Site-directed immobilization of a genetically engineered **anti-methotrexate** antibody via an enzymatically introduced biotin label significantly increases the binding capacity of immunoaffinity columns

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ABSTRACT

In this study, the effect of random *versus* site-directed immobilization techniques on the performance of antibody-based HPLC columns was investigated using a single-domain camelid antibody (VHH) directed against methotrexate (MTX) as a model system. First, the high flow-through support material POROS-OH was activated with disuccinimidyl carbonate (DSC), and the VHH was bound in a random manner via amines located on the protein's surface. The resulting column was characterized by Frontal Affinity Chromatography (FAC). Then, two site-directed techniques were explored to increase column efficiency by immobilizing the antibody via its C-terminus, i.e., away from the antigen-binding site. In one approach, a tetra-lysine tail was added, and the antibody was immobilized onto DSC-activated POROS. In the second site-directed approach, the VHH was modified with the AviTag peptide, and a biotin-residue was enzymatically incorporated at the C-terminus **using the biotin ligase BirA**. The biotinylated antibody was subsequently immobilized onto NeutrAvidin-derivatized POROS. A comparison of the FAC analyses, which for all three columns showed excellent linearity ($R^2 > 0.999$), revealed that both site-directed approaches yield better results than the random immobilization; the by far highest efficiency, however, was determined for the immunoaffinity column based on AviTag-biotinylated antibody. **As proof of concept**, all three columns were evaluated for quantification of MTX **dissolved in phosphate buffered saline (PBS)**. Validation **using UV-detection** showed excellent linearity in the range of 0.04 –12 μM ($R^2 > 0.993$). The lower limit of detection (**LOD**) and lower limit of quantification (LLOQ) were found to be independent of the immobilization strategy and were 40 nM and 132 nM, respectively. The intra- and inter-day precision was below 11.6%, and accuracy was between 90.7% and 112%. To the best of our knowledge, this is the first report of the AviTag-system

in chromatography, and the first application of immunoaffinity chromatography for the analysis of MTX.

Keywords: AviTag; Camelid antibody; Immunoaffinity chromatography; Methotrexate; Oriented antibody immobilization

1. Introduction

It has long been known that oriented immobilization of antibodies onto chromatographic support materials can result in a considerably higher binding capacity of immunoaffinity columns than random coupling [1,2]. Typically, site-directed strategies for the conjugation of classically produced immunoglobulins target their Fc region (fragment crystallizable region), which represents the stem of the Y-shaped molecule [3] and is spatially separated from the antigen-binding sites. Common methods include the formation of covalent bonds between the carbohydrate chains in this area and the support material, or non-covalent binding of the immunoglobulin to immobilized antibody-binding proteins such as protein A and protein G [4-8]. Using a monoclonal antibody that stereoselectively recognizes L-amino acids as a model system, we showed that even higher column efficiency can be achieved if an antibody is first biotinylated at its C-terminus using carboxypeptidase Y, and then non-covalently bound to a streptavidin-derivatized support material [4]. Compared to the 39 nmoles obtained with random amine-coupling, this site-directed approach increased the number of available antigen-binding sites by a factor of almost three, namely to 109 nmoles.

Advancements in the production of genetically engineered proteins, including antibodies and antibody-fragments, over the last few decades have opened up new avenues for the incorporation of functionalities and tags for numerous applications, e.g., detection, purification and conjugation/immobilization purposes [9-12].

In 1990, Cronan reported the post-translational biotinylation of proteins containing amino acid sequences that serve as recognition sites for naturally occurring biotin ligases [13]. Nine years later, Beckett et al. [14] showed that the 14 amino acid long peptide GLNDIFEAQKIEWH is sufficient to achieve efficient biotinylation of fusion proteins using the *E. coli* biotin protein ligase BirA [15]. This sequence, extended by a C-terminal glutamate, has been commercialized under the name AviTag [16],

and has found **application** ranging from protein purification and the investigation of protein-protein interactions to the detection of antigens in enzyme-linked immunosorbent assays [17,18].

