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Abstract

Affinity monolith columns of 375 nL (effective length 8.5 cm, internal diameter 75µm) were developed for protein-ligand affinity investigations needing only 3 µg of human serum albumin (HSA). To promote specific interactions and avoid non-specific ones, different combinations of monolithic supports and bio-functionalization pathways were evaluated. Silica and glycidylmethacrylate based monoliths were *in-situ* synthesized and grafted with HSA. Two direct grafting methods epoxy-amine and Schiff Base plus the streptavidin-biotin method were compared. The columns were evaluated by frontal analysis with ligands of known affinity for HSA. It is shown that a classical capillary electrophoresis instrument equipped with an external pressure device can be used to do weak affinity chromatography at low pressure (less than 1.2 MPa) in a fully automated way and with very low reagent consumption. The grafting pathways were compared in terms of (i) total and active amounts of immobilized protein, (ii) non-specific interactions, (iii) protein denaturation. According to these criteria, the organic monoliths combined with the streptavidin-biotin approach provided the best results. This immobilization pathway led to the highest active protein content (40 pmol of HSA per 8.5-cm column) with less than 10% non-specific interactions and 84% protein activity. The target grafting step lasts only 10 min and is UV-monitored, the UV breakthrough curve giving the exact amount of bound protein. This novel approach was validated by K_d measurements of 3 known ligands of HSA. Streptavidin generic monolith columns could be stored at 4°C for 3 months maintaining activity. µg of a biotin modified sensitive protein could be attached to a stable streptavidin monolith for immediate interaction studies avoiding stability problems. This development was subsequently extended to another protein of higher pharmaceutical interest: the N-terminal domain of HSP90. Affinity was measured for two known ligands and determined K_d values were in accordance with the literature, proving that our technique is applicable to other proteins.

Keywords: weak affinity chromatography, miniaturization, monoliths, protein-ligand interaction, HSA, HSP90

1. Introduction

Understanding and quantifying biomolecular interactions is at the very heart of life sciences. From a fundamental point of view, it allows for a better understanding of biological processes. Biomedical interactions are also needed for health applications, such as drug discovery applied to the development of new therapeutic molecules [1]. Recently, weak affinity chromatography (WAC) has been introduced as a chromatographic technique where biomolecules such as proteins are immobilized on the stationary phase [2,3]. WAC has been increasingly used to study interactions in biological systems [2–5]. The chromatographic retention of a ligand injected in WAC is linked to the protein-ligand interaction strength. Hence, WAC can be used to obtain fundamental and/or mechanistic information in a variety of fields such as: (i) chiral separations [6,7], (ii) fragment screening in fragment-based drug discovery [3], (iii) selection of site-selective probe for use in the high-throughput screening of drug binding to protein [8] and (iv) thorough thermodynamic and kinetic studies of protein-ligand interaction [9,10]. A study reported that the data obtained by WAC were coherent with what the more sophisticated NMR and Thermal Shift Assay techniques were producing [5]. Since running cost is an important factor, WAC appears to be very competitive for biomolecular interaction studies [3].

The WAC studies were mainly performed using “classical” columns with an internal diameters of 4.6 mm or 2.1 mm, a length varying from one to 20 cm, and using 5 µm silica particles with wide 30 nm pore size [4,8,9,11–14]. Few studies were also conducted with silica monoliths at classical scale (columns with a 4.6 mm i.d and with lengths ranging from a few centimeters [6] to a few millimeters [15]), or with organic polymer monoliths (4.6 mm i.d., length from a few centimeters [16] to a few millimeters [7]). The immobilization of the target protein onto the stationary phase allows for a significant decrease of the amount of protein needed for the interaction studies compared to what was needed with the *in vitro* solution studies. However, the preparation of a “classical” affinity column can require up to a few dozen milligrams of the target protein. This amount is still significant. Obtaining milligram amounts of stable and functionally folded proteins can be highly challenging. A first attempt to reduce the amount of protein needed in the sub-milligram range was described by the group of Ohlson who prepared 500 µm i.d. silica particles affinity columns [17]. Further down-sizing the column diameter should allow using even less target protein (µg for 75µm i.d.) but cannot be straightforwardly achieved. The packing of 75 µm i.d. capillary columns is very difficult so monolithic columns appear to be a viable alternative. Monoliths can be synthesized *in-situ* leading to

chromatographic capillary columns with high permeability. After the bare monolith synthesis, the protein grafting step has to be in-situ performed by flowing the protein solution.

The numerous bio-functionalization pathways of chromatographic supports with a target protein have been recently reviewed [18]. The simplest pathway is the direct covalent coupling of the protein onto an epoxy ring via the reaction of the primary amine of a lysine side chain of the protein. This grafting way has drawbacks: it is time-consuming (48 to 72 h) due to the low reactivity of the epoxy moieties [7,16]. Among the reviewed grafting methods, the Schiff base method stands out [7,16,19] as this method should produce 1.5 to 4 time higher grafting densities than the epoxy method for similar reaction times [7,16]. Although the epoxy and Schiff base grafting methods have proved to work well for model proteins such as HSA and few soluble proteins [3], they are less usable with fragile proteins (that may partially denaturate during the time consuming grafting step) or with proteins available in scarce amount (a great excess of protein is consumed). Therefore, we intended to develop an alternative method overcoming such drawbacks and to compare it with the previously reported methods. It relies on the instantaneous capture of the biotinylated-target protein onto a streptavidin-modified support. The streptavidin-biotin interaction (K_d=10^{-15} M [20]) should be strong enough to support dynamic affinity studies under mild conditions.

In this study, silica-based and organic-based monolithic nano-columns were prepared, investigated and compared in frontal analysis mode for nano-WAC applications. Human Serum Albumin (HSA) was used as model target protein and grafted according to three different immobilization pathways, namely: the epoxy-amine, Schiff base and streptavidin-biotin methods. The immobilization pathways were considered in term of (i) amount of total and active protein per column, (ii) secondary non-specific interactions and (iii) protein activity conservation. K_d were measured and compared to the literature in order to validate this new approach. For the streptavidin-biotin grafting path the stability of the columns was investigated in screening and storage conditions. After the development with HSA as target model, nano-columns were grafted with the N-terminal domain of HSP90, in order to confirm the applicability of our technique to another protein, and the K_d of three fragments (ligands) were measured.

