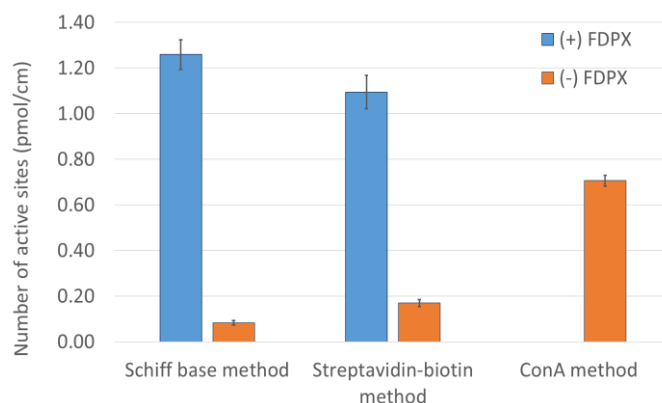


Figure 3. Number of active sites (pmol cm^{-1} of monolith) for the different grafting strategies studied.

Biochemical immobilization of AT III on Con A-functionalized monoliths: The last investigated method takes advantage of the fact that AT III is a glycoprotein and that its glycosylation pattern can be used to immobilize the protein onto lectin-affinity columns. Concanavalin A (Con A) is a member of this lectin family well-known for its specific interactions with rich-mannose glycoproteins. Direct immobilization of AT III on Con A-functionalized supports has been considered [7–9] as it preserves accessibility to the binding site (HBS). Moreover, it has been demonstrated that this link was stable over time in adapted buffer conditions [7]. It is worth noticing that for this last immobilization pathway protection of the HBS is unnecessary during the immobilization step as amine groups of the HBS are not involved. The loading of AT III on the Con A column can be UV monitored as for the streptavidin-biotin method. The loading profile of the AT III onto Con A functionalized column leads to a gentle slope profile that is attributed to slow kinetic interaction. It was shown that increasing the AT III infusion duration (overnight) is required to obtain the highest number of AIII active sites (data not shown). Nevertheless and as illustrated Figure 3, this “Con A” method, gives a quite low number of AT III active sites ($B_{\text{act,ATIII}} = 0.7 \pm 0.2 \text{ pmol cm}^{-1}$ ($n=3$)) despite the amount of active Con A on the column ($B_{\text{act,ConA}} = 1.7 \text{ pmol cm}^{-1}$ ($n=3$)). Considering a 1:1 interaction between AT III and Con A, a higher $B_{\text{act,ATIII}}$ (about 1.7 pmol cm^{-1}) could be expected (provided no steric hindrance issues occur). This discrepancy can be at least partially attributed to a partial denaturation of starting AT III before immobilization. Such a partial denaturation could also explain the relatively low activity ratio ($B_{\text{act,ATIII}}/B_{\text{tot,ATIII}}$) observed with the other grafting methods.

The Con A immobilization pathway is an easy and direct grafting method. Nevertheless, the method is limited to glycoproteins and requires the preparation of Con A columns. A specific feature of this interaction concerns its reversibility. Indeed, immobilized AT III proteins can be

removed from the Con A-support by elution with α -methyl glucoside [7]. However, few information is available about the experimental conditions (strength of the interaction, time to complete the protein-protein interaction).

Figure 3 compares the $B_{act,ATIII}$ measured for all columns prepared in absence or presence of FDPX (if relevant). Whatever the strategy used, the reproducibility of the column preparation is quite similar (coefficient of variation ranging from 9 to 16 % measured on at least 3 columns of different batches). AT III functionalized columns are reusable and support up to 7 successive capture/wash and elution cycle experiments. The behavior (evolution of the binding capacity) was not significantly different between the Schiff-base and the streptavidin methods. The loading capacity decreases slightly during these cycles (approx. 10 % after 7 cycles). We attribute this result to partial loss of activity of the AT III. On the Con A/AT III column, we observed a \sim 40% loss of capacity after the first elution cycle but afterwards the behavior was similar to the one observed with the two other methods. Due to the high binding capacity and to the lower number of grafting steps required to immobilize AT III, the Schiff base method was chosen for subsequent experiments.