Recently, Trilling et al. utilized the AviTag/BirA technology to achieve site-directed immobilization of the variable domains of two camelid heavy-chain only antibodies (VHHs) onto streptavidin-derivatized surface plasmon resonance sensor-chips [19]. Utilizing several variants of the original antigens, foot-and-mouth disease virus and a 16 kDa heat-shock protein of *Mycobacterium tuberculosis*, respectively, they investigated the effect of antibody-orientation and several analyte properties on biosensor sensitivity. While directed immobilization consistently led to sensitivity increases, ranging from modest (**1- to 2-fold**) to significant (14-fold), they came to the conclusion that the effect is more prominent for low-affinity than for high-affinity systems.

VHHs represent the antigen-binding domains of a class of antibodies that was first discovered in 1993 by Hamers-Casterman et al. in the sera of camels and llamas [20]. These camelid antibodies are composed of only two heavy chains, which do not require light chains for their interaction with antigens, as their three complementarity determining regions (CDRs) are sufficient for target recognition. However, as in conventional antibodies, the antigen-binding site is located towards the N-terminus of the heavy chains. While the complete heavy chain-only antibody has a molecular weight of about 100 kDa, a VHH is significantly smaller with a MW of about 15 kDa. Since VHHs are readily produced and modified by genetic engineering techniques, expressed in good **yield**, and relatively robust and stable, they are particularly suited for biotechnological applications, e.g., as capture reagents in biosensors and affinity chromatography [21,22]. In a previous publication, we demonstrated that camelid single-domain antibodies can also be used for the immunochromatographic separation of small molecules [23]. A VHH directed against caffeine [24,25] was linked to the high-

flow through support material POROS using random amine-coupling and utilized for the separation of several methylxanthines by HPLC [23].

In the present investigation, we immobilized an anti-methotrexate VHH onto POROS using three different conjugation approaches, examined the binding capacity of the respective immunoaffinity columns utilizing FAC, and evaluated the columns for quantification of MTX.

Methotrexate, a structural analog of folate (Vitamin B₉), is a potent inhibitor of dihydrofolate reductase, an enzyme which reduces folate to its active form 5,6,7,8-tetrahydrofolate (THF; Fig.1).

Since THF is an essential cofactor of a number of enzymes participating in one-carbon transfer reactions in, e.g., the *de novo* biosynthesis of purines and thymidylate, MTX has found widespread use for the treatment of various types of cancer, autoimmune diseases, and the medical termination of pregnancy [26,27]. Typical doses of weekly administered MTX range from 30 to 50 mg/m², but much higher doses > 1 g/m² may help to, e.g., overcome drug resistance [28]. In routine practice, therapeutic drug monitoring is only used after high-dose MTX infusions to assess drug elimination and avert excessive toxicity due to delayed elimination, which is defined as plasma levels of MTX above 10, 1, and 0.1 µM after 24, 48, and 72 h after administration, respectively [29]. A number of techniques have been utilized to separate MTX and its metabolites/structural analogs by reverse-phase, ion-pairing or ion-exchange chromatography, with reported lower limits of detection ranging between 2 and 200 nM using ultraviolet detection [30].