2. Material and methods

2.1. Reagents and chemicals

Ethylene dimethacrylate (EDMA, 97%), glycidyl methacrylate (GMA, 98%), 1-propanol, 1,4-butanediol, (3-methacryloxypropyl)-trimethoxysilane (γ-MAPS), tetramethoxysilane (TMOS), methytrimethoxysilane (MTMS), urea, polyethylene glycol (PEG, MW=10,000), methanol (HPLC

grade), triethylamine (TEA), sodium periodate, lithium hydroxide, dipotassium hydrogen phosphate (K_2HPO_4), o-phosphoric acid, dimethylsulfoxide (DMSO), acetic acid, coomassie brilliant blue G (CBB), (3-glycidyloxypropyl)trimethoxysilane (GPTMS), sodium cyanoborohydride, ibuprofen (racemic), warfarin (racemic), furosemide, adenosine 5'-diphosphate sodium salt, n,n-diethylvanillamide, 3-hydroxy-4-methoxybenzyl alcohol, magnesium chloride ($MgCl_2$), human serum albumin (HSA, fatty acid free, globulin free, ≥99%), streptavidin (from *streptomyces avidinii*, affinity purified, ≥ 13 $U.mg^{-1}$ of protein), were purchased from Sigma Aldrich (L'Isle d'Abeau Chesne, France). Ammonia solution (28-30 %) was from Merck (Darmstadt, Germany). Sulfuric acid (18 M) was from VWR (Fontenay-sous-bois, 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. An EZ-LinkNHS-PEG4-Biotinylation Kit was purchased from Thermo Scientific (VWR). HSP90 protein was produced by a partner laboratory (Institut de Biologie et Chimie des Protéines, Lyon France). The N-terminal fragment of HSP90 (9-236) was expressed in E.coli (BL21) with a histidine tag and purified by a monoQ column. Protein samples were delivered at 6.7 $mg.mL^{-1}$ in PBS buffer and stored at -80°C before use.

2.2. Instrumentation

Liquid flow into capillary columns was driven by pressure gradient using the microfluidic flow controller MFCS-EZ100 system (Fluigent, Villejuif, France). 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 capillaries in pressure mode (3 MPa). Nano-LC experiments (frontal weak affinity chromatography experiments) were carried out with a capillary electrophoresis Agilent HP3D CE system (Agilent Technologies, Waldbronn, Germany) equipped with external pressure nitrogen supply allowing to work up to 1.2 MPa. System control and data acquisition were carried out using the Chemstation software (Agilent).

2.3. Capillary monolith syntheses

2.3.1 Epoxy-functionalized silica monoliths

Polyimide-coated fused-silica capillaries (75 µm i.d.) were purchased from Polymicro Technologies (Molex, Phoenix, Arizona, USA). The silica monolith preparation was extensively described [21]. Briefly, a 18 mL mixture of TMOS/MTMS (85/15, v/v) was added to 40 mL 0.01 M acetic acid solution containing 1.9 g PEG and 4.05 g urea. The sol mixture was stirred at 0°C for 30 min. Then, the temperature was raised to 40°C and the mixture was loaded into 15-cm 75-µm i.d. fused-silica

capillary pieces. Next, the partially filled capillaries (approximately 10 cm on the total length of 15 cm), were end-plugged and kept at 40°C overnight for gelification. Mesopores were formed by urea decomposition through gently raising the capillary temperature up to 120°C maintained for 4 h. After cooling, the monoliths were thoroughly washed with methanol. GPTMS surface functionalization was done by flushing monoliths with a solution containing 5 % of GPTMS and 2.5 % of TEA in methanol/water (95/5, v/v). The reaction was conducted for 3 h at room temperature. Monoliths were then rinsed with methanol for 30 min (0.7 MPa).

2.3.2 Glycidyl methacrylate monoliths

Fused-silica capillaries with UV transparent coating (TSH, 75-µm i.d.) were purchased from Polymicro Technologies (Molex). A pre-treatment procedure was carried out in order to ensure a strong covalent attachment of monoliths to capillary walls. Capillaries were flushed with a 5 % (v/v) solution of γ-MAPS in methanol/water (95/5, v/v) with 2.5 % TEA for 1 h at 0.7 MPa. Next, they were rinsed with methanol for 15 min at 0.7 MPa and dried at room temperature under nitrogen stream. A polymerization mixture adapted from [22] was prepared by mixing 0.9 mL GMA, 0.3 mL EDMA, 1.05 mL 1-propanol, 0.6 mL 1,4-butanediol and 0.15 mL ultra-pure water. Prior to UV irradiation, potential oxygen in the solution was removed by 15 min ultrasonication, and 12 mg of the initiator 2,2-azobisisobutyronitrile were added under magnetic stirring. The 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 $J.cm^{-2}$. In order 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 column segment of the 15-cm capillary was rinsed with methanol for 1 h using a LC pump in pressure mode (3 MPa) to remove any unreacted reagents.

2.4. Protein immobilization protocols

2.4.1 Direct coupling methods

2.4.1.1 Epoxy method

Proteins can be directly immobilized onto epoxy-functionalized monolithic columns via reactions involving primary amines (-NH_2), such as the lysine side-chains, or the amino-termini of proteins. This grafting was carried out by circulating a 2 $mg.mL^{-1}$ HSA solution in phosphate buffer (67 mM, pH 7.4) for 48 h at 0.7 MPa. The column was then rinsed with phosphate buffer solution and stored at 4°C prior to use.

2.4.1.2 Schiff base method

The Schiff base method consists in the reaction of aldehyde reactive surface groups of the solid support with the amino-groups of the protein. Aldehyde reactive moieties are classically obtained by oxidation of diol functions. First, the epoxy groups of the epoxy-functionalized silica or GMA-co-EDMA monoliths were hydrolyzed into diols flowing 1 M sulfuric acid for 2 h at 0.7 MPa. After rinsing all residual acid, the diol-monolith was oxidized to aldehyde using a 0.12 M $NaIO_4$ solution at pH 5.5. Then a 2 $mg.mL^{-1}$ HSA and 8 $mg.mL^{-1}$ $NaBH_3CN$ solution in 67 mM pH 6 phosphate buffer was percolated through the column for 48 h at 0.7 MPa and room temperature. Sodium cyanoborohydride reduces imine groups, a necessary treatment in order to get reliable immobilization. After immobilization, the column was flushed with sodium borohydride (2.5 $mg.mL^{-1}$, phosphate buffer 67 mM pH 8 (2 h, 0.7 MPa) to reduce residual aldehydes. The HSA affinity columns were then rinsed with phosphate buffer and stored at 4°C. The same protocol was used to evaluate the impact of protein concentration (1 $mg.mL^{-1}$ and 0.5 $mg.mL^{-1}$ HSA were also tested) and grafting duration (12 h instead of 48 h was tested) on final protein content.

