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# Deciphering dynamic combinatorial libraries of glycoclusters with miniaturized weak affinity chromatography coupled with mass spectrometry (nano-FAC-MS)

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## Abstract

We report an original methodology based on affinity chromatography coupled with mass spectrometry to decipher the complexity of dynamic combinatorial libraries (DCLs) of glycoclusters. Such libraries are intended to boost the design of potential therapeutic anti-infectious agents targeting *Pseudomonas aeruginosa*, which is responsible for numerous diseases, mostly found in hospitals as major a cause of nosocomial infections. Dynamic combinatorial chemistry provides a rapid access to an equilibrating mixture of glycocluster candidates through the formation of reversible covalent bonds under thermodynamic control. Identifying each molecule in the complex mixture overcomes challenges due to the dynamic process. Selection of glycoclusters candidates was first realized on a model lectin (Concanavalin A, ConA). Home-made affinity nanocolumns, containing covalently immobilized ConA and have volumes in the microliter range, were used to separate DCLs of glycoclusters with respect to their specific lectin binding properties under buffered aqueous conditions. Miniaturization facilitates the inline coupling with MS detection in such purely aqueous and buffered conditions and reduces target protein consumption. Monolithic lectin-affinity columns prepared by immobilization of ConA were first characterized using a known ligand. The amount of active binding immobilized lectin is  $61 \pm 5$  pmol on 8.5-cm length column. We demonstrated the ability of our approach to evaluate individual dissociation constants of species directly in the complex mixture. The concept was then successfully applied to the screening of DCLs of more complex glycoclusters to identify (by mass spectrometry) and rank the ligands (by relative breakthrough curve delay) according to their affinity for the immobilized lectin in a single experiment.

**Keywords :** Dynamic combinatorial chemistry, Concanavalin A, affinity chromatography, multivalency, glycocluster, *Pseudomonas aeruginosa*

## 1. Introduction

Lectin-carbohydrate interactions govern a wide range of biological processes including simple recognition processes at the surface of cells, bacteria or viruses [1,2]. This receptor-ligand interaction between a bacterial/viral protein (also known as lectin or adhesion) and oligosaccharides displayed at the outer membrane of the host cells in their so-called glycocalyx is the very first event in bacterial or viral infection when a pathogen colonizes a specific organ [3]. The design of anti-adhesive molecular entities capable of blocking this adhesion is of paramount interest to provide innovative anti-pathogenic therapeutic strategies [4]. While a lectin-carbohydrate interaction is typically in the mM to  $\mu$ M range, the design of multivalent glycoconjugates such as glycoclusters, glycodendrimers, or glycopolymers to name a few, have generated high affinity ligands of this class of proteins and have thus found biomedical applications [5]. Their increased affinity due to the so-called glycoside cluster effect can then be measured by Isothermal Titration Calorimetry (ITC), Surface Plasmon Resonance (SPR) or BioLayer Interferometry (BLI).

The general strategy to study the binding properties of a set of multivalent glycoclusters is to synthesize a family of candidates and then evaluate their affinity for the lectin one by one and describe structure-activity relationships. Dynamic covalent chemistry, also called dynamic combinatorial chemistry (DCC for both) [6], is an alternative approach that allows the formation of a dynamic combinatorial library (DCL) of glycoclusters as a statistical mixture of all possible structures. Powerful analytical methods are then required to decipher the information from the DCL complex mixture *i.e.* to identify the best ligands for a specific lectin. These analytical methods must use the smallest possible amount of sample while providing robust and reliable data. The advantage of DCC, provided such powerful analytical methods are available, is that there is no need to prepare each glycocluster generated in the mixture, separately and as a pure sample.

In the established approach, a comparative HPLC-MS analysis of the DCL is performed in presence or absence of the target protein [7–9]. In the presence of protein in DCL, equilibria in solution are shifted due to the interactions of higher affinity ligands with the protein, leading to an amplification of the corresponding glycoclusters. The best ligands for a specific lectin are identified in the DCL by identifying glycoclusters with the highest amplification rates. However, this approach has some limitations as it requires the amplification of the equilibrated DCL with the lectin, which is an additional step to simply analyzing the equilibrated DCL. In addition, when amplification is performed with the lectin, a quenching treatment, typically with 1M HCl, is required to unfold the proteins and release the amplified glycoclusters for suitable HPLC-MS analysis. Some even use lectins immobilized on polymer beads to remove easily the protein from the DCL solution [7]. Also, this HPLC-MS analysis must be performed quickly to avoid any re-equilibration of the macrocyclic species. The amplification approach therefore requires a certain amount of lectin (mg scale) and a protein/ligand ratio of 1/1. The amount of protein required is usually in the milligram range with an equimolar ratio of protein to ligand in solution.

The present study aims to provide an alternative approach to identify the most suitable glycocluster ligands for a lectin by directly analyzing the equilibrated DCL through nano-affinity chromatography (nano-WAC) coupled to mass spectrometry (without any

amplification step)[10,11]. A schematic representation of the analytical workflow is shown Figure 1.

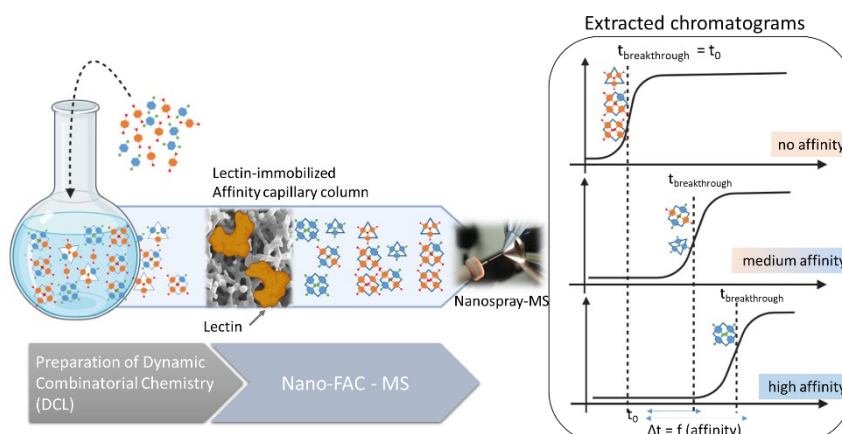


Figure 1: Schematic representation of the proposed workflow for the screening of affinity ligands from a dynamic combinatorial library by frontal affinity chromatography.