2. Materials and methods

2.1. Chemicals and reagents

The expression vector for the (His)₆-tobacco etch virus (TEV) protease was kindly donated by Anthony Kossiakoff (University of Chicago, Chicago, IL). pET21-BirA [31] was a gift from Alice Ting (Addgene, Cambridge, MA; plasmid #20857). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Denville Scientific (Metuchen, NJ). Tris(hydroxymethyl)aminomethane (Tris), sodium phosphate monobasic, LB Broth, Miller (molecular genetics granular), ampicillin sodium salt, and magnesium acetate tetrahydrate were purchased from Fisher Bioreagents (Fair Lawn, NJ), while sodium acetate tetrahydrate, sodium phosphate dibasic, sodium chloride, and ethylenediamine tetraacetate (EDTA) were from Fisher Chemicals (Fair Lawn, NJ). Adenosine 5'-triphosphate disodium salt (ATP), aminopterin, benzoic acid, D-biotin, dimethyl sulfoxide (DMSO), disodium sulfate, and methotrexate were purchased from Sigma (St. Louis, MO). POROS 20 OH was obtained from Applied Biosystems (Grand Island, NY). *N,N'*-Disuccinimidyl carbonate (DSC) and dimethylaminopyridine (DMAP) were from Nova Biochem (La Jolla, CA). NeutrAvidin was obtained from Thermo Scientific (Rockford, IL). Water was purified using a MilliQ water purification system (Millipore, Bedford, MA). All chemicals and reagents were of the highest purity available (i.e., chemical purity was $\geq 98\%$) and used as received.

2.2. Instrumentation

Expressed VHH was purified on either a Bio-Rad BioLogic Duo Flow 40 or a Biologic LP FPLC system (Bio-Rad Life Sciences, Hercules, CA) using GE HisTrap nickel IMAC columns, a HiPrep 26/10 desalting column, and Sephadex-75 size exclusion column (GE Healthcare, Piscataway, NJ). Cell sonication was performed using a Sonifier 450 digital ultrasonic processor (Branson, Danbury, CT). Protein concentrations were determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE).

HPLC separations of methotrexate and analogs, as well as FAC analyses, were carried out on a system consisting of a Hitachi L-7100 pump with degasser, an L-7400 UV-detector equipped with a semimicro flow cell, and a D-7000 interface with System Manager V 4.0 software. Injections were performed using a Rheodyne 7725i injection valve (Hitachi, Naperville, IL). **Detection of analytes was performed based on their UV-absorbance at 300 nm.**

Isothermal titration calorimetry (ITC) experiments were conducted using a MicroCal VP isothermal titration calorimeter (MicroCal, LLC, Northampton, MA).

2.3. Production and purification of camelid anti-methotrexate VHH antibodies

All three antibodies employed in this study are variants of a previously produced anti-methotrexate VHH [32]. Kunkel mutagenesis [33] was used to introduce a histidine at position 36 to produce anti-MTX VHH M36H. Kunkel mutagenesis was then used to introduce a C-terminal tetra-lysine tail and a C-terminal AviTag into anti-MTX VHH M36H to produce anti-MTX M36H (cLys)₄ and anti-MTX VHH M36H cAviTag, respectively.

The pET-21a vector containing the respective anti-methotrexate (His)₆-Flag-Tev-VHH variant sequence was transformed into BL21(DE3) *E. coli* and selected on LB-Amp (100 µg/ml) plates. An initial 5 ml overnight culture grown at 37 °C was used to start a 50 ml subculture, followed by a final inoculation of a 1 l culture (1:100 dilution). Antibody expression was induced with 0.1 mM IPTG at mid-log phase (OD₆₀₀ ~0.6-0.8). The culture was grown overnight at 20 °C with shaking (235 rpm) and the cells were harvested by centrifugation (11,300×g for 20 min at 4 °C). The pellet was frozen at -20 °C and subsequently thawed and resuspended in 10 mM Tris, pH 8.0. Sonication was performed for five 30 s on/30 s off cycles. The lysed cells were centrifuged (22,700×g for 20 min at 4 °C), and the soluble fraction was purified on a 5 ml HisTrap HP column using a stepwise elution between 10 mM

and 500 mM imidazole in 50 mM disodium hydrogen phosphate/HCl, pH 8, and 500 mM NaCl. Following buffer exchange to Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 8.0), the (His)₆-TEV-tag was cleaved off using (His)₆-TEV protease (1 mg/20 mg protein for 24 h at 4°C in the presence of 10 mM EDTA). The VHH was separated from (His)₆-TEV and the cleaved (His)₆ peptide tag product by running over a HisTrap HP column, collecting the VHH-containing flow through, and then further purified by size exclusion chromatography on a Sephadex-75 column using TBS as mobile phase.