2.4.2 Streptavidin-biotin method

The streptavidin-biotin method relies on the instantaneous capture of the biotinylated-target protein onto a streptavidin-modified monolith. The affinity of the streptavidin-biotin interaction is so high (in the femtomolar range, $K_d=10^{-15}$ M [20]) that this coupling may be considered as covalent. Streptavidin was grafted on the organic monolith following the Schiff base method described above using a 1 $mg.mL^{-1}$ streptavidin solution. Coupling of biotin to HSA was done following the instructions of the Thermo EZ-Link NHS-PEG4-Biotinylation Kit. Being a small molecule, biotin (M.W. 244 $g.mol^{-1}$) can be attached to many proteins without altering their biological activities [23]. The molar ratio of NHS-PEG_4-biotin to protein was adjusted to obtain the desired level of incorporation. Biotin incorporation (1.7 biotin/protein molecule) was estimated using the HABA assay. All excess of biotin reagent was removed using a desalting column. The concentration of the resulting biotin-HSA solution was calculated with the solution's absorbance at 280 nm (ε_{HSA}= 0.5982 $L.g^{-1}.cm^{-1}$). Finally, the biotin-HSA solution was loaded through the streptavidin immobilized column using the Agilent CE system at 1.2 MPa pressure, monitoring UV absorbance at 230 and 280 nm. The percolation was stopped when saturation of the support was reached, i.e. the biotin-HSA reached the detector (observation of a plateau at 230 and 280 nm). The breakthrough curve was used to estimate the quantity of captured protein on the streptavidin immobilized support. Similar protocol was used for the HSP90 protein. Biotin incorporation was 2.8 biotin/HSP90 N-ter and resulting biotinylated protein concentration was calculated using ε_{HSP90}= 0.5822 $L.g^{-1}.cm^{-1}$.

2.5. Columns evaluation

Affinity columns were evaluated by frontal chromatography using a capillary electrophoresis system (S.1). The experiments were conducted using the CE system in the so-called "short-end" injection mode. The system was exclusively operated in the pressurization mode by applying external pressure (no voltage applied). The inlet of the capillary column is simply immersed in the solution to be infused and the external pressure forces the liquid to flow inside the capillary column. 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. Experiments were conducted under controlled room temperature (25°C). After every analysis, the capillary column was rinsed with 20 column volumes of phosphate buffer to eliminate the infused compounds.

2.5.1 Determination of the number of actives sites (B_{act}) and protein-ligand dissociation constant (K_d) by in-capillary frontal analysis

The total number of actives sites (B_{act}) and the protein-ligand dissociation constant (K_d) were evaluated by frontal analysis [8]. Different ligands of known affinity for HSA (Fig. 1) were selected to characterize the columns : warfarin, furosemide and ibuprofen [6,11,24].

Fig.1.

10 mM stock solutions of the probe ligands were prepared in DMSO to ensure complete solubilization. Solutions of increasing ligand concentration (5, 7, 10 and 20 µM) were prepared in phosphate buffer (67 mM K_2HPO_4, pH 7.4) and percolated at 1.2 MPa (about 240 nL.min^{-1} or 0.1 cm.s^{-1} for 8.5-cm length columns). The flow rate, F, was calculated using a non-retained solute or the plateau of residual DMSO. Compounds were monitored at their characteristic wavelength (DMSO 214 nm, warfarin 308 nm, furosemide 280 nm, and ibuprofen 230 nm). The accurate determination of the saturation time, $t_{plateau}$, *viz* inflection point of the breakthrough curves, was determined using the 1st derivative of the UV-signal. For each ligand concentration, [L], the breakthrough curve is used to determine the amount of ligand captured by the HSA affinity support, q, using eq. 1:

$$q = \Delta t \times F \times [L] \qquad \text{(Eq.1)}$$

Δt being the time difference between dead time, t_0, and saturation time, $t_{plateau}$.

The total number of active sites on the affinity column, B_{act}, and the dissociation equilibrium constant, K_d, of the HSA-ligand complex were determined according to eq. 2 after plotting 1/q versus 1/[L]:

$$\frac{1}{q} = \frac{k_d}{B_{act}} \times \frac{1}{[L]} + \frac{1}{B_{act}} \quad \text{(Eq.2)}$$

B_{act} was first determined in pmol and then converted to pmol.cm^{-1} and nmol.mL^{-1} using the monolith density (300 and 320 mg.mL^{-1} respectively for silica and organic monolith).

2.5.2 Evaluation of non-specific interactions

Non-specific interactions were quantified by frontal analysis using a ligand concentration of 10 µM in phosphate buffer (67 mM K_2HPO_4, pH 7.4) on monolithic columns before the protein immobilization step. For each support, the amount of probe ligands captured by non-specific interaction was determined according to Eq.1. For non-specific interactions, the breakthrough volume is the same at all concentrations and the amount of ligand captured is directly proportional to the concentration. The contribution of non-specific interactions to total retention was also expressed as the fraction of ligand captured by non-specific interactions compared to the total amount captured by frontal affinity chromatography on the same monolith after the protein immobilization step (at the same ligand concentration).

2.5.3 Determination of the total amount of immobilized protein B_{tot}

In order to thoroughly characterize the influence of the immobilization process on the protein activity, the active amount of HSA, B_{act}, has to be compared to the total amount of immobilized protein, B_{tot}. So different methods were designed to determine *in-situ* the total amount of target protein immobilized according to the protein grafting process.

For monoliths bio-functionalized with the streptavidin-biotin coupling method, the UV-monitoring of the breakthrough curve of biotin-HSA at 230 and 280 nm during the dynamic grafting step allowed the direct quantification of biotin-HSA captured by the monolith.

For affinity monoliths prepared with the Schiff base method, the Amino Density Estimation by Colorimetric Assay (ADECA) method was adapted to micro-format and hydrodynamic mode from results previously obtained in a static mode on macro-monoliths synthesized in batch [25]. The on-line modified ADECA method use the Coomassie Brilliant Blue (CBB) dye and allows for *in-situ* determinations of the total amount of protein grafted inside miniaturized capillary column,

independently of its activity. The experiments were conducted using the CE system in the short-end injection mode using external pressuring device. The monolith is initially flushed with ultrapure water to remove the phosphate buffer, and a 10% methanol-5% acetic acid solution to protonate all amino groups, both at 1.2 MPa for 10 min. Then, a 100 $mg.L^{-1}$ CBB in 10 % v/v methanol-5 % v/v acetic acid solution is loaded under a 1.2 MPa pressure monitoring the corresponding breakthrough curve at 260 nm. Eq. 1 was used to determine the amount of captured CBB and the quantity of immobilized protein was calculated taking into account the 103 amino acids, distributed between lysine, histidine and arginine, in the HSA sequence. The rinsing step was performed by flushing the column with a 50/50 1 M ammonia/methanol solution at 1.2 MPa for 10 min.