The affinity chromatography column contains the lectin that is covalently grafted to the stationary phase. The DCL is then injected (zonal mode) or infused (frontal mode) into the column and the glycoclusters interact with the immobilized protein. The higher the affinity of the glycocluster for the lectin, the higher its retention time in zonal mode (the higher its breakthrough time in frontal mode). When affinity chromatography is hyphenated with mass spectrometric detection, the nano-FAC-MS can analyze complex mixtures of ligands with a reliable identification of each of them by MS [12–15]. Thus, information on the relative affinity of each glycocluster in the mixture can be directly provided by nano-WAC-MS (ranking of the best to weakest ligands for the protein immobilized on the stationary phase). This innovative method allows the rapid and efficient analysis of mixtures and the identification of the best ligands directly from the mixture, without any amplification step. Moreover, the miniaturization of the affinity column (with a volume of less than one microliter) makes it possible to reduce the quantity of immobilized protein (down to 1  $\mu\text{g}$ ) [16,17] and to reduce the amount of sample injected down to the microliter range (i.e. to a few picomoles of each glycocluster). This method is very well suited for the study and identification of micromolar ligands of proteins and can be adapted for better ligands with nanomolar dissociation constants in the “capture and release” mode (high-affinity ligands are strongly captured by the column and then released by disrupting the interaction with the protein, whereas weak affinity ones are eluted during the washing step preceding the releasing step) [18].

The proof of concept of this innovative approach has been validated using Concanavalin A (ConA) as a model lectin. First, we demonstrate the ability of home-made ConA affinity columns to capture mannose ligands and to determine their dissociation constant for the lectin. Then, the nano-WAC-MS methodology is implemented to study a simple mixture of glycoclusters and a complex DCL of glycoclusters obtained by equilibration of 1,4-dithiophenols by reversible disulfide bonds formation.

## 2. Materials & Methods

### 2.1 Chemicals

Glycidyl methacrylate (GMA, 98%), Methylene bisacrylamide (MBA, 97%), butanediol, DMSO, dodecanol, (3-methacryloxypropyl)-trimethoxysilane ( $\gamma$ -MAPS), methanol (HPLC grade), triethylamine (TEA), sodium periodate, lithium hydroxide, dipotassium hydrogen phosphate ( $K_2HPO_4$ ), *o*-phosphoric acid, acetic acid, sodium cyanoborohydride, 4-nitrophenyl  $\alpha$ -D-mannopyranoside (ManOPhNO<sub>2</sub>), Concanavalin A, were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), Sulfuric acid (18 M) was purchased from VWR (Fontenay-sous-bois, France). Aqueous solutions were prepared using >18 M $\Omega$  deionized water. Preparation of the phosphate buffer was performed by dissolving 1.17 g of  $K_2HPO_4$  in 100 mL of water and pH was adjusted to 7.4 with *o*-phosphoric acid. Fused-silica capillaries with polyimide coating (TSP, 75  $\mu$ m i.d.) were from Cluzeau Info Labo (Sainte foy la grande, France). Nanospray stainless steel emitters with a uniform 30  $\mu$ m-inner diameter were purchased from Pepsep (Marslev, Denmark).

### 2.2 Preparation of glycluster dynamic combinatorial libraries

The 1,4-dithiophenol functionalized with the mannose (**M**) or fucose (**F**) building blocks (Figure 2) have been synthesized according to previously reported procedures [19,20]. Dynamic combinatorial libraries (DCLs) of **M** and **M/F** were prepared from the corresponding **M** and **F** building blocks (4 mM) in TRIS buffer (200 mM, NaCl 100 mM, CaCl<sub>2</sub> 100  $\mu$ M, pH 7.4). The DCL was equilibrated by stirring in an open vial at room temperature during 24 h to afford the library of glyclusters to be studied by nano-FAC-MS. The DCL composed of only the mannose building block **M** will provide a statistical distribution of linear oligomers to the macrocyclic homoglycodynarenes (Figure 3).

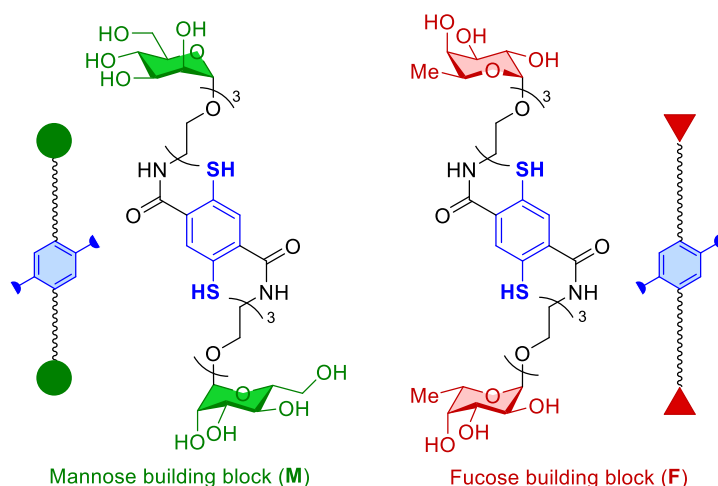


Figure 2: Structure of the 1,4-dithiophenol functionalized building blocks with mannose (**M**) or fucose (**F**)