VHH containing the AviTag peptide was biotinylated for 2 hours at RT using 40 µM VHH, 300 nM BirA in 10 mM Tris, pH 8.0, containing 10 mM ATP, 50 µM D-biotin, 10 mM magnesium acetate, and 150 mM sodium chloride. Labelled VHH was then purified by size-exclusion chromatography on a Sephadex-75 column.

To ensure that the VHH variants were still able to interact with MTX following purification, their antigen-binding activity was confirmed by isothermal calorimetry (ITC) prior to their conjugation to solid phase material. The ITC cell contained 10 µM VHH in phosphate-buffered saline (PBS; 20 mM disodium hydrogen phosphate buffer and 150 mM sodium chloride, adjusted to pH 7.4) and 0.8% DMSO. The syringe contained 100 µM methotrexate in PBS, 0.8% DMSO. Twenty-eight injections of 10 µL each were performed at 25 °C with a stirring speed of 300 rpm.

2.4. Stationary phase and column preparation

A batch of POROS-OH sufficient to pack three columns was activated for two hours with DSC and DMAP following published procedures [23,34], and subsequently washed successively with dry cold acetone, 5% dioxane in acetic acid, methanol, and dry isopropanol. The activated support material,

which contained 26 μmol active sites/g as determined spectrophotometrically [34], was split into three equal parts of 2 g (wet weight) each, which were derivatized as follows:

1) “Random” – The DSC-activated support material was incubated with 8.7 mg (equivalent to about 630 nmoles of antigen-binding sites) of the anti-methotrexate antibody named anti-MTX M36H (at 1.5 mg/ml in PBS containing 125 mM disodium sulfate) overnight at 4°C under rotation, followed by extensive washing with 100 mM Tris/HCl, pH 8, and PBS, respectively, on a glass frit. Amount of VHH not bound to the support was estimated spectrophotometrically by determining the absorbance of the flow-through at 280 nm.

2) “Tetra-lysine” – DSC-activated support was incubated with 9.2 mg (equivalent to about 630 nmoles of antigen-binding sites) of anti-MTX M36H (cLys)₄ (at 1.5 mg/ml in PBS containing 125 mM disodium sulfate), and then processed as described for the “Random” support.

3) “AviTag” – DSC-activated support was incubated with 14 mg of NeutrAvidin (equivalent to about 630 nmoles of biotin-binding sites) overnight at 4 °C under rotation (at 10 mg/ml in PBS containing 125 mM disodium sulfate), followed by extensive washing with 100 mM Tris/HCl, pH 8, and PBS, respectively, on a glass frit. Then, the NeutrAvidin-derivatized support was incubated overnight at 4°C under rotation with 10 mg (at 1 mg/ml in PBS) of biotinylated anti-MTX VHH M36H cAviTag (equivalent to about 630 nmoles of antigen-binding sites), followed by washing with PBS on a glass frit.

It should be noted that the in-batch-activation and immobilization employed here does not allow for an accurate determination of the amount of protein actually bound to the support material, as it is impossible to assess how much was lost during the process, e.g., by adsorption to the walls of the reaction containers. However, FAC was used to evaluate the number of active binding sites on each support.

Each of the three VHH-derivatized support materials was slurry-packed at 160 psi into a stainless steel column (3.2 mm x 50 mm) using an Alltech Slurry Packer Model 1666 (Alltech, Deerfield, IL).

No leakage of antibody was detected during column equilibration. The columns were stored under azide-containing PBS at 4 °C only when not used for an extended period of time.

