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Micropreparative ligand fishing with a cuvette-based optical mirror resonance biosensor

B. Catimel, J. Weinstock, M. Nerrie, T. Domagala, E.C. Nice*

Ludwig Institute for Cancer Research, Melbourne Tumour Biology Branch, Royal Melbourne Hospital, P.O. Box 2008, Parkville, Victoria 3050, Australia

Abstract

We have previously demonstrated the role of an optical biosensor (BIAcore 2000) as a specific detector to monitor chromatographic fractions during the purification and characterisation of ligands for orphan biomolecules. We have now extended this application to perform micropreparative ligand fishing directly on the sensor surface using an automated cuvette-based optical biosensor (Iasys Auto+) equipped with a high-capacity carboxymethyldextran surface (surface area 16 mm^2). Using a $F(ab)'_2$ fragment of the A33 monoclonal antibody as bait, we have recovered microgram quantities of essentially homogeneous A33 ligand from the sensor surface in a form suitable for subsequent sensitive and specific down stream analysis (micropreparative HPLC, sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blotting). The design of the cuvette-based system facilitates recovery of desorbed material from the constrained workspace in small volumes at high concentration. The use of on-surface detection allows the surface viability to be continuously monitored and permits direct quantitation of both bound and recovered material. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Interdisciplinary studies in the fields of chemistry, electronics and biology have led to the development of many novel sensor surfaces and detection systems [1–4]. This has resulted in the commercial availability of a number of multifunction instrumental biosensors, which are rapidly becoming a major tool in the field of biomedical research. These systems are now widely used to study biomolecular interactions between proteins and peptides, DNA, lipids, carbohydrates, drugs and more recently cells, and are suitable for a range of research applications including structure/function studies, epitope mapping, kinetic and equilibrium binding analysis, ligand searching and specific monitoring of chromatographic fractions [5].

The most widely used instrumental biosensors to date have been the BIAcore (BIAcore X, 1000, 2000 and 3000) range (Biosensor, Uppsala, Sweden, http://www.biacore.com) and IAsys (IAsys manual system, IAsys Plus and IAsys Auto+) systems (Affinity Sensors, Cambridge, UK, http://www.affinity-sensors.com). The BIAcore biosensors are flow-based instruments, which use the detection principle of surface plasmon resonance [6,7]. The sensor surface consists of a glass slide coated with a thin (50 nm) gold film to which is attached, by an inert (alkanethiol) linker layer, a chemical matrix onto which one of the binding partners can be immobilized using

*Corresponding author. Tel.: +61-3-3413-135; fax: +61-3-3413-104.

E-mail address: ed.nice@ludwig.edu.au (E.C. Nice)

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well defined chemistries [8]. The flow cells are formed by interfacing the sensor chip with a thermostatically controlled integrated fluidic cartridge (IFC). Four parallel channels (60 nl volume, 1.5 mm^2) are formed on the sensor surface and the microfluidics are used to deliver the reagents and samples over the sensor surface.

By comparison, the IAsys sensors are cuvette-based systems, which use a waveguide technique called a prism coupler or resonant mirror for detection [9,10]. The IAsys sensor surface (4 or 16 mm^2) is the bottom of an independent two-well format micro-cuvette, which has a choice of derivatised surfaces for ligand immobilisation. Samples (1 to 80 μl) can be added to, or removed from, the cuvette using a robotic dispenser.

The specific operating principles and design features of these instrumental biosensors suggest that they will be individually suited to particular applications (e.g., kinetic analysis, high throughput screening, ligand fishing). Biosensor analysis can be used in conjunction with other analytical techniques {e.g., micropreparative high-performance liquid chromatography (HPLC) [5,11,12], mass spectrometry (MS) [13]}, providing complementary information on the nature of the sample. The sensitivity and sample recovery volumes characteristic of micropreparative HPLC are compatible with biosensor requirements, allowing the two systems to be used in a complimentary manner that we have termed ''bidirectional synergy'' [12]. In one direction micropreparative HPLC generates well characterised homogeneous reagents for use on the biosensor, whilst in the other mode, the sensor provides specific information to aid the chromatographic process (e.g., development of affinity chromatography systems, vis-a-vis choice of antibody, optimisation of elution conditions [12,14], or specific immunodetection for chromatographic fractions [14,15]). Indeed, the biosensor can be considered as a micropreparative affinity surface with on-line detection comparable to a chromatographic immunoaffinity system. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis has recently been interfaced with biosensor analysis (BIA/MS) [13] and has been used to monitor affinity interactions occurring on the sensor surface (e.g., nature of the binding components, identification of unknown components, determination of specific and non-specific binding). The probe element surface of a laser desorption/ionisation TOF instrument has also been chemically and physically modified and used in both the binding process and the subsequent mass spectrometry analysis in a technique call affinity mass spectrometry or SELDI (surface enhanced laser desorption/ionisation) [16].

The interface between biosensor analysis, micropreparative HPLC and MS appears to be particularly attractive for the identification, purification and characterisation of ligands for orphan receptors identified by polymerase chain reaction (PCR) approaches or data base searching strategies [14]. For example, biosensor analysis has been used to identify sources of ligands for orphan proteins [17–22] and used as a specific affinity detector for the chromatographic fractionation [15,17,19,21,22]. The potential of BIA/MS has been demonstrated by using the BIAcore sensor chip [23] and BIAcore PROBE [24] directly as MALDI targets. Alternatively, the specifically bound ligand can be eluted from the sensor chip prior to MALDI analysis [25].