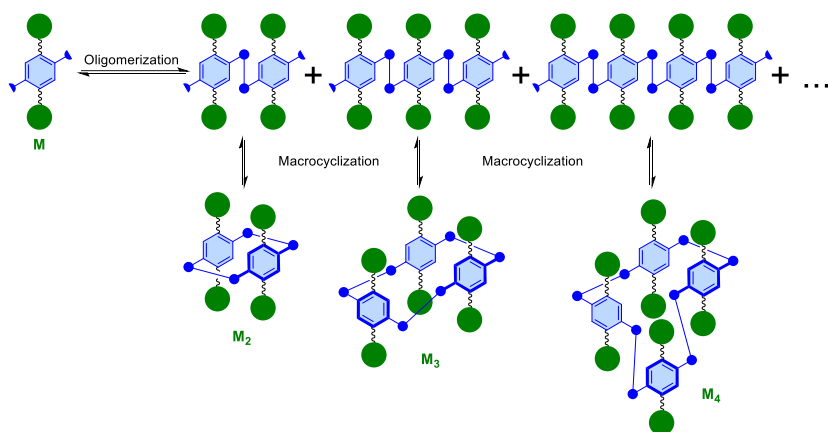


Figure 3: Schematic representation of the equilibrating DCLs composed of mannose building blocks (**M**)

### 2.3 Design and preparation of the ConA functionalized monolithic capillary columns

GMA-co-MBA monolithic capillary columns were synthesized as already described in a previous work [21]. Fused-silica capillaries were flushed (1 h, 0.5 bar) with a solution of  $\gamma$ -MAPS (5% v/v) and TEA (2.5% v/v) in methanol/water (95/5, v/v), then rinsed with methanol (15 min, 0.5 bar) and dried with a nitrogen stream. 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 was then filled with the polymerization mixture under 1 bar N<sub>2</sub> 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. Once the polymerization was completed, the columns were promptly rinsed with methanol for at least 1 h, then cut to reach a monolith length equal to 8 cm. Epoxy groups of the GMA-co-MBA monoliths were then hydrolyzed into diols by flowing sulfuric acid (1 M) for 2 h at 0.7 MPa (about 150 nL min<sup>-1</sup>). The resulting diol moieties were then oxidized into aldehydes using an aqueous solution of NaIO<sub>4</sub> (0.12 M, pH 5.5). ConA was then conjugated by percolation through the column during 12 h at 0.7 MPa (room temperature) of a solution containing ConA (1 mg/mL) and aqueous NaBH<sub>3</sub>CN (8 mg/mL) in phosphate buffer (67 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6). The column was then rinsed during 2 h at 0.7 MPa with NaBH<sub>4</sub> (2.5 mg/mL) in phosphate buffer (67 mM K<sub>2</sub>HPO<sub>4</sub>, pH 8) to reduce residual unreacted aldehydes. The affinity columns were then washed with phosphate buffer and stored at 4°C. Due to the dependence of ConA activity on the presence of calcium ions, phosphate buffer with Ca<sup>2+</sup> (67 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4, 100  $\mu$ M CaCl<sub>2</sub>) was used to store the column prior any further use.

### 2.4 Characterization of columns by nano-FAC-UV

The affinity columns were first evaluated by frontal affinity chromatography (FAC) using a nano-liquid chromatography (nano-LC, Thermo U3000) equipped with a UV-vis detector [22]. Solutions of 4-nitrophenyl  $\alpha$ -D-mannopyranoside (**ManOPhNO<sub>2</sub>**, 5 to 40  $\mu$ M in 20 mM ammonium acetate buffer, pH 7.4) were infused on the stationary phase at a flow rate of 400 nL min<sup>-1</sup> and detected at  $\lambda = 300$  nm. At each concentration [L] of the ligand, the breakthrough time is measured ( $t_{\text{breakthrough}}$ ) and the amount of ligand captured (q) can be experimentally determined according to equation 1.

$$q = \Delta t \times F \times [L] \quad (\text{Equation 1})$$

where F is the flow rate, and  $\Delta t$  the difference between the breakthrough time of the ligand and the dead time of the column ( $t_{\text{breakthrough}} - t_0$ ).

The amount of ligand captured (q) depends on its concentration [L], its dissociation constant with the lectin ( $K_d$ ) and on the number of active lectin's binding sites ( $B_{\text{act}}$ ) of the column according to equation 2:

$$q = \frac{B_{\text{act}} \times [L]}{K_d + [L]} \quad (\text{Equation 2})$$

Thus, plotting  $1/q$  vs  $1/[L]$  allows the determination of the amount of active binding sites  $B_{\text{act}}$  and of the  $K_d$  value according to Equation 3

$$\frac{1}{q} = \frac{K_d}{B_{\text{act}}} \times \frac{1}{[L]} + \frac{1}{B_{\text{act}}} \quad (\text{Equation 3})$$

## 2.5 Nano-FAC-MS Analysis

### 2.5.1 - Nano FAC-MS experimental conditions

Nano-FAC-MS analyses were carried out with a Nano-LC (from Eksigent) connected to an Electro-Nano-Spray Ionization source (from Agilent) with a TOF detector (Agilent 6230 TOF LC/MS). The aqueous mobile phase was ammonium acetate (5mM, pH 7.4) with a flow rate of 400 nL min<sup>-1</sup>. An additional make-up flow (400 nL/min, MeOH + 0.2% formic acid) was added at the exit of the affinity column with the loading pump of the nano-LC device and mixed with the chromatographic flow in a zero-dead volume union (Cluzeau info labo, France). The parameters of the ionization source were set as follows: Fragmentor = 300 V, Capillary voltage = 1400 V in the positive ionization mode with a drying gas (N<sub>2</sub>) at 300°C set at a flow rate of 5 L/min.