2.5. Chromatographic conditions

To confirm that the produced columns were capable of retaining methotrexate and purifying it from structural analogs, respectively, **methotrexate (at 1 μ M in PBS) was injected either alone or as a mixture with aminopterin and/or folate (at 0.5 μ M in PBS) in a volume of 20 μ l at RT and a flow rate of 1 ml/ml using PBS as mobile phase.** Bound analyte was eluted by switching to acetate buffer (20 mM sodium acetate, 150 mM sodium chloride), pH 4.0, followed by regenerating the columns with PBS. **Analytes were detected based on their UV-absorption at 300 nm.** The fastest baseline separations possible were obtained with the following protocol: 0-0.5 min PBS; 0.5-5.5 min acetate buffer; 5.5-9 min PBS.

For FAC analyses, columns were flushed at RT with methotrexate at concentrations ranging between 0.1 and 10 μ M at 0.4 ml/ml, followed by eluting bound analyte with acetate buffer, pH 4.0. These concentrations cover the range of about 1/10 to 10x the K_d for the interaction between the antibody and methotrexate as determined by ITC. Columns were subsequently regenerated with PBS. The 50% value of the maximum plateau was determined from the centroid of the first derivative of the breakthrough curve [35]. The retardation volume ($V-V_o$) was determined by subtracting the 50% value of benzoic acid, which was not retained on the columns, from the 50% value of the analyte. All data was determined at least in triplicate, and standard deviations were smaller than 3%. The number of available binding sites (B_t) and the dissociation constant (K_d) were then determined using the equation

$1/A_0(V-V_0)=K_d B_t \times 1/A_0 + 1/B_t$, where $[A]_0$ is the concentration of methotrexate, V is the volume at half saturation, and V_0 is the void volume. The intercept of the extrapolated straight line on the abscissa corresponds to $-1/K_d$, while the intercept on the ordinate corresponds to $1/B_t$. Furthermore, the slope of the line is equivalent to the ratio K_d/B_t . Thus, the total amount of immobilized active protein binding sites, B_t , and the dissociation constant, K_d , can easily be derived from the double-reciprocal plot using appropriate data evaluation programs [4,35].

2.6. Method validation

All three columns were evaluated for quantification of MTX dissolved in PBS (not containing other analytes), and the method was validated for linearity, accuracy, precision, LOD, and LLOQ.

A stock solution of MTX was prepared by dissolving 5 mg compound in 1 ml of DMSO, and samples were prepared by dilution with 10 mM PBS.

Accuracy was expressed as a percentage of the actual concentration divided by the known concentration. The actual concentration was determined using the linear regression curve of the calibration plot containing eight concentrations of MTX ranging from 0.04 to 12 μ M with five replications each. Precision was defined as percent coefficient of variance (CV, %) for each sample. Intra-day assays consisted of five replications of five concentrations (200 nM, 475 nM, 810 nM, 1.1 μ M and 4.74 μ M), while inter-day assays analyzed the consistency between injections performed on three different non-consecutive days. The LOD and LLOQ were determined as the concentrations resulting in a signal/noise-ratio of 3/1 and 10/1, respectively.

3. Results and Discussion

To demonstrate, for the first time, the utility of immunoaffinity stationary phases for separating MTX from structural analogs such as aminopterin and folate, we immobilized the single-domain camelid antibody anti-MTX M36H VHH onto the high-flow through support material POROS-OH. The antibody, in which a methionine in position 36 was mutated to a histidine, is a variant of a VHH that had been created originally by grafting the three hypervariable loops of an anti-MTX VHH antibody [36] onto the framework of an anti-RNase A VHH [32]. Using the X-ray crystallographic structure of the parent VHH [32], M36, due to its proximity to methotrexate, was identified as a position that could likely perturb methotrexate in a pH-dependent fashion [37]. M36H would be predicted to undergo an environmental change upon binding methotrexate, which may result in a change in its pK_a value that would produce a pH-dependent MTX binding event. While anti-MTX M36H VHH binds MTX at neutral pH values ($K_d \sim 1 \mu M$), the dissociation constant increases significantly under relatively mild acidic conditions, i.e., between pH 4 and 5.