3. Results and discussion

Two types of monolithic capillary supports were investigated for the development of miniaturized weak affinity experiments: silica and organic monoliths. Both types were first *in-situ* synthesized in 75 µm i.d. capillary and then bio-functionalized with HSA as model target protein. Depending on the type of monolith, several bio-functionalization pathways were considered: the direct "epoxy" and "Schiff base" methods and the "streptavidin-biotin" coupling method (Fig. 2).

Fig.2.

For the streptavidin-biotin coupling method described in this paper, streptavidin was grafted on the surface and biotin was attached to HSA. Then, due to the rapid and complete streptavidin-biotin interaction, the capture of the biotin-protein was monitored *in-situ* by UV spectrophotometry during its breakthrough percolation.

The HSA immobilized organic and silica monoliths were evaluated in terms of unwanted non-specific interactions and specific interactions (affinity), with the goal to minimize the former and to enhance the latter. Considering the high permeability of monoliths, the use of an expensive nano-LC set-up was put aside and all experiments were conducted in frontal affinity chromatography using a capillary electrophoresis system, operated in the pressurized mode (maximum external pressure of 1.2 MPa) in the short-end mode.

3.1. Affinity Silica monoliths

A first attempt to downsize weak affinity chromatography at nanoscale was made with silica monoliths that present similar chemical and textural features to that of silica particles used in the

literature. Bare silica monoliths were *in-situ* synthesized according to a previously described sol-gel protocol [21]. Such monolithic capillary columns have 2 µm through-pores with 8 nm mesopores giving a surface area of 480 $m^2.g^{-1}$ with a 1.5 µm skeleton diameter giving a permeability of 5.10^{-14} m^2 (determined by Darcy's law). For comparison, classical 3-5 µm silica particles have larger mesopores, specific areas between 100 and 300 $m^2.g^{-1}$, and permeabilities two orders of magnitude lower.

Only the two direct epoxy-amine and the Schiff base bio-functionalization processes of Fig. 2 were initially considered with silica monolithic columns. Indeed, the streptavidin-biotin coupling is not amenable with 8 nm nanopores. Bare silica monoliths were silanized with glycidoxypropyltrimethoxysilane (GPTMS) leading to epoxy-functionalized silica monoliths. Epoxy-ring opening was achieved in acidic medium to form diol groups that were oxidized into aldehyde groups. The silica monoliths were evaluated at different stages of modification in terms of non-specific and specific affinity interactions.

3.1.1 Non-specific interactions on silica monoliths.

Characterization of non-specific interactions was estimated through adsorption of three HSA-specific ligands: warfarin, furosemide and ibuprofen, at a unique concentration of 10 µM. Their adsorption on silica monolith surfaces was done at four different stages of modification: (i) bare silica monolith, (ii) epoxy-modified silica monolith, (iii) diol monolith after the epoxy-ring opening, and (iv) reduced-aldehyde monolith. Fig. 3 shows the absorbed amounts of three ligands on the 8.5 cm effective length 75 µm i.d. capillaries, expressed in $pmol.cm^{-1}$, obtained on bare monolith and at the different stages of modification. The epoxy-activated silica monoliths present the highest level of non-specific interactions, probably linked to the less hydrophilic character of the glycidoxypropyl group introduced during the silanization step. Hydrolysis of the epoxy-ring into diol groups significantly decreases the amount of non-specifically adsorbed ligands but the level remains twice higher than on bare silica (Fig. 3). The oxidation of the diol moieties into aldehydes, followed by their reduction by $NaBH_4$ to polar hydroxyl groups does not significantly decrease non-specific interactions.

Fig.3.

Since the log D values at pH=7.4 of these three negatively charged ligands, respectively around -1, 0.8 and 1 for furosemide, warfarin and ibuprofen [26], do not match the non-specific interaction levels, hydrophobicity is not the only contributing factor. This significant level highlights that non-specific interactions should not be neglected on silica affinity monoliths and that reference columns have to be prepared to quantify their contribution to overall retention. When the Schiff base method is used, diol columns can be used instead of a reduced-aldehyde ones as non-specific interactions are of the

same order of magnitude for both column types (Fig. 3). Diol columns are easier-to-synthesize references. If such a strategy, evaluation of non-specific interactions without any protein immobilized onto the surface, is usually recommended by several groups (Hage, Ohlson) it may not be fully representative of the final affinity column. Indeed, once proteins are grafted onto the support, the resulting surface is partially covered by the targets, thus probably limiting ligand accessibility to the underlying support. However, the grafting of another protein is not a satisfactory alternative at it may lead itself to other non-specific interactions (from electrostatic to hydrophobic ones).

3.1.2 Affinity on HSA-silica monoliths.

The epoxy-method is known to proceed through the quick adsorption of the protein onto the surface followed by a slower covalent links formation between epoxy and amino residues of biomolecules [27]. Adsorption of a protein onto a hydrophobic surface is known to partially denature it i.e. to alter its biological activity [28]. Thus the epoxy method was not further investigated on the epoxy silica and HSA-affinity silica monolithic columns were exclusively prepared though the Schiff base method. The performances of these affinity columns were assessed in frontal affinity chromatography (FAC) with the three ligands. These ligands target the two main interaction sites of HSA (Sudlow sites IIA and IIIA) [6,11]. For each solute, the breakthrough volume increased when the infused concentration decreased, which is characteristic of affinity. Both the experimental K_d values and the total amount of active binding sites (B_{act}) of immobilized target proteins were determined by plotting the reciprocal of the amount of captured ligand (1/q) versus the reciprocal of the ligand concentration (1/[L]) (Eq. 2). The values are reported in Table 1.

Grafting method	Columns tested	Binding site	Compound	K_d in literature (µM)	Calculated K_d (µM)	Active HSA content (B_{act})	
						(pmol.cm^{-1})	(nmol.mL^{-1})
Schiff base	n=3	IIA	Warfarin	2 – 7	20 ±8	10 ±2	212 ±47
			Furosemide	5 – 50	97 ±36	32 ±7	680 ±150
		IIIA	Ibuprofen	0.3 – 25	116 ±43	32 ±7	680 ±150

Table 1. Experimental K_d values and active HSA-content for silica monoliths bio-functionalized with HSA via the Schiff base method. Frontal analysis experiments implemented on silica monolithic columns (effective length = 8.5 cm, 75 µm i.d.). Relative standard deviations for the three experiments.

