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Studies on the purification of antibody fragments

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ABSTRACT

Antigen-binding fragments (Fabs) are becoming increasingly prevalent as an alternative to full-length monoclonal antibodies. In this work, the digestion of a mixture of different human antibodies isotypes was optimized in terms of different parameters, including the concentration of papain and cysteine, and the digestion time. The recovery of the Fab fragments was subsequently evaluated by designing four different downstream purification schemes, where the use of affinity chromatography (protein A and L) was the most efficient to isolate the Fab fragments after IgG digestion.

A rapid screening of optimal conditions for the binding of pure Fab fragments to four non-affinity chromatography resins was then performed using micro-columns fabricated in a PDMS microfluidic device. The goal of these studies was to screen and evaluate the performance of two cation exchange (carboxymethyl and heparin) and two multimodal (Capto MMC and phenyl boronate) ligands for the capture of Fabs under a wide range of pH (5–9) and conductivity (up to 8 mS/cm) conditions. Multimodal resins showed the best results in binding Fab fragments, particularly at pH 5, well below the range of isoelectric points of the target Fab molecules. In addition, these resins demonstrated to have a salt-tolerant behaviour, meaning that the binding of Fab fragments was not significantly impacted when the conductivity of the adsorption buffer was increased to near-physiological conditions (8 mS/cm).

1. Introduction

Monoclonal antibodies (mAbs) are playing a central role in the biopharmaceutical industry [1], with smaller antibody fragments, such as antigen-binding fragments (Fabs), currently emerging as viable alternatives to whole mAbs, with pharmacologic properties optimized for specific applications [2,3]. The increased interest in these molecules can be assessed not only by the number of Fab fragments already on the market, but also by the number of fragments currently in clinical trials [4,5]. Fabs are simpler and smaller structures that offer several advantages over intact antibodies, such as potentially higher sensitivity in antigen detection, reduced nonspecific binding derived from absent Fc interactions, lower immunogenicity and higher penetration in tissues [6].

Fab products already approved by regulatory agencies (FDA, EMA) are produced by two different routes: either through proteolytic digestion of full-length mAbs produced by animal cell culture and partially purified by protein A chromatography (e.g., abciximab, ReoPro®, Centocor; sulesomab, LeukoScan®, Immunomedics GmbH), or through microbial fermentation of *E. coli* cells transformed with Fab encoding genes (certolizumab pegol, Cimzia®, UCB S.A.; ranibizumab, Lucentis®, Genentech, Inc.) [7]. Regardless of the production route, purification of Fab fragments always requires extensive optimization, since the traditional mAbs purification platform cannot be applied as it is based on the affinity interaction between the protein A ligand and the antibodies' crystallisable fragment (Fc)-region and Fabs lack the Fc fragment. Currently, there are affinity ligands, such as protein L from *Peptostreptococcus magnus* that were developed to bind this type of fragments, however, those are specific only to kappa light chain Fab fragments. There is still no universal method to bind all the types of Fabs, including lambda light chain Fab fragments [8]. Other frequently employed strategies to purify Fab fragments are based on the use of general chromatography techniques, such as ion exchange [9], size-exclusion [10], and multimodal chromatography [11].

Since there is no established platform to purify Fab fragments, it is essential to develop effective high-throughput process development

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methods to perform a rapid evaluation of binding conditions of these molecules to existing or newly developed chromatography ligands. Current techniques for performing these studies typically include the use of multi-well plates, micropipette tips and mini-columns operated in automated liquid handling stations [12,13], which still require laborious operation and expensive instrumentation. Micro-columns fabricated in a microfluidic platform are an alternative approach to expedite process development that allows the optimization of chromatographic conditions, with minimal amounts of mAb molecules (μg-range) and other reagents (μL-range), whilst delivering results in a few minutes [14].

As Fab products currently on the market are either produced by enzymatic digestion of full-length mAbs or directly produced through microbial cell fermentation, the present work has been divided in two main sections. The first section is focused on Fabs produced by enzymatic digestion and aims at revisiting conventional antibody fragment production and purification techniques. In order to design a digestion protocol that is able to efficiently cleave different antibodies, different variables (digestion time, amount of papain and cysteine) were studied and tuned. After establishing the digestion protocol, four different downstream schemes were designed, using protein A, protein L and centrifugal ultrafilters, in order to isolate the Fab fragments produced in the digestion protocol. The second section pictures the purification of recombinant Fabs and aims at assessing different capture steps using a rapid and innovative screening methodology of binding conditions to alternative non-affinity chromatography resins anchored on a microfluidic platform. Four chromatography resins – two cation exchange ligands (carboxymethyl and heparin) and two multimodal ligands (Capto MMC and phenyl boronate) – were assessed for their ability to efficiently bind fluorescently-labelled Fab fragments over a wide range of pH (5 – 9) and conductivity (up to 8 mS/cm) conditions. These studies were performed in 210 nL micro-columns, fabricated in a polydimethylsiloxane (PDMS) microfluidic device. With the advent of microfluidics in the biotechnology paradigm, it is important to evaluate their applicability. Herein we tested the use of this approach to access the best working conditions for a downstream process of a specific protein in a novel and high-throughput manner. Fab fragments, as a class of emerging proteins, were used as model, and traditional chromatographic processes (cation exchange) were directly compared with new and less explored processes (multimodal).

