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Dual fluorescence confocal imaging of the accessibility and binding of $F(ab')_2$ to an EBA resin with various immobilised antigen densities

Rihab Boushaba *, Clemens F Kaminski, Nigel KH Slater

Department of Chemical Engineering, University of Cambridge, Pembroke Street, Cambridge CB2 3RA, UK

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Abstract

Dual fluorescence confocal laser scanning microscopy has been used to visualise the binding of a fluorescently labelled polyclonal ovine anti-fluorescein $F(ab')_2$ antibody to immobilised fluorescein. The fluorescent ligand was immobilised on a Streamline quartz base agarose matrix; a resin used industrially for expanded bed chromatography, using two different fluorescein initial concentrations in order to obtain two batches of immunogen-affinity adsorbent with different immobilised ligand densities. The fluorescein specific $F(ab')_2$ were purified from anti-fluorescein serum pepsin digest by adsorption on immobilised antigen chromatographic resin, followed by conjugation to the fluorescent probe Alexa Fluor 660. The dual fluorescence signals from the immobilised antigen and the immuno-specific $F(ab')_2$ were used to map the progressive depth of the bound $F(ab')_2$ layer within individual adsorbent beads. In addition, the labelled anti-fluorescein $F(ab')_2$ was diluted to identical antigen binding activity concentrations in crude serum digest and in blank buffer and the resulting fluorescence intensity profiles were comparatively assessed for any detectable differences in binding patterns that might be caused by processing the more complex mixture of crude serum digests. It was observed that the relative immobilised ligand utilisation was higher when using the immuno-adsorbent with lower immobilised antigen density. Furthermore, the progression of the adsorbed $F(ab')_2$ front inside the immuno-adsorbent beads displayed closer agreement with the postulates of the shrinking core mechanism (SCM) when the immuno-adsorbent with lower immobilised antigen was used. The confocal images did not reveal any differences between the depth of the adsorption fronts of crude serum digest and pre-purified $F(ab')_2$ samples.

Keywords: Confocal scanning laser microscopy; $F(ab')_2$; Fluorescein; Immuno-affinity chromatography; Adsorbent; CSLM

1. Introduction

The specificity shown by polyclonal IgG's and their active Fab or $F(ab')_2$ fragments towards their corresponding antigens makes them ideal for many therapeutic applications [1]. Purification of $F(ab')_2$ fragments may be achieved by precipitation (ammonium sulphate or caprylic acid [2]) or chromatographic methods [3–5]. Several ways have been proposed to improve the purity and neutralising efficiency of $F(ab')_2$ fragments and to increase their safety [5,7]. However, many of these have resulted in minor improvements in $F(ab')_2$ quality and higher cost of production due to their reliance upon chromatography [8].

Antigen immuno-affinity chromatography is one way of ensuring an antibody product of high specific activity, as it is highly selective towards the sub-population of antibodies directed towards the antigen of interest. The possible advantages of the use of affinity ligands in expanded bed adsorption (EBA) have been reported by a number of authors; in particular, the combination of EBA as a direct recovery operation with the enhanced selectivity of affinity ligands may prove in some cases to be the most efficient method for the selective capture of the target product from complex feedstocks [9]. Streamline matrices were developed by Amersham Biotech with the purpose of allowing the formation of stable fluidised beds at high operating flow velocities. Streamline beads are inter-dispersed with inert quartz particles in order to increase the settling velocity of the matrix and increase its mechanical rigidity. These quartz particles introduce a higher degree of non-homogeneity compared with regular chromatographic adsorbents such as Sepharose.

In the present study, a dual fluorescence antibody-antigen system was used in order to visualise the accessibility of Alexa Fluor 660-labelled anti-fluorescein antibody fragments $F(ab')_2$ to

* Corresponding author.
E-mail address: r.boushaba@ucl.ac.uk (R. Boushaba).

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immobilised fluorescein inside epoxy-activated agarose Streamline beads by means of confocal microscopy. The fluorescence intensity profiles of immuno-affinity adsorbents carrying two different immobilised ligand loadings were compared in order to develop insights on the spatial distribution of the bound anti-fluorescein $F(ab')_2$ fragments in the two cases. Confocal scanning laser microscopy has been demonstrated by several published studies to be a useful technique for visualising binding events inside chromatographic matrices due to its high resolution characteristic [10]. However, the majority of the studies achieved on chromatographic matrices have centred on homogeneous gel phase adsorbents such as Sepharose [10]; which are more useful for packed bed chromatography operations. Less homogeneous adsorbent such as Streamline matrices are more challenging to characterise using confocal microscopy, however the high resolution power of confocal microscopy might still be used to compare the binding of a target protein to various batches of Streamline adsorbent containing different immobilised ligand loadings. To achieve this, we have used the fluorescein/anti-fluorescein $F(ab')_2$ system since it allows the visualisation of the binding process via two independent fluorescence processes: (i) fluorescence quenching of fluorescein resulting from binding of anti-fluorescein $F(ab')_2$ to the immuno-affinity adsorbent and (ii) fluorescence of the bound Alexa Fluor 660-conjugated anti-fluorescein $F(ab')_2$. This dual fluorescence detection method allowed the visualisation of immobilised ligand usage relative to the immobilised ligand density on the beads by imaging two types of immuno-affinity adsorbents having different immobilised fluorescein concentrations.