The fact that the three ligands exhibit a measurable affinity for the HSA-column indicates that the two HSA binding sites are active. However, the experimental K_d values obtained in this work (Table 1) are slightly higher than those reported in the literature [24] (Fig. 1). The high surface area of the silica

monolith (480 $m^2.g^{-1}$) did not produce a higher number active sites B_{act} compared to the number of sites found on silica particles with only 100 $m^2.g^{-1}$ [15]. The relative small size of the monolith mesopores (8 nm) combined to the high total monolith porosity may account for this relatively low active site density. The native HSA protein is quite large with a hydrodynamic radius of 3.5 nm likely to produce steric hindrances in mesopores that make most of the surface area. In addition, the small phase ratio V_s/V_m of silica monoliths, that are about 90 % porous, is less favorable to binding than that of silica particles with only 70-80 % total porosity.

Considering the relative amounts of solutes captured at the same concentration (10 µM concentration) on the HSA-silica monoliths and on reduced-aldehyde monoliths, non-specific interactions make a substantial contribution to the overall retention (respectively 7%, 18% and 41% for ibuprofen, furosemide and warfarin at 10 µM). This level of non-specific interactions to overall retention is similar to what was observed by several authors on silica particles [15]. The difference in active HSA-content (B_{act}) obtained for the same IIA binding site with two different ligands (Table 1) suggests that these values could be partially biased by non-specific interactions that are more important for warfarin. Considering (i) the relatively high part of non-specific interactions and (ii) the impossibility to enlarge the mesopore diameter of our silica monoliths to increase access to the pore volume, further investigation was focused on organic monoliths.

3.2. Glycidyl methacrylate affinity monoliths

Among the great diversity of organic monoliths [18], the GMA ones were selected for the simplicity and rapidity of their photochemically induced synthesis leaving directly available epoxy reactive groups at their surface. They were *in-situ* synthesized in UV-transparent capillaries according to a described protocol [22]. UV-masks allow for an easy selection of monolith length and location in the capillary. This monolith had a specific area of 4 $m^2.g^{-1}$ and a permeability of 10^{-13} m^2.

All the protein immobilization pathways described in Fig. 2 were possible with the GMA monoliths. The systematic non-specific interaction study was done in order to estimate the benefits of each pathway with respect to affinity measurement on GMA-based monolith.

3.2.1 Non-specific interactions on organic GMA monoliths

Possible non-specific interactions were investigated at the different stages of the monolith elaboration: (i) as-synthesized, (ii) after the epoxy-ring opening to form diols, (iii) after diol oxidation into aldehyde and reduction of the aldehyde with $NaBH_4$, and (iv) after the covalent grafting of streptavidin. Fig. 4 presents the non-specific adsorption of the three ligands at each step.

The highest adsorption value in Fig. 4 (0.43 pmol.cm^{-1}) is lower than the adsorption values on silica monolith (Fig. 3). Clearly, organic monoliths, at all elaboration stages, show less non-specific interactions than silica monoliths. The dramatically lower amounts of ligands adsorbed onto GMA and GMA-modified monoliths compared to silica monoliths may be due to the much lower surface area of the organic monoliths. Among the GMA-based monoliths, epoxy monolith exhibits the highest level of non-specific interactions. As expected, the conversion of epoxy into diol groups led to a significant decrease in non-specific interactions that remain quite identical after oxidation. (Fig. 4). After the grafting of streptavidin, the level of non-specific interaction slightly increases, thus demonstrating that the grafting of a protein onto the surface is not a better alternative to hide the underlying surface and may lead to small non-specific interactions.

3.2.2 Affinity on GMA-based monoliths

Frontal affinity chromatography was used to characterize the immobilized-HSA activity and accessibility with respect to the three HSA immobilization pathways. The K_d values and amount of active binding sites B_{act} are gathered in Table 2. An illustration of the affinity experiments conducted on a streptavidin-biotin monolith (breakthrough curves and plot of 1/q vs 1/[L]) is given in S.2. For HSA-columns prepared through the Schiff base and streptavidin-biotin methods, the total HSA content was *in-situ* determined. The colorimetric ADECA method was implemented to quantify the total amount (B_{tot}) of HSA immobilized onto the aldehyde monolith via the Schiff base method. The total amount of biotin-HSA captured on the streptavidin-column was directly quantified through the breakthrough curves (S.2). The ratio between active and total HSA contents gives the HSA activity yield.

Grafting method	Columns tested	Binding site	Compound	Calculated K_d (µM)	Active HSA content (B_{act})		Total HSA content (B_{tot})		Activity yield
					(pmol.cm^{-1})	(nmol.mL^{-1})	(pmol.cm^{-1})	(nmol.mL^{-1})	
Epoxy	n=3	IIA	Warfarin	17 ±9	5 ±1	103 ±27	Not determined (high non-specific interactions)		
			Furosemide	24 ±13	4.0 ±0.8	91 ±24			
		IIIA	Ibuprofen	21 ±11	2.6 ±0.5	59 ±15			
Schiff base	n=13	IIA	Warfarin	11 ±5	1.9 ±0.5	43 ±11	2.4 ±0.8	54 ±18	71%
			Furosemide	17 ±3	1.7 ±0.6	38 ±14			
		IIIA	Ibuprofen	12 ±1	1.5 ±0.4	34 ±9			
Streptavidin	n=13	IIA	Warfarin	13 ±4	4 ±1	91 ±22	4.9 ±0.6	111 ±14	84%
			Furosemide	18 ±7	4.2 ±0.8	95 ±18			
		IIIA	Ibuprofen	16 ±4	4.0 ±0.5	91 ±12			

Table 2. Experimental K_d values, active HSA-content (B_{act}) and total HSA-content (B_{tot}) for GMA-based monoliths bio-functionalized with HSA via different immobilization pathways. Frontal

analysis experiments implemented on monolithic columns (effective length = 8.5 cm, 75 µm i.d.). Relative standard deviations for the n experiments.

The K_d values obtained for the three test ligands are in good agreement with previously reported values and show that the two binding sites of immobilized HSA are active whatever the grafting method used.

Another important point is the relative amount of ligand captured by non-specific versus that captured by specific interactions. The level of non-specific interactions (at 10µM) ranges from 16 to 34 % (epoxy), 0 to 8 % (Schiff base) and 0 to 7 % (streptavidin monoliths). These results show that GMA-modified monoliths present a significantly lower contribution of non-specific interaction than their silica counterparts.