### 2.5.2 - Determination of ligand binding affinity by competition experiments in nano-FAC-MS

Ligand binding affinity ( $k_d$ ) of the **M<sub>3</sub>/M<sub>4</sub>** compounds in mixture were evaluated by competition experiments. In such experiments, the breakthrough time of the indicator **ManOPhNO<sub>2</sub>** with a known  $K_d$  value (monitored at 318.47 Th as [**ManOPhNO<sub>2</sub>+NH<sub>4</sub>**]<sup>+</sup> adduct) was first determined in absence of **M<sub>3</sub>/M<sub>4</sub>**. Then, **ManOPhNO<sub>2</sub>** was infused in presence of increasing concentrations of **M<sub>3</sub>/M<sub>4</sub>** as competitors (40 μM and 200 μM). The  $K_d$  value of the (**M<sub>3</sub>/M<sub>4</sub>**) compounds was calculated using equation 4.

$$K_{d,comp} = \frac{k_{d,Ind} \times [C]}{\frac{B_{act}}{(V_r - V_m)} - k_{d,Ind} - [Ind]} \quad (\text{Equation 4})$$

Where,  $K_{d,comp}$  is the apparent dissociation constant for the competitor (**M<sub>3</sub>/M<sub>4</sub>**),  $K_{d,Ind}$  the dissociation constant for the indicator (**ManOPhNO<sub>2</sub>**), [C] the concentration of the competitor,  $B_{act}$  the quantity of active sites of the protein (ConA),  $V_r$  the retention volume of the indicator (**ManOPhNO<sub>2</sub>**),  $V_m$  the retention volume of the indicator in presence of a large excess of competitor saturating the protein's binding sites.

For competition experiments, the operating conditions were optimized for the detection of **ManOPhNO<sub>2</sub>**: the voltage of the fragmentor was set to 100 V while the other conditions remained unchanged.

### 3. Results and Discussion

#### 3.1 Evaluation of the ConA affinity columns with ManOPhNO<sub>2</sub>

The monolithic capillary columns functionalized with ConA were first characterized using Nano-FAC-UV and **ManOPhNO<sub>2</sub>** as a test ligand with a known  $K_d$  value for ConA. Infusion of increasing concentrations of **ManOPhNO<sub>2</sub>** allowed the amount of active binding sites of ConA ( $B_{act}$ ) to be determined as well as the  $K_d$  value by plotting the reciprocal of the amount of ligand captured at each concentration vs the reciprocal of the ligand concentration (Lineweaver-burk plot) (Fig S1). The affinity columns have a mean  $B_{act}$  of  $61 \pm 5$  pmol of active sites of ConA ( $n = 3$  columns) (Column length 8.5cm). The dissociation constant measured for **ManOPhNO<sub>2</sub>** in these conditions is  $K_d = 39 \pm 3$   $\mu$ M, which is in good agreement with the values reported with other methods ( $K_d = 40.9 \pm 6.2$   $\mu$ M [23] and 14-17  $\mu$ M [24,25]). These results confirm that the monolithic affinity capillary columns are successfully and reproducibly prepared and can provide useful data in terms of dissociation constants.

#### 3.2 Analysis of a mixture of two glycoclusters $M_3/M_4$ by nano-WAC-MS

1,4-Dithiophenol functionalized with a carboxamide-triethyleneglycol- $\alpha$ -D-mannopyranoside moiety (**M**) was used as the equilibrating building block. The dynamic combinatorial libraries (DCLs) of this building block evolve (by disulfide formation and exchange) and equilibrate to form a nearly equimolar mixture of **M<sub>3</sub>** and **M<sub>4</sub>**, two homoglycodynarenes composed of three and four building blocks respectively. These **M<sub>3</sub>** and **M<sub>4</sub>** multivalent species have a valency in terms of mannose units of six and eight respectively. The purification of the **M<sub>3</sub>** and **M<sub>4</sub>** macrocycles was successfully carried out by preparative HPLC but unfortunately, each isolated **M<sub>3</sub>** or **M<sub>4</sub>** fraction systematically re-equilibrates into a nearly equimolar mixture of **M<sub>3</sub>** and **M<sub>4</sub>** upon evaporation (results not shown). Therefore, the relative affinity of each homoglycodynarene **M<sub>3</sub>** and **M<sub>4</sub>** for ConA cannot be determined by any classical bioanalytical techniques (e.g. ITC, BLI, SPR) that would require each pure macrocycle. In this case, the use of nano-FAC is crucial to be able to determine the relative affinity of each glycocluster **M<sub>3</sub>** and **M<sub>4</sub>** without the need to purify and isolate them separately.

As UV-VIS properties of **M<sub>3</sub>** and **M<sub>4</sub>** are very similar or even identical, detection must be performed by mass spectrometry for specific identification of each glycocluster. The coupling of frontal affinity chromatography (FAC) with mass spectrometry requires a modification of the experimental set-up as purely aqueous mobile phases and the presence of non-volatile salts are not compatible with a direct and robust mass spectrometric measurement. Coupling with nano-LC was achieved with a nanospray source in positive ionization mode with an additional flow of MeOH + 0.2% formic acid to stabilize the spray. Optimization of the detection parameters (source, flow and choice of adducts) was performed with the **M<sub>3</sub>/M<sub>4</sub>** solution in direct infusion in ammonium acetate buffer at 40  $\mu$ M (**M<sub>3</sub>+M<sub>4</sub>**) (Figure S2). The trimeric macrocycle **M<sub>3</sub>** was detected with several adducts such as  $[M_3+H+NH_4]^{2+}$ ,  $[M_3+2NH_4]^{2+}$ ,  $[M_3+Na+NH_4]^{2+}$ , and  $[M_3+K+NH_4]^{2+}$  while the tetrameric glycodynarene **M<sub>4</sub>** could be observed with a single adduct  $[M_4+H+K]^{2+}$  (Table 1).



