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Abstract

Affinity chromatography is a powerful technique to identify and quantify weak ligand-protein interactions (Kd in the range of mM to 0.1µM). In some fields such as Fragment Based Drug Discovery, the detection of very weak affinities (mM) is of utmost importance since weak ligands can be good starting points for the conception of high affinity ligands. However, the identification of such weak ligands can be hampered by the limited bulk density of active target grafted onto the support. At the same time, downscaling the chromatographic column is of utmost interest when scarce and/or expensive proteins are targeted. In this context, we herein present a novel approach of protein immobilization to improve the bulk density of active protein grafted onto organic capillary monolithic columns. The proposed approach is based on the streptavidin-biotin interaction and consists of successive grafting steps of biotinylated target protein onto streptavidin layers through a multi-layering process. Concanavalin A was used as model protein. The study focuses on the optimization of the grafting conditions to maximize the amount of active protein during the multi-layering process and highlights the impact of the biotinylation ratio of the protein. It is demonstrated that a 3-layer grafting process allows to improve the bulk density of active sites by a 2-fold factor compared to a single layer. This improvement in protein density allows to increase the affinity range of this technique to the millimolar range.

Keywords: Frontal Affinity Chromatography, Organic monolith, Biofunctionalization, Miniaturization, Nano-liquid Chromatography.

1. INTRODUCTION:

Affinity chromatography is widely used in sample preparation to purify and pre-concentrate analytes in complex matrices [1]. This method is furthermore proving to be just as interesting in a niche domain such as the identification/characterization of weak interactions by Weak Affinity Chromatography (WAC).

In the latter, the principle is based on the observation of differences in retention between species exhibiting different affinities with targets grafted on the surface of the chromatographic stationary phase. The higher the affinity (i.e. the lower the Kd value), the higher the retention time of a binding ligand [2–5].

Weak Affinity Chromatography operated either in zonal or frontal mode has shown to be a powerful and potentially money-saving tool, allowing both the ranking of ligands' affinity [4–6] and the latter determination [7], while reducing resources consumption and analysis time by means of miniaturization [8] and hyphenation with Mass Spectrometry [9,10]. The advantages over other biophysical methods are the ability to analyze complex ligand mixtures such as natural extracts, together with the possibility to re-use the target that is immobilized onto the chromatographic support, thus reducing consumption of targets that can be produced only in minute amounts [11,12].

Weak affinity chromatography is suitable for the study of weak affinity interactions (Kd in the range of mM to 0.1µM). The identification of ligands of very weak affinity (mM) can be of great interest, for example in the context of Fragment Based Drug Discovery (FBDD) where such weak affinity ligands (also called fragments) are the starting points for the conception of high affinity ligands [13]. However, the identification of such starting points can be limited by the amount of active target of interest grafted onto the support. Indeed, for a given Kd value, the retention factor of a ligand is directly related to the bulk density of active target grafted on the support. The weaker the affinity, the higher the required bulk density of active target to detect the affinity i.e. to get a detectable retention. It is therefore of great interest to increase this bulk density in order to extend the detectable affinity range [2,8,14–16].

We have recently demonstrated the interest of miniaturizing weak affinity chromatography (using monolithic chromatographic columns in capillary format) to identify weak affinity ligands targeting proteins available in scarce amounts [11]. Targets can be grafted by a traditional direct immobilization (e.g. via aldehyde route) which does not require any protein modification. An alternative approach for the in-situ grafting of such supports is the immobilization of the biotinylated target onto "generic columns" pre-functionalized with streptavidin. The instantaneous and quantitative capture of biotinylated targets by these generic columns makes it possible (i) to immobilize them extemporaneously (a great advantage for targets with low stability), (ii) to reduce

the amount of target consumed to what is strictly required thanks to the UV-monitoring of the grafting step [11]. With this approach, we have shown that it is possible to consider fragment screening against membrane proteins embedded in biotinylated biomimetic membranes [7].