3.2.2.1 Epoxy method

Regarding the active sites content (B_{act}), different values were obtained for the two HSA binding sites. Whereas about 4-5 pmol.cm^{-1} of active sites is measured on the site IIA, only about 2.5 pmol.cm^{-1} of actives sites is afforded for the site IIIA (n=3) (Table 2). This difference may be partially ascribe to non-specific interactions on the epoxy-monolith and/or to a partial loss of activity of the Sudlow site IIIA consecutive to the HSA immobilization process as already observed on silica [9]. Several attempts were made to reduce or suppress non-specific interactions on epoxy monoliths using different post-grafting reactions. Post-grafting modification proposed in the literature were tested unsuccessfully: the monoliths were treated with tris(hydroxymethyl)-aminomethane (Tris) (0.1 M, pH 8.6, 48 h), cysteine (1 M, 48 h), or ethanolamine (1 M, 15 h) [7,16]. FTIR spectroscopy was used to follow the reaction of epoxy groups. No reduction of the intensity of the C-O vibration band of the epoxy at 907 cm^{-1} was observed, which confirms the lack of reactivity of Tris and ethanolamine at room temperature. Increasing the temperature to 60°C was effective in opening the epoxy groups but it was not practical due to obvious protein stability issues. Conversely, the reaction of epoxy groups with cysteine was efficient by totally eliminating non-specific interactions at room temperature, but it also suppressed all HSA-specific interactions with the ligands. According to the literature, cysteine may form a disulfide bond with the free ^{34}Cysteine of HSA impairing all activity of the binding sites by oxidation cascade [29]. Despite the simplicity of the direct grafting of HSA onto poly(GMA-co-EDMA) monoliths, this modification pathway was no further investigated due to excessive non-specific interactions and potential partial deactivation of site IIIA.

3.2.2.2 Schiff base and streptavidin-biotin method

Very coherent active sites B_{act} were obtained with the three ligands on affinity monoliths prepared by immobilization of HSA using the Schiff base method or the streptavidin-biotin coupling (Table 2). This accurate determination of B_{act} is probably related to the very low level of non-specific interactions (in the low percent range). Also, the concordant values obtained for the two sites indicate that no partial deactivation of the IIIA site occurs with these two bio-functionalization methods. This outlines the proper accessibility of immobilized proteins with equivalent activities for the two binding sites (IIA and IIIA).

There is more than twice less HSA on the Schiff base monoliths *viz* 1.7 pmol.cm^{-1} (n=13) compared to the streptavidin-biotin one with 4 pmol.cm^{-1} (n=13) (Table 2). Also, the amount of active HSA, ratio (B_{act})/ (B_{tot}) is 84% for the streptavidin monolith, slightly better than the 71% for the Schiff base monoliths. Assuming that reduced-aldehyde monoliths bind the same amount of streptavidin than of HSA (these two proteins have similar features in terms of mass, hydrophobicity and isoelectric point), it was estimated that one tetramer of streptavidin binds to two biotin-HSA.

3.2.3 Grafting duration and protein consumption considerations

Optimization of the protein immobilization concerns the reaction time and the quantity of target protein used for the immobilization step. In the literature, grafting durations with the Schiff base method range from 3 to 6 days (0.5 mL.min^{-1}) for HSA concentrations of 5 mg.mL^{-1} [6,7,16]. Our protocol uses a twice to ten times less concentrated HSA solution (2 down to 0.5 mg.mL^{-1}) and the grafting time is six to ten times faster completed in 48h then downed to 12 h in dynamic mode at a linear velocity of about 0.06 cm.s^{-1}. No significant difference in active HSA content was observed between the different columns, validating the grafting under flow conditions (dynamic mode) [4,9]. Another real advantage, beside time and protein mass savings, is the greatly reduced risk of loss of protein activity and/or denaturation.

The streptavidin-biotin coupling process is faster than the two other modes which is a great advantage with proteins of low stability. The coupling of biotinylated-target protein can be achieved in few minutes, e.g. about 15 min with a 1 mg.mL^{-1} protein concentration and a 8.5 cm effective length 75 µm i.d. column at a percolation pressure of 1.2 MPa. This coupling can be applied just before the affinity experiments: the column is installed in the CE cartridge, grafted in dynamic mode (under UV monitoring) and ready to use. Last but not least, the UV monitoring during the grafting step of the biotin-protein onto the streptavidin support allows for (i) minimizing the protein consumption to a few pmol per centimeter of capillary column, and (ii) the exact determination of

the amount of grafted protein which is a tremendous asset for the quality control of each column before its use.

3.2.4 Stability of the streptavidin-biotin affinity columns

Stability of the bio-functionalized columns is of great concern. Indeed, screening affinity of unknown ligands against a specific target protein implies preserving the protein activity during the screening process as well as the protein content constant during successive analyses. Long-term stability and storage conditions must be also examined.

3.2.4.1 Stability of streptavidin-functionalized monolithic columns

The stability of streptavidin-functionalized columns was not considered so far. So, it was checked if it is possible to prepare and to stock streptavidin-functionalized columns for a long time at 4°C before their coupling with the biotinylated-target protein.

Six streptavidin-functionalized columns synthesized through the Schiff base method were prepared. Three were coupled with biotin-HSA and used immediately. The other three were stored at 4°C for three months. The columns stored at 4°C kept their trapping ability intact as per biotin-HSA content after 3 months. Thus, the streptavidin-functionalized columns can be prepared in advance and stored for at least three months before use, knowing that protein coupling is rapid and easy and can be done freshly before the affinity study.

3.2.4.2 Stability of affinity-HSA columns (prepared by the streptavidin-biotin coupling)

The stability of HSA-affinity columns may be a critical parameter especially in the case of a screening campaign as it will determine the useful lifetime of the column. The stability of the HSA affinity columns was evaluated in phosphate buffer (67 mM, pH=7.4) using frontal analysis with warfarin to determine the remaining amount of active protein (B_{act}) under various temperature conditions, namely: (i) at 4°C i.e. in storage conditions, (ii) at room temperature in static mode and (iii) at room temperature in dynamic mode (screening conditions).

At 4°C, no variation of the active HSA content (B_{act}) was observed within 3 months, thus indicating that affinity columns can be prepared in advance and used later. At room temperature and in dynamic conditions, the column being continuously flushed with buffer at 0.06 $cm.s^{-1}$, 80% of active HSA were remaining after 24 hours and around 550 column volumes. 64% remained after 80 hours of extensive use or over 1800 column volumes. In order to assess if the partial loss of activity was due to protein stability or to the sustained released of poorly or unbound HSA, the same experiment was carried out in static mode. After 80 h of storage at room temperature in static mode, the remaining

activity was only 55%. This result indicates that exposure to room temperature is responsible for the loss of activity and that there is no loss of protein by leaching.