Table 1. Main adducts detected for **M<sub>3</sub>/M<sub>4</sub>** glycodynarenes in direct infusion (ESI, positive mode)

Glycodynarene	Monoisotopic peak (Th)	Identification
<b>M<sub>3</sub></b> C <sub>96</sub> H <sub>150</sub> N <sub>6</sub> O <sub>54</sub> S <sub>6</sub>	1230.934	[ <b>M<sub>3</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
	1239.449	[ <b>M<sub>3</sub></b> +2NH <sub>4</sub> ] <sup>2+</sup>
	1241.870	[ <b>M<sub>3</sub></b> +Na+NH <sub>4</sub> ] <sup>2+</sup>
	1249.897	[ <b>M<sub>3</sub></b> +K+NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>4</sub></b> C <sub>128</sub> H <sub>200</sub> N <sub>8</sub> O <sub>72</sub> S <sub>8</sub>	1648.203	[ <b>M<sub>4</sub></b> +H+K] <sup>2+</sup>

The **M<sub>3</sub>/M<sub>4</sub>** mixture was then analyzed at different total concentrations of about 5, 20 and 200  $\mu\text{M}$  *i.e.*, about 2.5, 10 and 100  $\mu\text{M}$  for each glycocluster, on a nano-FAC column (12.8 cm in length) grafted with ConA (sample solubilized in ammonium acetate buffer). Warfarine, as a ligand not binding to ConA, was added to the infused solutions as an internal reference to measure the dead time ( $t_0$ ) of the column. The nano-FAC-MS analysis of the **M<sub>3</sub>/M<sub>4</sub>** mixture allows the unambiguous identification of each glycodynarene with the two following adducts [**M<sub>3</sub>**+2NH<sub>4</sub>]<sup>2+</sup> et [**M<sub>4</sub>**+H+K]<sup>2+</sup> (Figure 4). As expected, when molecular recognition occurs between the infused solute and the immobilized lectin target, the breakthrough time of each glycodynarene shifts to a lower value with increasing concentrations. At the highest concentration (200  $\mu\text{M}$ ), the breakthrough time is equal to the dead time ( $t_0$ ), confirming the absence of non-specific interaction of the **M<sub>3</sub>** or **M<sub>4</sub>** glycodynarenes with the stationary phase. At each concentration, identical breakthrough times are measured for **M<sub>3</sub>** and **M<sub>4</sub>** thus indicating comparable affinity for the target (same  $K_d$  value). The trimeric macrocycle **M<sub>3</sub>** has a valency of 6 mannose units displayed on its core scaffold, and the **M<sub>4</sub>** glycodynarene displays 8 mannosides. The lectin ConA cannot distinguish either glycodynarene because the difference in valency is too small. A strong increase in binding properties is typically observed when the valence increases from 1 to 4 or 2 to 6-8, but not from 6 to 8 [5].

Nevertheless, measurement of individual dissociation constants ( $K_d$ ) for **M<sub>3</sub>** and **M<sub>4</sub>** was considered to test the performance of the nano-FAC-MS system designed.

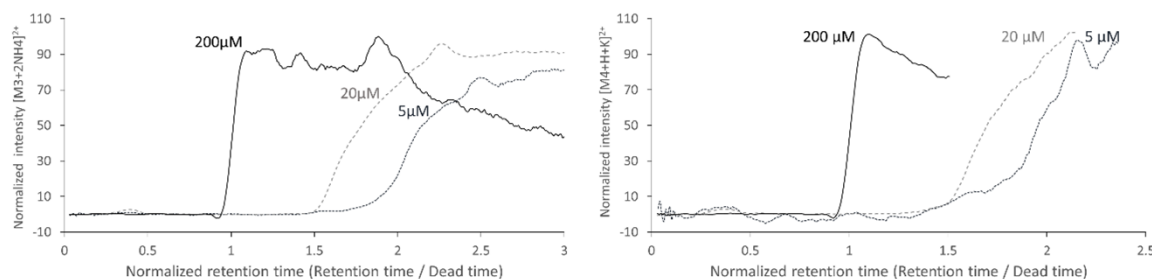


Figure 4: Nano-FAC-MS experiments on **M<sub>3</sub>/M<sub>4</sub>** mixture. Extracted Ion chromatograms (EIC) for [**M<sub>3</sub>**+2NH<sub>4</sub>]<sup>2+</sup> (left) and [**M<sub>4</sub>**+H+K]<sup>2+</sup> (right) at three concentrations (5, 20 and 200  $\mu\text{M}$ ). Eluent : ammonium acetate buffer (5 mM, pH 7.4) with additional MeOH+0.2% formic acid at 400 nL/min. Source parameters : capillary source potential 1400 V, source temperature 300°C, fragmentor potential 300 V. Polarisation : positive. Drying gas : 5 L/min. Skimmer : 90V.

The Lineweaver Burk plot used for the determination of the  $K_d$  value of the test ligand **ManOPhNO<sub>2</sub>** was used for the determination of the  $K_d$  values of **M<sub>3</sub>** and **M<sub>4</sub>** (assuming an equimolar proportion of the compounds in the mixture).  $K_d$  values of 37  $\mu\text{M}$  were obtained for both compounds.