To extend the reach of WAC and to allow the identification of ligands of ever weaker affinity (a challenge for FBDD), we envisioned to expand on the already adopted streptavidin-biotin interaction and build tridimensional layers of target with the goal of increasing the final number of active sites available. This can be done by employing biotinylated targets with a higher than usual biotin incorporation ratio (>1.5). A first layer of biotinylated target is first immobilized on the streptavidin chromatographic support (Layer 1). At the end of this first step, residual biotin residues are available on the target. A solution of streptavidin is then infused, leading to the capture of an additional layer of streptavidin by the residual biotin residues of the immobilized target (Mid-Layer). Since streptavidin has four active sites, it can capture a next layer of biotinylated target (Layer 2). This layering process can be repeated several times as shown in Figure 1. Biotinylated Concanavalin A was used as target protein model to investigate the impact of multi-layering on affinity chromatography. This protein, belonging to the lectin family, is used in affinity chromatography for its ability to bind molecules containing mannose.

Figure 1

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Concanavalin A (ConA) and biotinylated Concanavalin A from *Canavalia ensiformis*, streptavidin (from streptomyces avidinii, affinity purified, ≥ 13 U mg^{-1} of protein), (3-methacryloxypropyl)-trimethoxysilane (γ-MAPS), ethylene dimethacrylate (EDMA, 97%), glycidyl methacrylate (GMA, 98%), 1-propanol, 1,4-butanediol, dimethylsulfoxide (DMSO), sodium periodate, lithium hydroxide, dipotassium hydrogen phosphate (K_2HPO_4), o-phosphoric acid, acetic acid, sodium cyanoborohydride, triethylamine (TEA), Azobisisobutyronitrile (AIBN), p-nitrophenyl-α-D-mannopyranoside were purchased from Sigma Aldrich (L'Isle d'Abeau Chesne, France). All aqueous solutions were prepared using >18 MΩ 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 ortho-phosphoric acid. Thermo Scientific EZ-Link™ NHS-PEG4 Biotinylation Kit from thermo (Thermofischer Scientific, France).

2.2. Biochemistry methods:

2.2.1. Biotinylated Concanavalin A:

Concanavalin A was biotinylated with a long chain (29 Å), water soluble biotinylating reagent using the Thermo Scientific EZ-Link™ NHS-PEG4 Biotinylation Kit, following the manufacturer's instructions. The biotinylation molar excess was varied to control the biotinylation ratio.

2.2.2. Biotin incorporation ratio evaluation:

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.

2.3. Instrumentation

A microfluidic flow controller MFCS-EZ100 system (Fluigent, Villejuif, France) was used to flow liquids into capillary columns. An LC pump LC 10AD (Shimadzu, Tokyo, Japan) was used to rinse the freshly prepared capillaries.

Frontal Weak Affinity Chromatography experiments were carried out with a 7100 capillary electrophoresis Agilent system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and an external nitrogen tank to reach pressures up to 12 bar. System control and data acquisition were carried out using the Chemstation software (Agilent). All experiments were

carried out at 25°C in “short-end” injection mode, with the inlet of the capillary immersed in the solution to be infused.

2.4. Capillary monolith synthesis and biofunctionalization

2.4.1. Poly GMA-co-EDMA monolith synthesis:

Poly(GMA-co-EDMA) monoliths were synthesized inside fused-silica capillaries presenting a UV transparent coating (TSH, 75 µm i.d., Cluzeau Info Labo, France) as already described in previous works [17,18]. Capillaries were pre-treated by flushing them (1 h, 0.5 bar) with a solution composed of 5% (v/v) γ-MAPS and 2.5% (v/v) TEA in methanol/water (95/5, v/v). They were then rinsed with methanol (15 min, 0.5 bar), and finally dried with a nitrogen stream. The polymerization mixture was prepared by adding to 12 mg of AIBN initiator, 0.9 mL GMA, 0.3 mL EDMA, 1.05 mL 1-propanol, 0.6 mL butan-1,4-diol and 0.15 mL ultra-pure water. The polymerization mixture was then agitated (15 minutes) and sonicated (15 minutes), to be then used to fill the pre-treated capillaries at a nitrogen pressure of 1bar. To localize the monolith on a specific length of the capillaries, PEEK tubings (380 µm i.d.) were used to mask non-irradiated areas, limiting the monolith length to about 10 cm. A Bio-link UV cross-linker (VWR International, France) was used to carry out the photopolymerization under 365 nm UV light for a total energy of 6 J/cm^2. After polymerization, the column was immediately rinsed with methanol for 1 h, then cut to reach a monolith length of 8.5 cm.