3.3. HSP90 affinity columns

As HSA affinity columns gave promising results with organic monolith grafted by the streptavidin-biotin approach, affinity columns were then prepared by the same method with another soluble protein: the N-terminal domain of HSP90. Grafted quantity of HSP90 (5.8±0.7 $pmol.cm^{-1}$, n=11 columns) is similar to HSA (4.9±0.6 $pmol.cm^{-1}$, Table 2). Two known ligands were studied by frontal affinity chromatography to validate the technique on a different protein: adenosine diphosphate (ADP, K_d=10-15 µM [30]) and n,n-diethylvanillamide (K_d=790 µM [30]). Specific interactions were detected for both ligands and calculated dissociation constants (14±2 µM and 150±70 µM respectively for ADP and n,n-diethylvanillamide) were in the literature range. It may be noted that the weaker the affinity, the higher the incertitude on K_d determination. Specific affinity was also detected for a third ligand, 3-hydroxy-4-methoxybenzyl alcohol and the affinity was estimated at about 40 µM (no reference K_d was found in the literature). It seems therefore possible to graft different proteins with the streptavidin-biotin approach while maintaining their specific binding capacity.

4. Conclusion

Results obtained during this evaluation of two types of monoliths, a silica based and an organic glycidyl methacrylate based one, and different ways of protein grafting led us to the following conclusions: silica based monoliths can load a higher amount of active protein, but a high level of non-specific interactions is noted when interacting with ligands. The silica grafting of reactive moieties requires a first step that is silanization with glycidoxypropyltrimethoxysilane. It is thought that this silanization step is responsible, at least partly, for the non-specific interactions. Non-specific hydrophobic interactions with the propyl linker are possible. Due to a higher adsorption and a lower permeability on silica monoliths, it seems better for our purpose to work with GMA monoliths.

GMA monoliths may be directly grafted with proteins. However, some residual epoxy moieties are left over after target grafting. These reactive epoxy groups cannot be efficiently suppressed under reaction conditions compatible with the grafted protein stability. Protein grafting using the Schiff base method or the streptavidin-biotin method produced the best organic monoliths: negligible non-specific interactions and high active protein content. The very low protein consumption is another significant advantage for the streptavidin-biotin method.

The method requires a first step that is attaching biotin to the target protein, which is straightforward. This said the method presents several advantages over the Schiff base method: (i) it binds up to twice more active sites allowing targeting lower affinity ligands. (ii) The streptavidin column can be loaded in a few minutes with the target protein just prior its use thus limiting stability issues. (iii) For each column, the exact amount of biotinylated-target protein captured during the grafting step is exactly known, which is a major asset for quality control. (iv) The amount of bound protein (a few ten picomoles per column) is just what is needed. (v) The method does not denature the protein giving an activity rate passing 80%. Such streptavidin-biotin grafting way is generic, as it may be implemented to any other biotinylated target. Indeed, it has been shown that this method was successfully applied to the N-terminal domain of HSP90.

As an example of the benefits of 8.5 cm effective length 75 µm i.d. miniaturized monolith columns modified by the streptavidin-biotin method, the grafting requires only 40 pmol of the target protein (3 µg for a 70 kDa protein). It is achieved in about 10 min with a pressure drop of 1.2 MPa giving a flow rate of 240 $nL.min^{-1}$ and a 1 $mg.mL^{-1}$ protein concentration. The great monolith permeability of these nano-columns can be operated in a very simple and automated way with the external pressure device of a common capillary electrophoresis system using its in-situ UV detection avoiding all nano-LC problems with high pressure particle-filled columns.

In the frame of screening process (using zonal affinity chromatography analysis coupled to mass spectrometry), it will be possible to screen mixtures of about 10-20 compounds in one analysis of about 30 min, which gives around 48 runs/day i.e. around 500-1000 compounds/day (namely an entire fragment library in the fragment-based drug discovery (FDBB) approach). Thus, boosting the fragment screening step using a generic method (applicable to any biotinylated proteins) is of great interest in FBDD.

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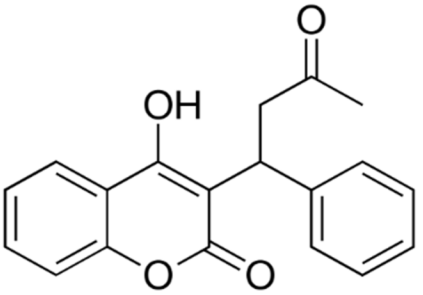

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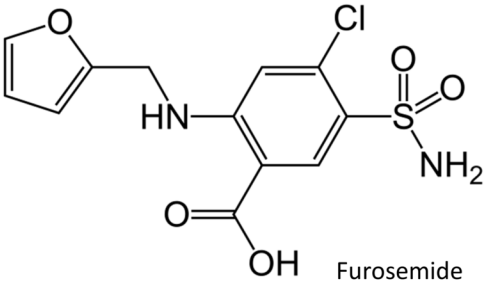
K_d = 2-7 μM



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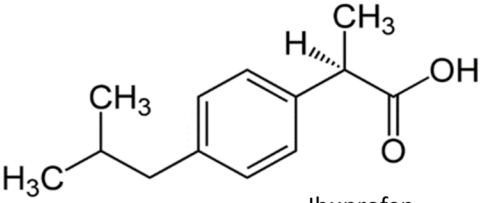