As the concentration of each glycocluster is not known with accuracy, we used a second method that does not require the knowledge of the concentration of the ligand. The **M<sub>3</sub>/M<sub>4</sub>** mixture was analyzed at two different concentrations  $[L]_i$  and  $[L]_j$  with  $[L]_i = n [L]_j$  (here 5 and 20  $\mu\text{M}$ ). The corresponding amounts of each ligand captured for the two concentrations ( $q_i$  and  $q_j$ ) were calculated. The knowledge of the number of ConA binding active sites ( $B_{act}$ ) and of the flowrate  $F$  allows the determination of the  $K_d$  value according to equations 5 and 6.

$$\frac{1}{q_i} - \frac{1}{q_j} = \left( \frac{K_d}{B_{act}} \times \frac{1}{[L]_i} + \frac{1}{B_{act}} \right) - \left( \frac{K_d}{B_{act}} \times \frac{1}{[L]_j} + \frac{1}{B_{act}} \right) \quad (\text{Equation 5})$$

$$K_d = \frac{1}{n-1} \times B_{act} \times \left( \frac{n \times (t_{breakthrough_j} - t_0) - (t_{breakthrough_i} - t_0)}{(t_{breakthrough_j} - t_0) \times F \times (t_{breakthrough_i} - t_0)} \right) \quad (\text{Equation 6})$$

where  $t_{breakthrough,i}$  and  $t_{breakthrough,j}$  are the breakthrough times measured for the two concentrations. With this method, it is possible to determine the  $K_d$  values without taking into account the concentrations in each ligand (only the ratio  $n$  between these two concentrations is required). The obtained  $K_d$  values (monitoring the  $[\text{M}_3+2\text{NH}_4]^{2+}$  and  $[\text{M}_4+\text{H}+\text{K}]^{2+}$  adducts) are  $46 \mu\text{M} \pm 17\%$  and  $44 \mu\text{M} \pm 13\%$  for **M<sub>3</sub>** and **M<sub>4</sub>** respectively.

### 3.3 Competition experiments

Nano-FAC-MS competition experiments between **M<sub>3</sub>/M<sub>4</sub>** and **ManOPhNO<sub>2</sub>** (which has a known  $K_d$  value) were also implemented. The decrease in breakthrough time of the indicator **ManOPhNO<sub>2</sub>** with increasing concentration of **M<sub>3</sub>/M<sub>4</sub>** (Figure 5) indicates that the binding site on ConA is identical for **ManOPhNO<sub>2</sub>** and **M<sub>3</sub>/M<sub>4</sub>**.

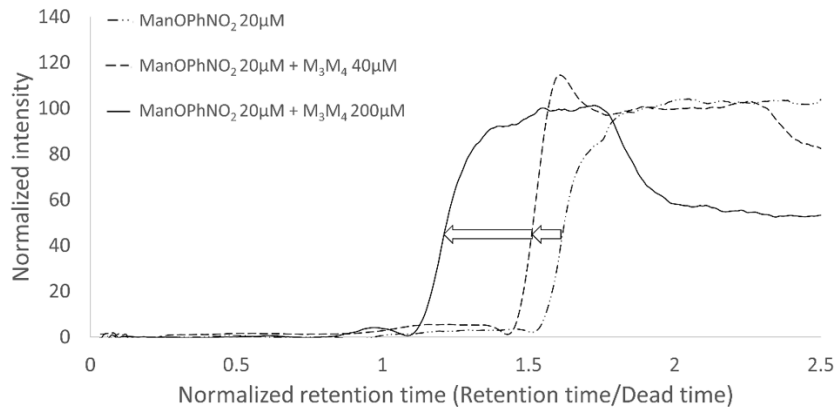


Figure 5: Nano-FAC-MS competition experiments. **ManOPhNO<sub>2</sub>** infused as indicator and monitored at 318.47 Th as  $[\text{ManOPhNO}_2+\text{NH}_4]^+$  adduct, in absence and in presence of increasing concentrations of competitors **M<sub>3</sub>/M<sub>4</sub>** (total concentration of **M<sub>3</sub>/M<sub>4</sub>** 20  $\mu\text{M}$  or 100  $\mu\text{M}$ ).

Competition experiments using FAC-MS allow to obtain information on the protein binding site but also to estimate the dissociation constant of the competitor ( $K_{d,comp}$ ) according to Equation 4 [26,27]. Given that **M<sub>3</sub>** and **M<sub>4</sub>** are (i) competitors with **ManOPhNO<sub>2</sub>** for the same

protein's binding sites and that (ii)  $M_3$  and  $M_4$  display similar affinities for ConA, both compounds can be considered as a single molecular entity in competition with  $\text{ManOPhNO}_2$ . The apparent  $K_{d,\text{comp}}$  values obtained from Equation 4 were estimated to 29  $\mu\text{M}$  and 24  $\mu\text{M}$ , for total competitor concentrations of 20  $\mu\text{M}$  and 200  $\mu\text{M}$  respectively, which is in line with the values calculated above. In all cases, a dissociation constant for  $M_3$  and  $M_4$  or an apparent dissociation constant for  $M_3/M_4$  in the low micromolar range has been estimated, either through the analysis of the ligands at various concentrations or through a competition experiment.

### 3.4 Analysis of a complex mixture of heteroglycodynarenes and ranking of their binding properties towards ConA

The full potential of the nano-FAC-MS system designed was then further evaluated with the analysis of complex mixtures of putative lectin ligands (without the need to purify them) and with the aim of ranking them according to their binding properties. When an equimolar mixture of  $M$  and  $F$  1,4-dithiophenol building blocks was equilibrated, a complex mixture of oligomers was obtained in the DCL and macrocyclized homo- ( $M_n$  or  $F_n$ ) and heteroglycodynarenes ( $M_nF_m$ ) were formed as illustrated (Figure 6). The inherent complexity of the equilibrating DCL composition is largely increased by the use of two different carbohydrates in the building blocks.