2.4.2. Preparation of streptavidin-functionalized monolithic capillary columns:

Columns were functionalized with streptavidin as described in the work of Lecas et al. [11]. The epoxy groups present on the GMA-co-EDMA monolith were first hydrolyzed into diols by flushing the columns with 1M sulfuric acid (2h, 6.5 bar) and then washing the column with ultra-pure water (1h, 6.5 bar). A periodic acid solution at pH 5.5 was then percolated (1h, 6.5bar) to oxidize the diol groups into aldehyde groups, followed by a quick rinse (67 mM phosphate buffer, pH 6, 15 minutes, 6.5 bar). A 1mg/mL streptavidin and 4mg/mL $NaBH_3CN$ solution in 67 mM pH 6 phosphate buffer was then percolated through the column overnight (18 h, 4.5 bar, room temperature). After streptavidin grafting, the column was flushed with a solution of sodium borohydride 2.5mg/mL in phosphate buffer 67 mM pH 8 (2h, 6.5bar), to reduce residual aldehydes. The resulting streptavidin columns were then stored at 4°C.

2.4.3. Immobilization of biotinylated Concanavalin A on streptavidin-functionalized monolithic capillary columns:

Dynamic grafting was carried out by infusing a diluted biotinylated ConA solution (concentration in the µM range in phosphate buffer 67mM, pH 7.4) and monitoring it by *in-situ* UV detection, to stop the grafting once completed (column saturated).

2.4.4. Multi-layer grafting procedure:

Columns presenting a single layer of ConA were dynamically grafted with streptavidin by flowing a solution of streptavidin at a concentration ranging between 5 and 15 µM in phosphate buffer 67 mM, pH 7.4), monitoring the grafting by in-situ UV detection (Mid-layer). The same process was then repeated with the same sample of biotinylated ConA used for the first layer grafting. This layering process was repeated for up to 3 layers of ConA, evaluating the column by frontal affinity chromatography (FAC) at each step, using HABA as test ligand for the streptavidin mid-layers, and p-nitrophenyl-α-D-mannopyranoside for ConA layers.

2.5. Frontal affinity chromatography (FAC) experiments

FAC was used to determine the amount of ConA active sites *($B_{act,ConA}$) at each layer and mid-layer and the amount of* Streptavidin active sites *($B_{act,Strepta}$) at each mid-layer.* For the results presented in this manuscript, a variation of FAC addressed as "staircase FAC"[19] (similar to that seen in the work of He et al. [20]) was employed (see S1 for the rationale of the staircase approach). Solutions of increasing concentrations of the test ligand were percolated consecutively in the column (without any rinsing step in-between the percolations of the different concentrations).

2.5.1. Determination of the amount of Concanavalin A active sites ($B_{act,ConA}$)

P-nitrophenyl-α-D-mannopyranoside was used as a weak affinity test ligand to determine the amount of ConA active sites at each step of the grafting (after each layer of ConA and each mid-layer of Streptavidin). Due to the dependence of ConA activity on the presence of Calcium ions, before any activity evaluation, phosphate buffer 67mM enriched with Ca^{2+} (67 mM K_2HPO_4, pH 7.4, 100 µM Ca^{2+} was percolated through the column for at least 40 minutes, to ensure the maximal activity of the ConA grafted. Solutions of increasing concentrations (5, 7.5, 10, 15, 20, 40 and 500 µM) of P-nitrophenyl-α-D-mannopyranoside were percolated successively. Rationality of the results was ensured by comparing the obtained Kd value with literature value (Kd ≃ 30-50 µM [21,22]. DMSO was used as dead time marker.