Ibuprofen
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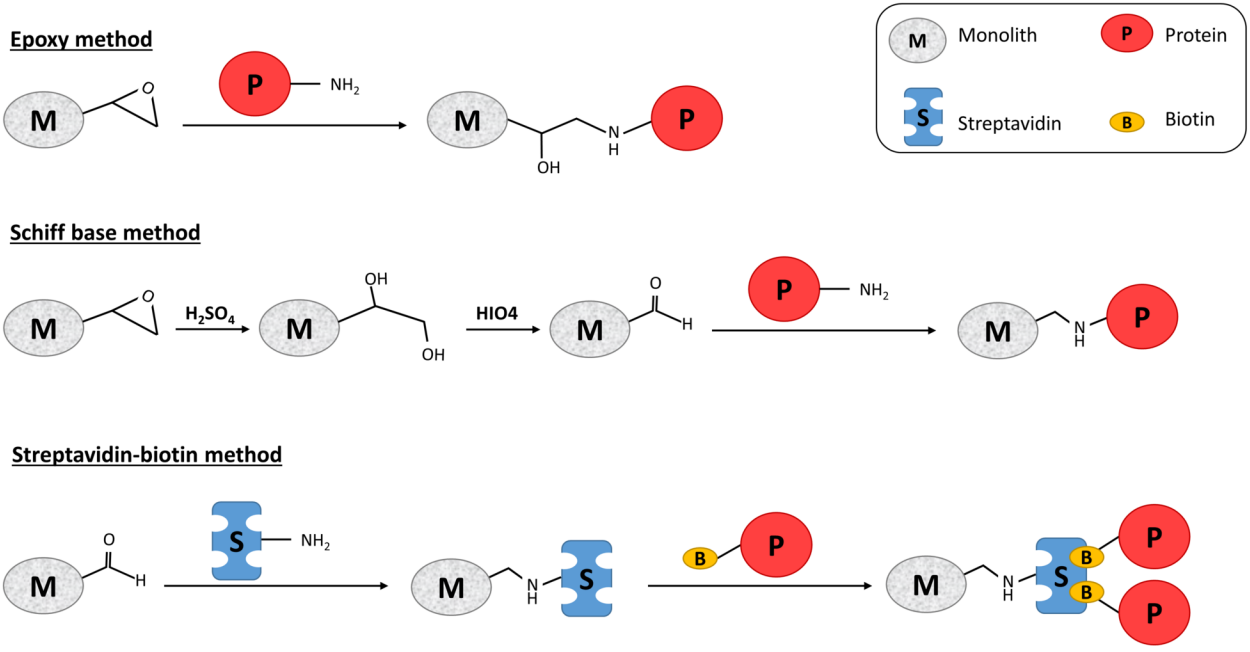
K_d = 0.3-25 μM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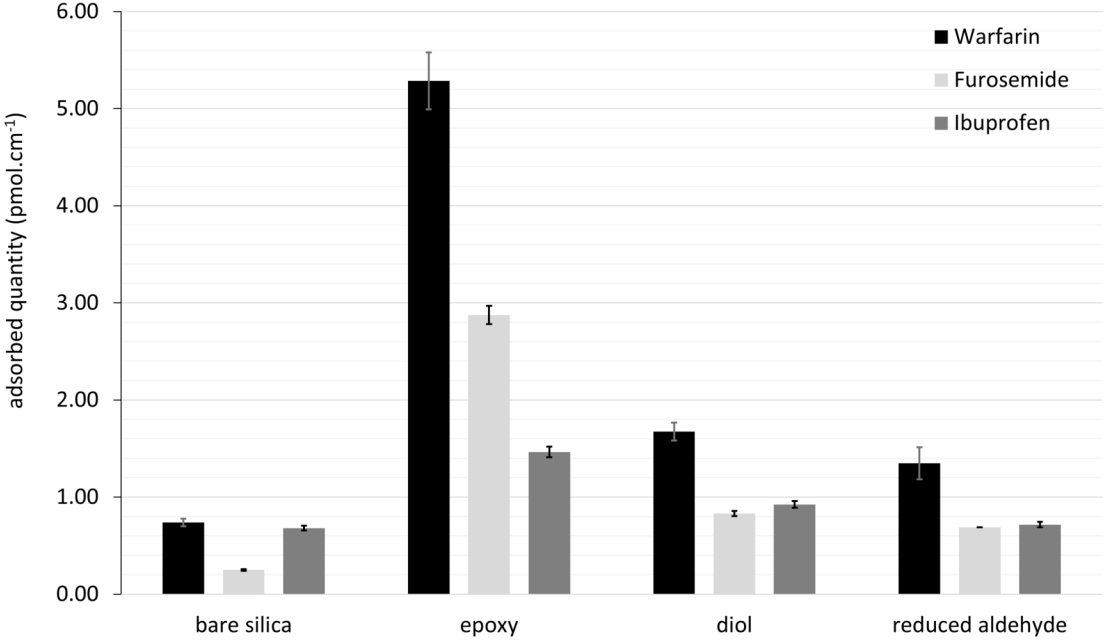
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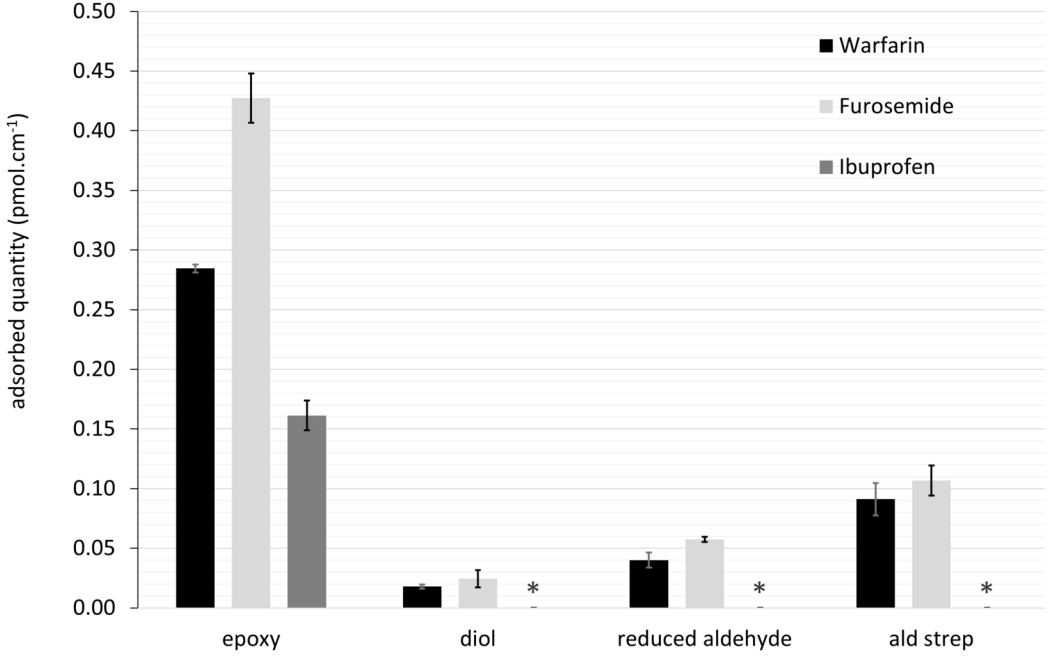
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Furosemide
Ibuprofen
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epoxy
diol
reduced aldehyde
ald strep