Journal of Chromatography B, 1021 (2016) 114–121

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Kaitlynn R. Davenport[1], Christopher A. Smith[1], Heike Hofstetter[2], James R. Horn, Oliver Hofstetter*

Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, IL 60115-2862, USA

ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 11 January 2016
Accepted 12 January 2016
Available online 15 January 2016

Keywords:
AviTag
Camelid antibody
Immunoaffinity chromatography
Methotrexate
Oriented antibody immobilization

ABSTRACT

In this study, the effect of random vs. 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.

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

* Corresponding author. Fax: +1 815 753 4802.
E-mail address: ohofst@niu.edu (O. Hofstetter).
[1] Both authors contributed equally to this work.
[2] Permanent address: Department of Chemistry, University of Wisconsin—Madison, 1101 University Avenue, Madison, WI 53706, USA.

http://dx.doi.org/10.1016/j.jchromb.2016.01.021
1570-0232/

39 nmol 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 nmol.

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 *Escherichia 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 B_9), 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

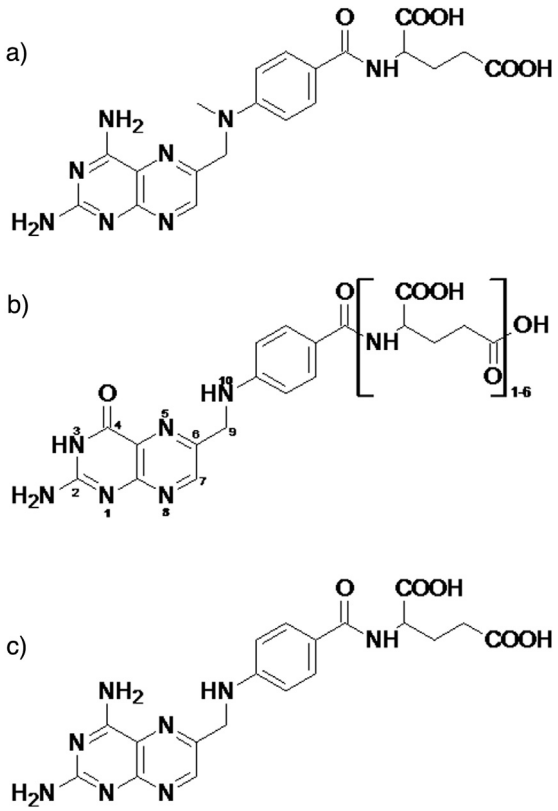

Fig. 1. Chemical structures of a) methotrexate, b) folate, and c) aminopterin.

to 50 mg/m^2, but much higher doses >1 g/m^2 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 following 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