2.5.2. Determination of the amount of Streptavidin active sites ($B_{act,Strepta}$)

For the determination of the amount of streptavidin active sites available at each mid-layer, HABA (K_d = 100 µM) was used as test solute with increasing concentrations (5, 10, 50, 100 and 200 µM).

Rationality of the results was ensured by comparing the obtained Kd (range 90-120 μM) with literature values (100 μM) [23].

3. RESULTS AND DISCUSSION

3.1. Influence of the ConA biotinylation ratio on the number of ConA active sites for a monolayer grafting (layer 1)

The ConA biotinylation ratio may impact the amount of resulting Concanavalin active sites. Concanavalin was biotinylated with different molar excesses of biotinylation reagent, leading to different biotinylation ratios addressed as “Low”, “Medium” and “High”, relatively to their biotin incorporation ratio (Table 1).

Table 1 - Biotinylation incorporation ratios obtained at different biotinylation conditions

The influence of the biotinylation ratio on the amount of ConA active sites ($B_{act,\ ConA}$) after a single grafting layer has been evaluated for the three biotinylation ratios. $B_{act,\ ConA}$ values were determined by Frontal Affinity Chromatography, in the staircase mode (see experimental section and S1) that allows obtaining data related to the quantity of active sites in a column (B_{act}) and the dissociation constant of the ligand (K_d).

Figure 2

As illustrated Figure 2, the number of ConA active sites is maximum (around 55 pmol per column i.e 6.4 pmol cm^{-1} of monolith i.e. 146 pmol μL^{-1}) for “low” or “medium” biotinylation ratios. An increase in the ConA biotinylation ratio leads to a significant decrease (about 20%) in the number of ConA active sites.

Indeed, when the ConA biotinylation ratio increases, the probability that several biotins of the same ConA are engaged with multiple underlying streptavidin sites increases. Such a multi-point anchoring of the biotinylated ConA on the streptavidin layer limits the amount of ConA that can be grafted (one ConA occupies several streptavidin active sites).

3.2. Influence of the ConA biotinylation ratio on the number of ConA active sites for a multi-layering grafting

The impact of the biotinylation ratio on the number of ConA active sites over a multi-step approach was studied by considering 3 successive layers. The amount of ConA active sites was determined at each step and results are presented in Figure 2. The variation of the amount of ConA active sites ($B_{act,\ ConA}$) through different layers is not comparable for each biotinylation ratio.

For the « low » biotinylation ratio, the multi-step approach does not significantly increase the amount of ConA active sites. Indeed, if a gain of about 30% is observed at the end of the second step, a third one doesn't increase the quantity of active sites anymore. After the second grafting cycle (layer 2) , it seems that no free and accessible biotin residue is available on ConA to bind a new streptavidin mid-layer necessary to initiate a third layer of ConA.

The behavior is different for the two higher biotinylation rates. Indeed, the amount of ConA active sites increases during the three successive grafting steps.

The best grafting outcome (two-fold increase) was observed for the medium biotin incorporation level sample. A medium biotin incorporation ratio is the best compromise to get a first layer with a high ConA density, while making possible to build several layers.

To better understand the layering process, the amount of ConA active sites was determined at each layer and mid-layer (Fig 3 up, in green) in order to highlight a potential deactivation of the already grafted ConA (reduction of the number of active sites) consecutive to the addition of a new streptavidin mid-layer. At the same time, the amount of streptavidin active sites *($B_{act,Strepta}$)* added at each mid-layer was also evaluated (Figure 3, bottom, in blue).