- *Use of the miniaturized ATIII-columns for fondaparinux capture/release experiments*

Optimization of fondaparinux capture and release

Main objective of the direct coupling of a miniaturized AT III column with mass spectrometric detection is to carry out the in-line ligand capture, release and detection of high affinity oligosaccharides contained in complex mixtures. The analytical workflow is composed of 4 steps. The first step concerns the capture of AT III ligands within a set of oligosaccharides. It is followed by a thorough wash of the affinity column to remove weak-affinity oligosaccharides. The two last steps are the elution of the captured high-affinity oligosaccharides from the affinity column and their MS detection. Usually, elution of high affinity oligosaccharides from affinity column is triggered by an increase of the ionic strength using high salt concentration mobile phases. Elution with 1M NaCl concentration or more (up to 3 M NaCl)[7,17,18,27] is usually used on ATIII affinity columns. The use of such high salt concentration mobile phases is precluded with in-line MS detection and requires finding an MS compatible alternative. Given the two main contributions that promote the specific FDPX/ATIII interaction, viz a specific conformation of the HBS of ATIII and electrostatic interactions [28], elution in basic conditions was investigated. Indeed, it was shown that the affinity of ATIII for heparin starts to decrease markedly at pH 8.5 with complete loss of affinity above pH 10. This behavior has been attributed to a combined effect of a reversible change in the AT III conformation and of a modification of its ionization state (deprotonation of amino

and hydroxyl residues, which become respectively neutral or partially negatively charged). Two MS compatible basic solutions were thus evaluated for the elution step, ammonium carbonate buffer (20 mM) and ammonia solution. The detectability of FDPX in ammonium carbonate buffer was greatly reduced due to a large increase in background noise. In NH_4OH medium, the FDPX detectability was comparable to that observed in ammonium acetate buffer (data not shown). The elution of FDPX was therefore studied by percolating a 0.1M ammonia onto the column.

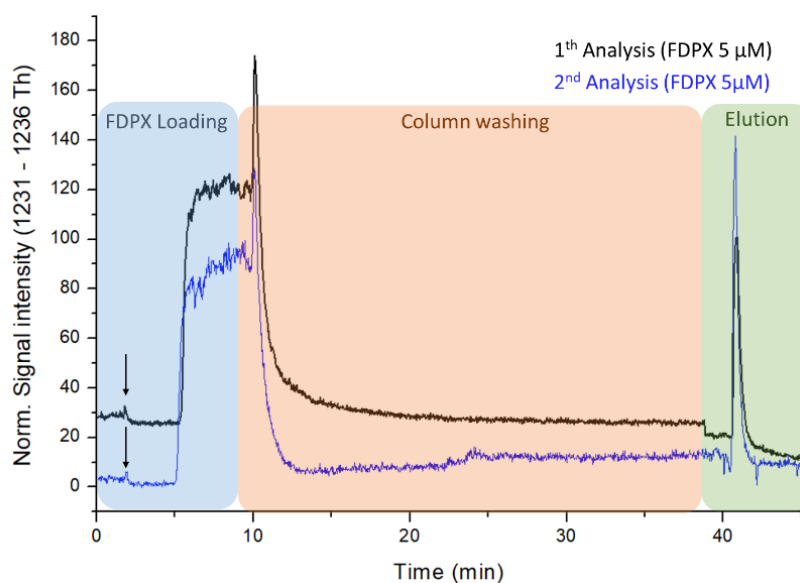


Figure 4. Chromatograms of the different steps (loading, washing and elution) of FDPX analysis using a 8-cm length AT III affinity capillary column (Schiff base method) coupled to TOF-MS detection. Mobile phase flow rate is 300 nL min^{-1} , HFIP/PTA make-up phase flow rate is 300 nL min^{-1} and detection of FDPX is done by extracting the signal between 1231 and 1236 Th. The loading is done with a solution of fondaparinux $5\mu\text{M}$ diluted in ammonium acetate solution, the washing with ammonium acetate solution and elution with ammonia 0.1M. Dead time is marked by an arrow.

To evaluate the compatibility and the efficiency of the elution (or release) of the captured FDPX, an AT III column was first loaded with FDPX and then rinsed with a 30-column volumes of ammonium acetate buffer. It was then eluted by injecting a one-column-volume plug of ammonia (0.1 M) onto the affinity column coupled to the MS detector. A FDPX sharp signal was detected in this elution plug, indicating that a basic ammonia solution successfully elutes fondaparinux from ATIII affinity columns in front of this large plug (Figure 4). The repetition of the capture and elution steps showed that the use of ammonia does not denature ATIII and allows the chromatographic support to be reused in-line with MS detection. Indeed, the FDPX breakthrough times are the same i.e., the amount of FDPX captured are comparable. In these conditions the method allows to detect as low as 1 pmol of FDPX in the elution peak (see supplementary material S2).