2. Materials and methods

2.1. Chemicals and biologicals

Tris (hydroxymethyl)aminomethane (Tris), NaCl, citric Acid, D-sorbitol, Na_2CO_3, $NaHCO_3$, EDTA, L-cysteine, iodoacetamide and papain (≥10 units/mg protein) were obtained from Sigma Aldrich (St. Louis, MO/USA). NaH_2PO_4, K_2HPO_4 and KH_2PO_4 were purchased from Panreac Quimica Sau (Barcelona, Spain). Sodium acetate was obtained from Merck (Darmstadt, Germany). Acetic acid 100% (AnalaR Normapur®) was purchased from VWR BDH Prolabo (Radnor, PA/USA). Sodium dodecyl sulfate (SDS) and glycine were obtained from Bio-Rad (Hercules, CA, USA). Water used in all experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA/USA). Human immunoglobulin G (IgG) for therapeutic administration (product name: Gammanorm®) was obtained from Octapharma (Lachen, Switzerland), as a 165 mg/mL solution.

2.2. Chromatographic resins and filtration devices

HiTrap™ Protein L and HiTrap™ Protein A HP were purchased as pre-packed 5 mL columns from GE Healthcare (Uppsala, Sweden). Carboxymethyl Sepharose™ Fast Flow, Capto™ MMC and HiTrap™ Heparin Sepharose™ HP were purchased as bulk resins, also from GE Healthcare. Aminophenylboronate P6XL bulk resin was purchased from ProMetic Life Sciences Inc. (Cambridge, UK). Amicon® Ultra-15 centrifugal filter units (NMWL of 10 kDa) were purchased from Merck Millipore.

2.3. Labelling of human IgG

IgG molecules present in the Gammanorm® mixture were conjugated before digestion to the amine-reactive dye Alexa Fluor® 430 (A430) NHS ester, obtained from Thermo Fisher Scientific, whose maximum excitation and emission wavelengths are 430 nm and 545 nm, respectively. The IgG mixture was first diluted in 0.1 M sodium bicarbonate buffer to a concentration of 20 mg/mL and it was added to the reactive dye solution in a volume ratio of 4:1. The reaction was incubated for one hour in the dark at room temperature. The non-conjugated dye was then removed in a series of 10 diafiltration steps with phosphate buffer saline (PBS, 0.01 M phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4) using Amicon® Ultra-0.5 centrifugal filter units (NMWL of 10 kDa), centrifuged at 14,000*g* for 10 min, until a clear permeate was obtained.

2.4. Digestion protocols

The digestion protocol selected for this study was adapted from that reported in literature by Andrew et al. [15]. To evaluate the effect of digestion time and papain concentration the following conditions were selected: 2 g/L of IgG, 0.02 M of cysteine and 0.02 M of EDTA. The time of digestion was varied (4, 6, 8, and 24 h) and for each digestion time, three concentrations of papain were tested (0.01, 0.02 and 0.1 mg/mL). The digestion occurred at 37 °C. To stop the digestion, a solution of 0.3 M of iodoacetamide was added to the digestion mixture to a final concentration of 0.03 M. To study the effect of cysteine, a concentration of 0.02 mg/mL of papain and a digestion time of 8 h were chosen. The tested concentrations of cysteine were: 0, 0.01, 0.02 and 0.05 M.

To produce the Fab fragments for the downstream processes (DSP), the same protocol was followed and a digestion volume of 40 mL was processed. The labelled Fab fragments used in the microfluidic experiments were also produced using this digestion protocol, but a mixture of Alexa 430 labelled IgG at a concentration of 2 g/L was digested.

2.5. Chromatographic runs

All chromatographic experiments were performed in an ÄKTA™ Purifier 10 system (GE Healthcare). In all chromatographic runs, the conductivity, pH, and UV absorbance at 280 nm were continuously monitored. The data was acquired and processed by the software Unicorn 5.1. The flow-through and elution fractions were collected on a Fraction Collector Frac-950 (GE Healthcare).

2.6. Downstream processing

Four different DSP sequences were evaluated for the isolation of Fab fragments from the digestion mixture. DSP 1 included a protein A step followed by a protein L step; DSP 2 included a diafiltration step before purification by protein L; DSP 3 included a protein L step followed by a protein A step; and DSP 4 included a protein A step followed by concentration.

In the chromatographic runs (protein A or protein L), 2 mL of sample were injected, either from the digestion mixture or collected pools. Adsorption occurred at pH 7.4 using a 20 mM sodium phosphate buffer with 150 mM NaCl; and elution was triggered by decreasing the pH to 2.5 using 0.1 M citrate buffer. In all runs, both flow-through and elution peaks were collected until the UV 280 absorbance reached the baseline. All elution fractions were neutralized with 3 M Tris pH 9. Regarding DSP 2, an initial ultrafiltration/diafiltration was performed in a 10 kDa centrifugal filter, in which 3 mL of digestion mixture was 4× diafiltered against PBS. For the DSP 4, the protein A flow-through