It can be observed that the 1st streptavidin mid-layer deactivates part of the previously grafted ConA. The higher the degree of biotinylation of ConA, the stronger this partial deactivation (10 % for the "medium" biotinylation rate and more than 50 % for the "high" one). This loss of activity may be due to a decrease in accessibility to ConA due to the grafting of the streptavidin mid- layer. Indeed, the loss ConA active sites is higher for greater amounts of streptavidin added at the mid-layer (loss of 10 % and 50 % for amounts of added streptavidin actives sites of 22 pmol and 42 pmol respectively). However, regardless of the extent of this loss of activity, the total amount of active ConA after the second grafting step (layer 2) is greater than that obtained after the first layer.

Figure 3

The same trend is observed during the third grafting step *viz* partial deactivation of the grafted ConA following the addition of streptavidin (2d middle-step) and overcompensation of the total amount of ConA active sites by the final ConA grafting step. The best compromise is obtained with the medium biotinylation rate, which limits the deactivation effect while allowing for further grafting of a consistent amount of active ConA at each step.

With a medium biotinylation ratio, a 3-layer grafting process allows to improve the active protein grafting density by a 2-fold factor compared to a single layer. It is worth noticing that such a multi-layering process is automatically implemented, the successive protein solutions being infused with the pressurization system of the capillary electrophoresis device. The overall duration time of a

three-layer process is about 3h (30 min for a monolayer). Although it is possible to make more layers, we have estimated that a 3-layer process was the best compromise between the number of active sites and grafting duration. This improvement in bulk density of active sites should allow to increase the affinity range that can be addressed by this technique.

3.3. Detection of very weak affinity

As stated earlier in the text, frontal weak affinity chromatography is useful to identify very weak affinity ligands in early stages of drug discovery processes. For a compound with no affinity for the immobilized target, the breakthrough time t_p measured is independent of the concentration of the infused ligand. ($t_p = t_0$ if no non-specific interactions occur and $t_p > t_0$ in the case of non-specific interactions). For a compound with affinity for the target, the breakthrough time depends on the ligand concentration that is infused. The ability to detect weak affinity ligands in FAC is based on the shift in breakthrough times Δt_p that can be non-equivocally detected between two extremely different ligand concentrations (concentrations of about 0,1Kd and 10Kd). For comparison purposes, this difference in plateau time Δt_p must be normalized by dividing it by the dead time and can be then expressed as $\Delta t_p/t_0$.

The reduced breakthrough time difference $\Delta t_p/t_0$ is directly correlated to both the strength of the affinity interaction and the quantity of protein target interacting in the column. Increasing the quantity of protein target available for the interaction in the column consequently allows to detect weaker interactions (higher Kd).

To illustrate the improvement regarding the extension of the detectable affinity range by the proposed grafting strategy, a weaker affinity ligand (p-nitrophenyl-α-D-glucopyranoside Kd > 200 µM [22,24]), was used as test ligand.

Figure 4 shows the frontal affinity chromatograms obtained for two different concentrations (10 µM and 2000 µM) of p-nitrophenyl-α-D-glucopyranoside for a single-layer (B_{act} = 52.4 ± 0.5 pmol) and on a 3-layers ConA column (B_{act} = 95 ± 0.7 pmol). The plot shows how the difference in breakthrough time difference changes greatly due to the number of ConA active sites increase due to the layering strategy.

Figure 4

Empirically, the limit of affinity detection (the minimum value of Δtp/t0 needed to be able to confidently recognize specific affinity) was evaluated to be 0.1 . Considering the gain in the amount of active protein on the column (about 2-fold), the affinity range is extended allowing the detection

of lower affinity. The lowest affinity observable with the resulting Bact values can be estimated being in the range of 1mM in Kd.