Based on the structure of the parent VHH [32], anti-MTX M36H VHH contains five surface-exposed amino-groups, namely four ϵ -amines of lysine residues and the amino-terminal end of the protein (Fig. 2). Immobilization of the antibody in a random manner onto DSC-activated support via these functional groups resulted in a stationary phase that was capable of separating MTX from folate (data not shown) and its structural analog aminopterin (Fig. 3), which lacks the methyl-group at N^{10} (Fig. 1c). While binding of MTX was achieved using PBS at pH 7.4, the analyte readily eluted after switching to a sodium acetate buffer of pH 4. The elution conditions did not harm the structural integrity or antigen-binding activity of the antibody as the column performance did not significantly change after more than 1000 separations (data not shown).

The number of available binding sites and the apparent affinity of the VHH for methotrexate were determined by FAC. Flushing the column with the analyte at varying concentrations at a flow rate of

0.4 ml/min, elution profiles with fronts and plateaus typical for FAC were obtained (Fig. 4a). The elution volume V at each analyte concentration was determined from the centroid of the first derivative of the breakthrough curve, while V_o was obtained using the volume at half-maximal saturation of the elution front of benzoic acid, which was not retained on the column. A Lineweaver-Burk-type double-reciprocal plot of $1/[A]_o (V-V_o)$ as a function of $1/[A]_o$ gave a straight line as shown in Fig. 4b ($y = 0.185x + 0.126$; $R^2 = 0.999$). The amount of immobilized active binding sites, B_t , and the dissociation constant, K_d , were determined from the extrapolated intercepts on the ordinate and abscissa, respectively, and from the slope of the straight line. It was found that random immobilization of the VHH yielded about 8 nmoles of available antigen-binding sites with an apparent K_d of $1.4 \pm 0.2 \mu\text{M}$, a value comparable with and within experimental error of the one determined by ITC ($1.2 \pm 0.1 \mu\text{M}$).

In an attempt to improve the binding capacity of VHH-based anti-methotrexate columns, genetic engineering techniques were used to introduce a four-residue long tail of lysines at the antibody's C-terminus. It was expected that the larger number of amines in this region (Fig. 2) would increase the probability of immobilizing the antibody in a way that the antigen-binding site – which is located at the opposite, i.e., N-terminal side of the protein – would be accessible for interaction with the analyte. The addition of lysine residues in a desired area of a protein's surface by genetic engineering was previously utilized to facilitate site-directed immobilization, improve stability, and increase the activity of some enzymes [38,39]. Using FAC analysis (Fig. 4c and 4d; $y = 0.097x + 0.0799$; $R^2 = 0.999$), we found that immobilization of the tetra-lysine containing VHH onto DSC-activated POROS led to only a slight increase in the number of available **antigen**-binding sites to 10 nmoles, which displayed an apparent affinity comparable to that determined by ITC ($1.0 \pm 0.2 \mu\text{M}$ vs. $1.3 \pm 0.1 \mu\text{M}$).

It should be noted that immobilization of proteins via surface-exposed amines using amine-reactive chemistry is, in reality, not a truly random process, as the reactivity of individual groups may depend

on steric factors influencing their availability, and their electrostatic environment. Since the N-terminal amino group, generally, has a pK_a of 7.6-8, it is – compared to the ϵ -amines (pK_a of 9.3-9.5) – more likely to be deprotonated at pH values typically employed with N-reactive chemistries, which makes it a better nucleophile [40]. However, a direct comparison of the methotrexate elution profiles obtained with the anti-MTX M36H-based column and the one prepared with the tetra-lysine variant shows a clear increase in retention time on the latter (Fig. 5).