This work followed on from previous studies which were concerned with the assessment of disposable process technology as a less expensive alternative to traditional packed bed chromatography, for the manufacture of therapeutic $F(ab')_2$ fragments [11]. Magnetisable beads were used to synthesise the chromatographic adsorbent, which was subsequently introduced aseptically into disposable bio-processing bags containing the $F(ab')_2$-rich serum digest (resulting from a pepsin proteolysis step). The purification of $F(ab')_2$ was thus performed in batch-mode, with the separation of the solid phase being achieved by the application of a magnetic field in order to rapidly sediment the adsorbent beads. Streamline adsorbent was used in these studies because its design makes it inherently suited for applications where good fluidisation properties, mechanical rigidity and rapid settling velocities are essential; such as in the case of bioprocessing in disposable bags.The study described herein aimed at using confocal imaging in order to gain further insights on the binding characteristics of the $F(ab')_2$ under these batch purification conditions and therefore finite batch experiments were carried out as opposed to the more traditional packed bed configuration.

2. Materials and methods

2.1. Materials

Streamline agarose quartz matrix, PD-10 Sephadex G-25 columns and high resolution Superose 12 10/30 HR column for size exclusion chromatography were purchased from GE Healthcare (formerly Amersham Biosciences) (Amersham, UK). FLEXBOY® pre-sterilised, disposable bio-processing bags were a generous gift from Stedim (Aubagne, France). Ultra-filtration membranes Vivaflow 50 were purchased from Sartorius Ltd. (Epsom, UK). Alexa Fluor 660 succinimidyl ester and Fluorescein-5-thiosemicarbazide were purchased from Invitrogen (formerly Molecular Probes) (Leiden, Netherlands). Sheep anti-fluorescein serum and anti-fluorescein-free serum were supplied by Micropharm Ltd. (Emlyn, UK). Pepsin and all other reagents were obtained from Sigma–Aldrich Company Ltd. (Gillingham, UK).

2.2. Instrumentation

Confocal microscopy analysis was performed with an Olympus/FluoView FV300 laser scanning microscope (Olympus, UK) equipped with a multi-line Argon ion laser and red and green Helium neon lasers as excitation sources. Up to 3 detection channels could be acquired simultaneously. Image acquisition and analysis were performed using the built-in Olympus Fluoview software. Each image was the result of an overlay of the images obtained in the two fluorescence channels corresponding to fluorescein (ligand) emission and Alexa Fluor 660 emission ($F(ab')_2$).The detection settings were kept constant for both batches of adsorbents to allow direct comparison to be made between the depth of penetration of the labelled anti-fluorescein $F(ab')_2$ in the case of high immobilised ligand load and low immobilised ligand load. Therefore, only the fluorescence emitted by the Alxa Fluor 660 dye was used to estimate the penetration depths. The fluorescence of the immobilised ligand was only used to visualise the immobilised ligand distribution within the adsorbent particles.

2.3. Preparation of immuno-affinity adsorbent

Agarose Streamline quartz base matrix was epoxide activated by reaction with oxirane 1,4-butanediol diglycidyl ether in 2:1 (v/v); according to the method described by Sundberg and Porath [12]. Activated agarose beads were washed with distilled water and subsequently reacted with solutions of an amine-derivative analogue of fluorescein (fluorescein-5-thiosemicarbazide). Fluorescein-5-thiosemicarbazide was used in solutions of 6.25 or 0.625 mM concentrations, prepared in 0.5 M sodium carbonate pH 11.0. Throughout the manuscript, fluorescein-5-thiosemicarbazide will be referred to as fluorescein for simplicity. The mixtures were incubated at 37 °C for 18 h. Blocking of unreacted active sites was achieved by adding 2 volumes of 1 M ethanolamine pH 10.0 and incubating overnight at 25 °C. The adsorbent was washed with 0.5 M sodium carbonate + 0.15 M NaCl, then with 50 mM glycine-HCl, pH 2.5 and finally with 10 mM sodium phosphate + 0.1 M NaCl, pH 8.0. The extent of fluorescein immobilisation was determined from the absorbance of the supernatant solutions at 491 nm. Two sets of immuno-affinity adsorbents were synthesised in this way, a high ligand loading adsorbent with 8.2 μmol immobilised fluorescein/mL beads and a low ligand loading adsorbent with 0.92 μmol immobilised fluorescein/mL beads.

2.4. Preparation of anti-fluorescein $F(ab')_2$ by pepsin digestion and immuno-affinity

Whole hyperimmune serum from sheep was diluted in 2 volumes of 20 mM glycine-HCl and the pH adjusted to pH 3.5 with 1 M HCl. Pepsin lyophilised powder was added at a ratio of 1:25 mg pepsin/mg protein, where the protein content of serum was determined with the Bradford assay. The mixture was incubated at 37 °C for 16 h, after which the proteolysis reaction was terminated by the addition of 1 M Tris and raising the pH to 7.0; at which pH pepsin is irreversibly inactivated. The digestion efficiency was visualised using SDS-PAGE of digested serum as compared with whole undigested serum and the results were published elsewhere [13]. The digest was filtered and diluted with 2 volumes of 10 mM sodium phosphate + 0.1 M NaCl, pH 7.0. A volume of 2 L of this serum digest solution was pumped into a 5 L capacity FLEXBOY® pre-sterilised bag and 45 mL immuno-adsorbent with 0.92 μmoles immobilised fluorescein/mL beads were injected. The batch mixture was incubated for 2 h with gentle mixing, after which the supernatant solution was filtered and the immuno-adsorbent beads were washed with sodium phosphate buffer until no detectable absorbance at 280 was noted on spectrophotometer readings of the wash supernatant. Elution of bound $F(ab')_2$ was carried out by the addition of