4. CONCLUSIONS

A novel approach to increase the amount of active protein that can be immobilized on organic monolithic capillary column was presented. This approach implements a multi-step process of protein immobilization based on the streptavidin-biotin interaction and involves the use of multi-biotinylated proteins. Biotinylation of proteins is quite easily done and controlled using a commercial biotinylation reagent kit. The streptavidin-biotin interaction is fast and quantitative allowing to prepare multi-layers without any loss of protein sample and for a limited increase in preparation time. However, this straightforward approach requires to carefully control the number of biotin molecules borne by proteins, to be efficient. Whereas a low biotinylation ratio i.e. < 2 is optimal to prepare dense monolayers using a single grafting step, a higher biotinylation ratio (higher than 2) is required for the multilayering approach. The most interesting biotinylation rate for a multi-step approach is the intermediate one. Compared to a single layer, optimal grafting condition allows twice the density while doubling retention properties and extending the affinity range.

This approach is simple and can easily be applied to other biomolecules that can be biotinylated and/or to other methods requiring the preparation of a biofunctionalized layer (e.g biosensors, microfluidic lab-on-chips).

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the Institut de Recherche Servier IDRS (France). We thank Ian Ramtanon for his final english proof reading.

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Figure captions

Figure 1:

Schematic illustration of the immobilization process through streptavidin-biotin multi-layering.

Figure 2:

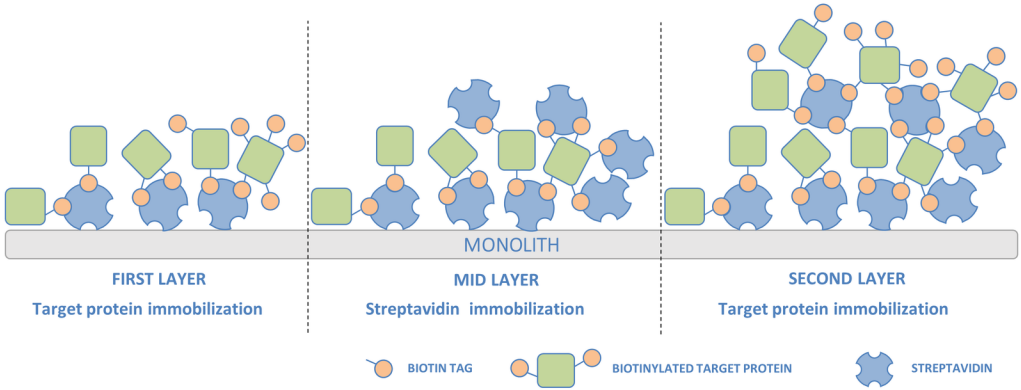
Effect of different biotinylation ratios on $B_{act,ConA}$ by multilayering approach ($B_{act,ConA}$ evaluated after each layering step) ($n \geq 3$).

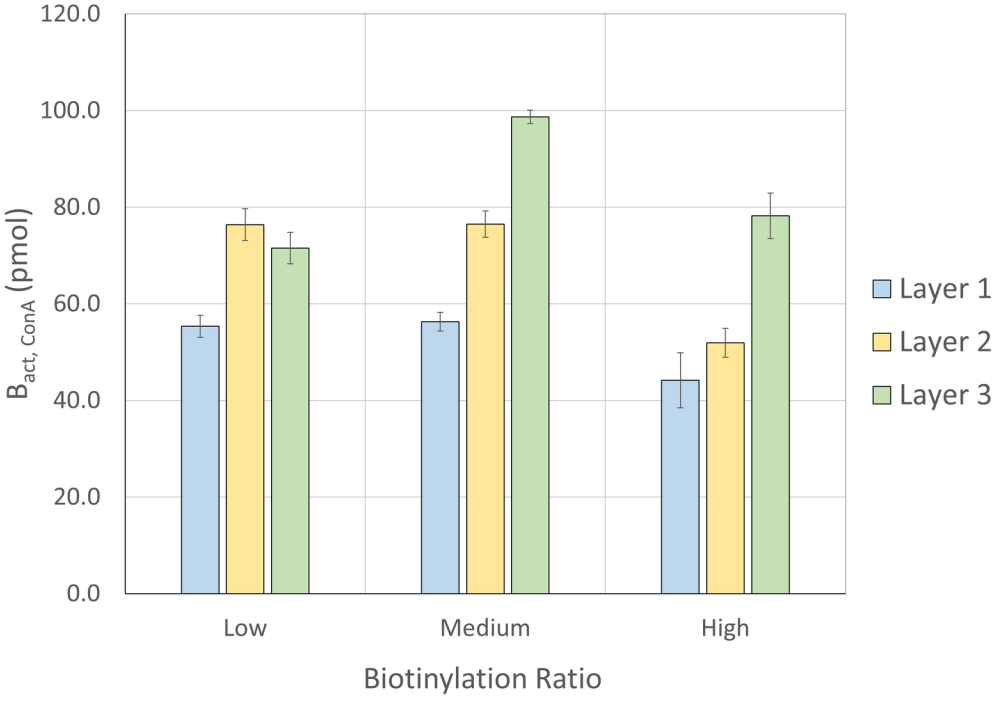
Figure 3:

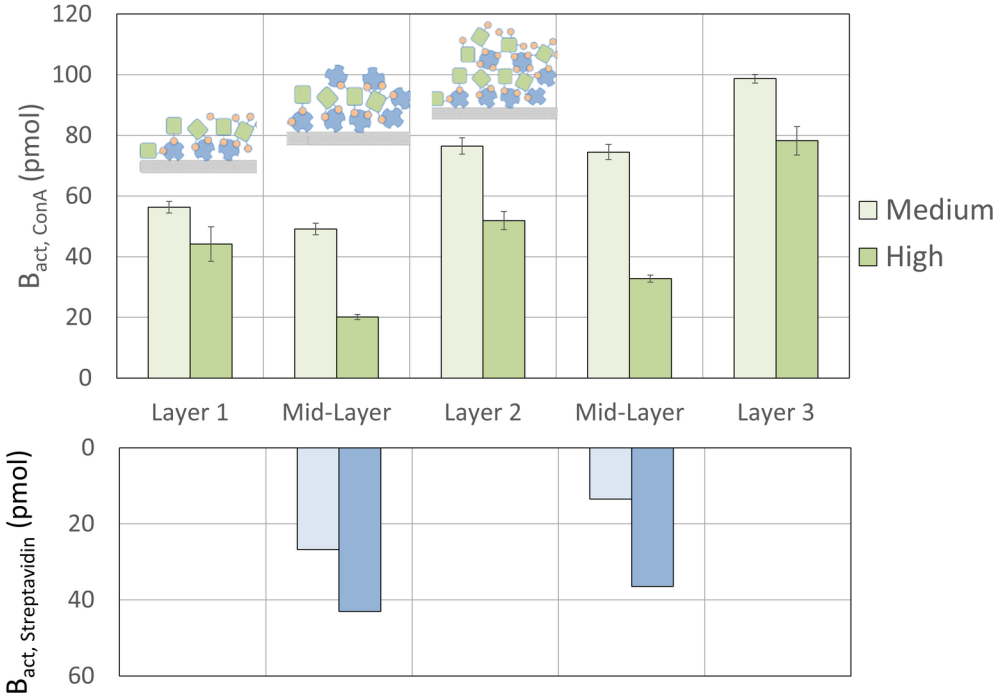
Plot representing the evolution of the number of ConA active sites ($B_{act,\ ConA}$) and the quantity of streptavidin (Bact,Strepta) added on monolithic columns at each step of the 3-step grafting process using Medium and High biotinylation ratios.(light color for a medium biotinylation ratio and dark color for a high one)

Figure 4:

Frontal affinity chromatograms of p-nitrophenyl-α-D-glucopyranoside at two concentrations, obtained for a column grafted with a single layer of ConA (solid line) and a column grafted with 3 layers of ConA (dotted line) using a medium biotin incorporation level.