The strong interaction (K_d ca. 10^{-15} M) between biotin and the tetrameric protein avidin or its analogs (such as streptavidin and NeutrAvidin) has found countless applications in biotechnology, e.g., for the purification of biomolecules by affinity chromatography [41]. Here, we utilized the avidin-biotin system to immobilize a biotinylated variant of anti-MTX M36H non-covalently onto the chromatographic support material. In an attempt to obtain a number of biotin-binding sites sufficient to immobilize the same amount of VHH as in the other two columns, we incubated DSC-activated POROS with 14 mg of NeutrAvidin, a deglycosylated form of avidin, but found that the support was already saturated with half the amount. Rather than employing random, chemical labelling of the VHH using one of the many available biotinylation reagents, we decided to introduce the AviTag at the C-terminus and perform site-specific enzymatic biotinylation of the lysine contained in this 15 amino acid long sequence with BirA. Following incubation of the NeutrAvidin-derivatized support with the labelled VHH, FAC was performed with the resulting column using MTX as analyte. Evaluation of the obtained elution profiles at varying analyte concentrations (Fig. 4e) using a double-reciprocal plot (Fig. 4f; $y = 0.028x + 0.024$; $R^2 = 0.999$) revealed a 4- to 5-fold increase in the number of available antigen-binding sites (40 nmoles) compared to the other two columns. The apparent affinity was determined to be comparable to that measured by ITC (1.2 ± 0.1 μ M vs. 1.5 ± 0.2 μ M). Taking into account that, compared to the other columns, only about half the amount of VHH could be immobilized due to the

shortage of biotin-binding sites on the NeutrAvidin-derivatized support, one can argue that site-directed immobilization via a C-terminal biotin could result in as much as a 8- to 10-fold increase in binding capacity. The effect of the greater number of binding sites on the retention time is particularly evident in the overlay plot that compares the elution profile of methotrexate on all three columns (Fig. 5).

As proof of concept, all three columns were evaluated for quantification of MTX dissolved in PBS.

The linearity of the method was evaluated to demonstrate a proportional relationship of response versus sample concentration over the working range. Calibration curves were prepared for all columns using 8 different concentrations. Regression analysis showed that all curves obtained by plotting the area of the peak (y) versus the MTX concentration (x) are linear in the concentration range between 0.04 and 12 μ M, which covers the range relevant for therapeutic drug monitoring [29].

The following regression equations were determined – Random column: $y = 27.74x - 2.26$ ($R^2 = 0.993$); Tetra-lysine column: $y = 26.64x - 1.52$ ($R^2 = 0.999$); AviTag column: $y = 24.77x - 0.75$ ($R^2 = 0.999$).

Accuracy and precision of the method using the three different columns were investigated using five replicate determinations of five concentrations on the same day (intra-day), and a total of three non-consecutive days (inter-day). Results are shown in Table 1. Intra- and inter-day precision values were less than 11.6% CV for the random column, 7.9% CV for the tetra-lysine column, and 10.7% for the AviTag-column.

Intra- and inter-day accuracy values were 12.7% or lower for all columns. The standard acceptance criterion for each back calculated concentration was a 15% deviation from the normal value.

It is noteworthy that the LOD and LLOQ were not dependent on the immobilization strategy and were found to be 40 nM and 132 nM, respectively. This, however, is not surprising as it is reasonable to

assume that, in a classical affinity chromatography system with separate binding and elution steps, a higher number of available binding sites resulting from oriented vs. random immobilization should primarily affect the maximum amount of analyte that can be retained on a column, but have a lesser or no discernible effect at lower analyte concentrations.