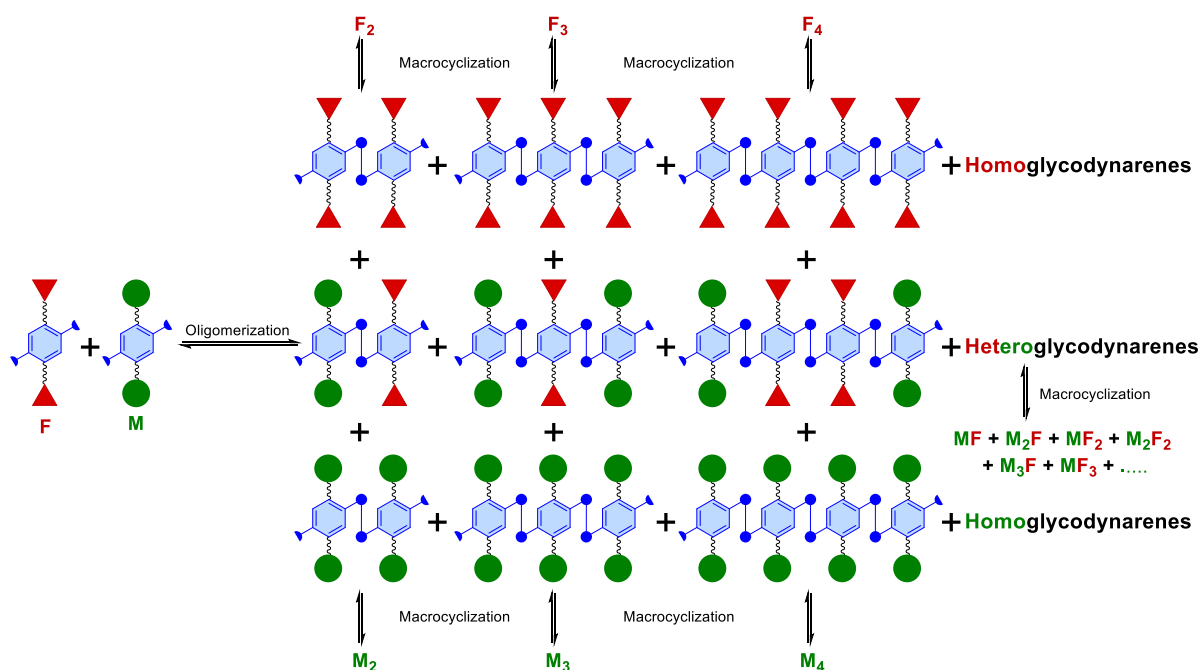










Figure 6: Schematic representation of the equilibrating DCLs composed of mannose ( $M$ ) and fucose ( $F$ ) building blocks.

This complex mixture of homo- and heteroglycodynarenes  $M+F$  was directly analyzed by nano-FAC-MS. The aim was to differentiate each macrocycle according to its affinity for ConA, without prior purification of each species. To our knowledge, there is no such bioanalytical technique that would allow for this direct analysis. The mass spectrometric data collected from the nano-FAC-MS provide a series of mass spectra in two different mass ranges with the trimeric macrocycles doubly charged ions between 1180 and 1240 Th, while the tetrameric counterparts are observed between 1580 and 11640 Th (Figure S3). While Figure 6 shows all possible combinations of 1,4-dithiophenols as linear or cyclic oligomers, not all of these

molecules may be present in the equilibrating DCL or, if present, maybe at very low levels in the mixture. The molecular ions observed after nano-FAC-MS provide the real composition of the DCL while Figure 6 would depict all possible combinations. The composition and structure of the corresponding adducts (Table 2) confirm that homo- and heteroglycodynarenes are indeed formed in the equilibrated DCL with various compositions.

Table 2 : Assignments of adducts with structures of the homo- and heteroglycodynarenes obtained after equilibration of an equimolar mixture of **M+F**.

Glycodynarene		Monoisotopic peak (Th)	Identification
<b>F<sub>3</sub></b> C <sub>96</sub> H <sub>150</sub> N <sub>6</sub> O <sub>48</sub> S <sub>6</sub>		1182.82	[ <b>F<sub>3</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
		1191.31	[ <b>F<sub>3</sub></b> +2NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>1</sub>F<sub>2</sub></b> C <sub>96</sub> H <sub>150</sub> N <sub>6</sub> O <sub>50</sub> S <sub>6</sub>		1198.77	[ <b>M<sub>1</sub>F<sub>2</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
		1207.26	[ <b>M<sub>1</sub>F<sub>2</sub></b> +2NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>2</sub>F<sub>1</sub></b> C <sub>96</sub> H <sub>150</sub> N <sub>6</sub> O <sub>52</sub> S <sub>6</sub>		1215.25	[ <b>M<sub>2</sub>F<sub>1</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
		1223.23	[ <b>M<sub>2</sub>F<sub>1</sub></b> +2NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>3</sub></b> C <sub>96</sub> H <sub>150</sub> N <sub>6</sub> O <sub>54</sub> S <sub>6</sub>		1231.25	[ <b>M<sub>3</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
		1241.71	[ <b>M<sub>3</sub></b> +Na+NH <sub>4</sub> ] <sup>2+</sup>
<b>F<sub>4</sub></b> C <sub>128</sub> H <sub>200</sub> N <sub>8</sub> O <sub>64</sub> S <sub>8</sub>		1587.25	[ <b>F<sub>4</sub></b> +2Na] <sup>2+</sup>
		1595.70	[ <b>F<sub>4</sub></b> +Na+K] <sup>2+</sup>
<b>M<sub>1</sub>F<sub>3</sub></b> C <sub>128</sub> H <sub>200</sub> N <sub>8</sub> O <sub>66</sub> S <sub>8</sub>		1611.67	[ <b>M<sub>1</sub>F<sub>3</sub></b> +Na+K] <sup>2+</sup>
		1590.26	[ <b>M<sub>1</sub>F<sub>3</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>2</sub>F<sub>2</sub></b> C <sub>128</sub> H <sub>200</sub> N <sub>8</sub> O <sub>68</sub> S <sub>8</sub>		1627.66	[ <b>M<sub>2</sub>F<sub>2</sub></b> +Na+K] <sup>2+</sup>
		1606.52	[ <b>M<sub>2</sub>F<sub>2</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
<b>M<sub>3</sub>F<sub>1</sub></b> C <sub>128</sub> H <sub>200</sub> N <sub>8</sub> O <sub>70</sub> S <sub>8</sub>		1622.17	[ <b>M<sub>3</sub>F<sub>1</sub></b> +H+NH <sub>4</sub> ] <sup>2+</sup>
		1643.65	[ <b>M<sub>3</sub>F<sub>1</sub></b> +Na+K] <sup>2+</sup>