MONOLITH
FIRST LAYER
Target protein immobilization
MID LAYER
Streptavidin immobilization
SECOND LAYER
Target protein immobilization
BIOTIN TAG
BIOTINYLATED TARGET PROTEIN
STREPTAVIDIN


120.0
100.0
80.0
60.0
40.0
20.0
0.0
$B_{act,\ ConA}$ (pmol)
Low
Medium
High
Biotinylation Ratio
Layer 1
Layer 2
Layer 3


120
100
80
60
40
20
0
$B_{act,\ ConA}$ (pmol)
Medium
High
Layer 1
Mid-Layer
Layer 2
Mid-Layer
Layer 3
0
20
40
60
$B_{act,\ Streptavidin}$ (pmol)

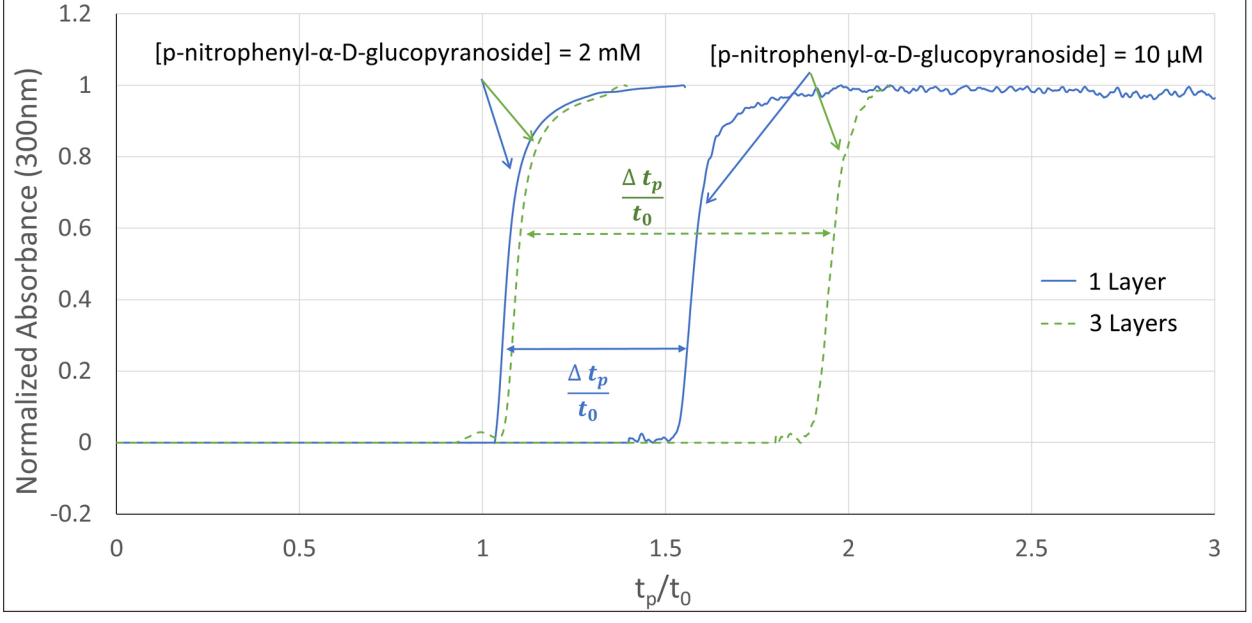

[p-nitrophenyl-α-D-glucopyranoside] = 2 mM
[p-nitrophenyl-α-D-glucopyranoside] = 10 µM
Normalized Absorbance (300nm)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.5
1
1.5
2
2.5
3
t_p/t_0
$\frac{\Delta t_p}{t_0}$
$\frac{\Delta t_p}{t_0}$
1 Layer
3 Layers

Table 1 - Biotinylation incorporation ratios obtained at different biotinylation conditions

Sample name	*Biot. molar excess*	*Mean Biot. inc. ratio*	*Std (n = 5)*
Low	10x	1.9	± 0.4
Medium	15x	2.8	± 0.7
High	Commercial sample	7.5	± 0.9