4. Conclusions

The ability to generate immunoaffinity capture reagents utilizing molecular biological techniques offers a variety of advantages compared to traditional, animal-based production methods. Specificity and affinity of such antibodies or antigen-binding fragments, which can be expressed in large quantities and at comparatively low cost in, e.g., bacterial cell cultures, may be modulated using mutagenesis techniques based on rational design or library approaches. In addition, unnatural amino acids with unique reactive chemical properties [42] or amino acid sequences that serve as recognition sites for other capture reagents or enzymes capable of adding labels [9-12] can be incorporated at basically any position in the protein. Here, we modified an anti-methotrexate VHH at its C-terminus to achieve site-directed immobilization onto chromatographic support materials via a tetra-lysine tail or an enzymatically biotinylated AviTag, and compared the resulting columns with one that was produced using random amine-coupling. While both site-directed approaches yielded a higher number of active binding sites, the effect was particularly pronounced for the column produced using the AviTag/BirA approach. **To the best of our knowledge, this constitutes the first reported use of AviTag/BirA-based site-directed biotinylation for chromatographic separations.** As the AviTag can readily be incorporated into basically any genetically engineered protein, this method should have wide general utility and be of great value for other affinity chromatography systems. The increased number of binding sites that

can result from the oriented immobilization of capture reagents should be of particular use for preparative applications.

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Figure Legends

Fig. 1 Chemical structures of a) methotrexate, b) folate, and c) aminopterin.

Fig. 2 Ribbon structure of the anti-methotrexate VHH in complex with methotrexate (green).

The N-terminus as well as the side chains of the intra-chain lysines are shown in red, while the C-terminus is highlighted in yellow.

Fig. 3 Chromatographic separation of aminopterin (first peak) and methotrexate (second peak) at 1 ml/min on a column (3.2 mm x 50 mm) produced by randomly immobilizing anti-MTX M36H onto DSC-activated support material. The injection volume was 20 μ l, with aminopterin at 0.5 μ M and methotrexate at 1 μ M. The baseline disturbance indicates the time when the mobile phase changed from PBS, pH 7.4, to acetate buffer pH 4, for elution of methotrexate.

Fig. 4 FAC elution profiles and plots of $1/[A]_0(V-V_0)$ as a function of $1/[A]_0$ for methotrexate on columns obtained by immobilizing anti-MTX M36H randomly (panels a and b), by immobilizing a variant of the VHH via a C-terminal tetra-lysine tail (panels c and d), or by immobilizing a variant that contains a C-terminal, biotinylated AviTag onto NeutrAvidin-derivatized support (panels e and f). V_0 was determined using benzoic acid.

Fig. 5 Overlay plots of FAC elution profiles obtained with 1 μ M methotrexate on columns created by immobilizing a) anti-MTX M36H randomly, b) a variant of the VHH via a C-terminal tetra-lysine tail, and c) a variant that contains a C-terminal, biotinylated AviTag onto NeutrAvidin-derivatized support.

Table 1

Accuracy and precision for the determination of MTX in buffer

Concentration	Random Column Precision (CV, %)		Tetra-lysine Column Precision (CV, %)		AviTag Column Precision (CV, %)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
200 nM	1.8	11.6	4.7	7.9	2.9	10.7
475 nM	2.9	4.9	3.4	6.2	1.6	7.2
810 nM	1.9	4.9	4.2	6.4	1.5	2.7
1.1 μ M	1.8	5.6	2.5	5.5	3.6	5.9
4.74 μ M	0.5	4.6	2.8	5.9	3.7	4

Concentration	Random Column Accuracy (%)		Tetra-lysine Column Accuracy (%)		AviTag Column Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
200 nM	99.8	93.1	99.0	99.2	101.6	101.2
475 nM	100.2	104.6	92.4	90.9	104.3	95.6
810 nM	109.2	112.8	97.0	98.3	102.6	103.9
1.1 μ M	97.9	106.5	99.0	94.7	99.1	100.0
4.74 μ M	92.0	90.7	101.0	96.4	100.4	102.0

Fig. 1

a)

b)

c)

Fig. 2

Fig. 3

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

Fig. 4e

Fig. 4f

Fig. 5