Proof of feasibility: Capture and release of fondaparinux in complex matrix

A complex mixture of oligosaccharides composed of low molecular weight heparins (LMWHs) spiked with FDPX was used to investigate the capacity of the miniaturized AT III-affinity column to selectively bind and elute high affinity ligands in a soup of oligosaccharides. A low molecular weight heparin sample produced by partial depolymerization of heparin with heparinase I (Hep 1) was used for this study. Hep I cleaves the glycosidic linkage between N-sulfated glucosamine and 2-O-sulfated uronic acid. It was shown that Hep 1 cleaves at the AT-III binding site [29] and generate truncated (low affinity) and inactive sequences [18,30]. 5- μM FDPX solutions prepared in 25 to 200 μM LMWH matrices (using an average molecular weight of 4000 Da) were tested. The corresponding chromatograms are presented figure 5. While MS detection was useful to follow the breakthrough of FDPX during the capture step in pure solution (figure 4), addition of LMWH in the sample solution leads to the suppression of the MS signal (ionic suppression). For the highly concentrated LMWH samples ($> 100 \mu\text{M}$), FDPX was no longer detectable during the loading step. Whatever the LMWH concentration during the percolation, the peak areas of the FDPX eluted peaks are comparable. This result demonstrates that the capture of FDPX in a complex mixture of highly concentrated oligosaccharides is successful and that the column is reusable.

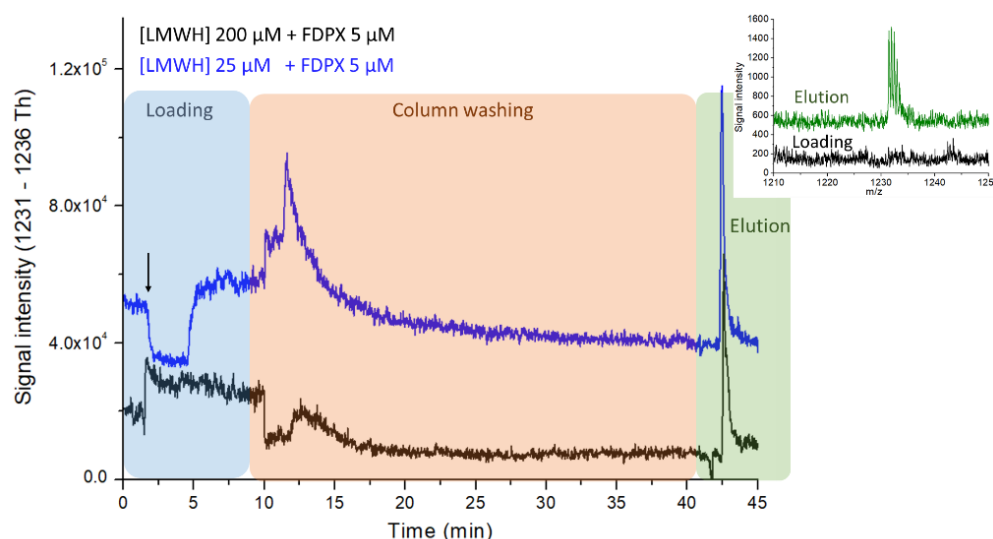


Figure 5. Chromatograms of the different steps (loading, washing and elution) of FDPX analysis in mixture with 25 μM and 200 μM LMWH. Analysis using a 8-cm length AT III affinity capillary column coupled to TOF-MS detection. Mobile phase flow rate is 300 nL min^{-1} , HFIP/PTA make-up phase flow rate is 300 nL min^{-1} and detection of FDPX is done by extracting the signal between 1231 and 1236 Th. The loading is done with a solution of FDPX 5 μM diluted in ammonium acetate solution, the washing with ammonium acetate solution and

elution with ammonia 0.1M. Zoom of the spectra (1210 – 1250 Th range) extracted during the loading and elution steps.

Conclusion

Elaboration of affinity columns functionalized with AT III were implemented at the capillary scale using three different grafting pathways. The affinity columns were characterized by frontal affinity chromatography hyphenated with TOF-MS detection with FDPX as specific high affinity ligand of AT III. The direct grafting of AT III by the Schiff base and the streptavidin-biotin methods led to the highest ATIII grafting density. For both methods, protection of the HBS during the grafting or the labelling step is compulsory to maintain the protein activity. The elution of FDPX from the affinity column with an aqueous ammonia solution (0.1M) allows the direct in-line coupling of the miniaturized affinity column to MS detection (which is not possible with the classical NaCl elution conditions). It was demonstrated that FDPX can be specifically captured from a small sample volume (few μL) and in a complex mixture of oligosaccharides and then detected with a limit of detection estimated to 1 pmol. This overall process is useful to process in-line small sample volumes of oligosaccharide mixtures with MS detection.

Main limitations of the method would come from the amount of active binding sites and MS detection. The potential loss of activity consecutive to the immobilization process as well as a low density of active protein (low quantity / unit volume) could limit the quantity of ligand that can be captured and hence detected. This concern must be addressed for each new target. Regarding the MS detection, an increase in size of the oligosaccharides leads to the formation of a higher number of ionized species and hence to a decrease in their detectability. This may discriminate the ligand species by their size. The method is thus more appropriate for short oligosaccharides. Such an in-line coupling could be used for deciphering the complex structure of oligosaccharide binders. The structure elucidation could be facilitated by its coupling with Infrared multiple photon dissociation-mass spectrometry (IRMPD-MS)[31].

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