Nano-FAC-MS analysis of the equilibrated DCL obtained from an equimolar mixture of **M+F** was performed and breakthrough curves observed for the identified structures are illustrated (Figure 7). The ConA-functionalized capillary column can readily distinguish between lectin-recognised mannose species and unrecognised fucose species. Hence, the first compounds eluted from the column are fucose-rich glycodynarenes such as **F<sub>3</sub>** and **F<sub>4</sub>** followed by two different groups of ligands with drastically higher breakthrough times. The first group displaying affinity towards ConA is composed of mannose-based glycodynarenes **M<sub>1</sub>F<sub>3</sub>**, **M<sub>1</sub>F<sub>2</sub>** et **M<sub>2</sub>F<sub>2</sub>**. The second group with a higher breakthrough time and hence a higher affinity towards ConA, is composed of **M<sub>2</sub>F<sub>1</sub>**, **M<sub>3</sub>F<sub>1</sub>** et **M<sub>3</sub>**. These results demonstrate a higher affinity of the glycodynarenes for ConA with the increasing mannose content **M** in their structure. The general ranking based on the retention time and affinity towards ConA is therefore **F<sub>3</sub>**, **F<sub>4</sub>** << **M<sub>1</sub>F<sub>3</sub>**, **M<sub>2</sub>F<sub>2</sub>**, **M<sub>1</sub>F<sub>2</sub>** < **M<sub>2</sub>F<sub>1</sub>**, **M<sub>3</sub>F<sub>1</sub>**, **M<sub>3</sub>**. The group of ligands observed (**M<sub>1</sub>F<sub>3</sub>**, **M<sub>2</sub>F<sub>2</sub>**, **M<sub>1</sub>F<sub>2</sub>**) has a lower affinity than the other group (**M<sub>2</sub>F<sub>1</sub>**, **M<sub>3</sub>F<sub>1</sub>**, **M<sub>3</sub>**) but the difference in affinity cannot be addressed with precision within the same group of ligands.

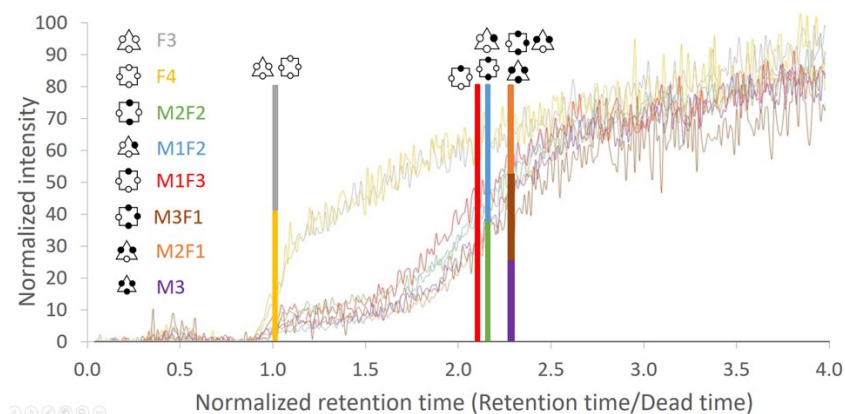


Figure 7: Extracted Ion chromatograms (EIC) for the equilibrated DCL of M+F Eluent : ammonium acetate buffer (5 mM, pH 7.4) with additional MeOH+0.2% formic acid at 400 nL/min. Source parameters : capillary source potential 1400 V, source temperature 300°C, fragmentor potential 300 V. Polarisation : positive. Drying gas: 5 L/min. Skimmer: 90V.

#### 4. Conclusion

The analysis of mixtures obtained by dynamic combinatorial chemistry involves many analytical challenges (nature and number of molecules, identification, characterization or affinity ranking). In this work, the nano-FAC-MS study of equilibrating dynamic combinatorial libraries of glycocluster mixtures obtained by dynamic combinatorial chemistry on columns functionalized with Concanavalin A has made it possible to highlight the potential of this technique.

Selection of fitted candidates has been realized on a model lectin (Concanavalin A) using home-made affinity nanocolumns with immobilized protein to separate molecular entities with respect to their specific lectin binding properties. Miniaturization facilitates the on-line coupling with MS detection in such purely aqueous and buffered conditions and allows reduced target protein consumption. Monolithic lectin-affinity columns prepared by immobilization of ConA were first characterized using a known ligand. The amount of active binding immobilized protein is  $61 \pm 5$  pmol on 8.5-cm length column. We demonstrate the capability of our approach to evaluate individual dissociation constants of species in mixture. The concept was then successfully applied to the screening dynamic combinatorial libraries (DCLs) of tri- and four-membered macrocycle glycoclusters.

Nano-FAC-MS can be applied to the study of any mixture in dynamic equilibrium and provide, in a single experiment, identification and affinity ranking of potential ligands for the target protein. This tool will be very useful in DCC and supramolecular chemistry as it uses very little material.

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