The success of ligand fishing experiments depends critically on the ability to conveniently recover in high yield the bound ligand for subsequent characterisation. Therefore, a recent area of biosensor development has been the automated elution and recovery at high concentration in small volumes (≥1 μl) of specifically bound material from the sensor surface in a form suitable for sensitive and specific downstream analysis [e.g., MS, N-terminal sequence analysis, two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE)]. The latest generation of BIAcore (BIAcore 3000, Biosensor) has been designed to minimise the dilution effect during recovery following desorption by reversing the flow through the flow cell to return the sample back to the auto-sampler. However, the surface area of the flow cells (1.6 mm^2) in this instrument limits the overall capacity and hence the amount of material that can be recovered at each cycle. The use of the BIAcore probe will overcome this limitation (surface area 12–20 mm^2) although this instrument is not automated. The IAsys cuvette based system appears to be particularly suited for ligand fishing due to the potential for long binding contact times and the confined environment which facilitates recovery post-desorption in small (μl) eluent volumes. The

availability of a high-capacity carboxymethyldextran sensor surface (CMD-SELECT cuvette, 16 mm^2 surface) will also increase the level of recovered ligand, further facilitating post-elution analysis.

We present herein the use of a cuvette based biosensor (IAsys Auto+) for preparative ligand fishing using the A33 antibody–antigen interaction [15] as a model system. A33 antibody (IgG) or antibody fragment [$F(ab)'_2$] were immobilised onto a preparative CMD-SELECT cuvette and used to purify the A33 epithelial antigen from Mono Q fractions generated from transfected Sf9 cell lysates or detergent extracts of LIM1215 colonic cells lines [26]. The recovered ligand was subsequently analysed and characterised using sodium dodecyl sulphate (SDS)–PAGE, Western blot analysis and micropreparative HPLC. Using automated repetitive injection and recovery, microgram quantities of essentially homogeneous ligand could be recovered in high yield using the $F(ab)'_2$ surface.

2. Experimental

2.1. Cell culture techniques

The LIM1215 colonic carcinoma cell line was grown in RPMI medium (Irvine Scientific, Santa Ana, CA, USA) containing 10% foetal calf serum. Confluent cells ($2.2 \cdot 10^5/cm^2$) were passaged using Trypsin-Versene solution (Life Technologies GIBCO BRL, Gaithersburg, MD, USA). Cells were seeded 1/10 into tissue culture dishes (150×20 mm, Nunclon, Roskilde, Denmark) containing 25 ml RPMI 1640 supplemented with 10% foetal calf serum, 1 μg/ml hydrocortisone, 0.025 U/ml insulin and 10.82 μg/ml α-thioglycerol. Dishes were incubated at 37°C in an atmosphere of 5% CO_2 for five days. After removing the media, cells were washed with phosphate-buffered saline (PBS) before being recovered from the surface using a cell scraper (Costar, Cambridge, MA, USA). Cells were washed in PBS and resuspended at 10^9 cells/ml.

A33 antigen was expressed using the recombinant baculovirus expression system (Invitrogen, Carlsbad, CA, USA). Sf9 insect cells were grown in spinner flasks at 26°C, in 1-l Graces Insect Cell Medium (Life Technologies GIBCO BRL) containing glutamine, streptomycin/penicillin and 10% fetal calf serum, to a density of $2 \cdot 10^6$ cells/ml. The cells were infected with recombinant baculovirus and cultured for three days, then harvested by centrifugation.

2.2. Extraction of A33 antigen from LIM1215 cells with Triton X-114

LIM1215 colonic cells ($2 \cdot 10^9$ cells) or A33 expressing Sf9 cells ($0.5 \cdot 10^9$ cells) were solubilised (10^8 cells/ml) for 30 min at 4°C using 1% (v/v) Triton X-114 in 15 m*M* Tris–HCl (pH 7.4) containing 1 m*M* phenylmethylsulphonyl fluoride, 1 m*M* pepstatin, 0.1 m*M* leupeptin and 0.01 U/ml aprotinin. The Triton X-114 extracted supernatant was layered over 6% sucrose in 15 m*M* Tris–HCl (pH 7.4) with 0.06% (v/v) Triton X-114 and containing the protease inhibitors listed above. The tubes were incubated at 37°C for 30 min and then centrifuged at 25°C for 15 min at 5000 *g*. The detergent phase was collected for chromatographic purification.

2.3. Chromatographic purification

2.3.1. Anion-exchange HPLC

The Triton X-114 detergent phase of A33 expressing Sf9 cells or LIM1215 colonic cells was loaded at 4°C onto a Mono Q HR 10/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) connected to a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) previously equilibrated in 10 m*M* Tris–HCl (pH 7.4) containing 0.1% (w/v) 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS). Retained proteins were eluted using a linear 0–1 *M* NaCl gradient generated over 40 min at a flow-rate of 1 ml/min. Fractions (1.5 ml) were collected automatically (FRAC 100, Amersham Pharmacia Biotech). Proteins were detected by absorbance at 280 nm. The A33 antigen in eluent fractions was detected using both biosensor analysis and Western blot analysis under non-reducing conditions, as described in Experimental.

2.3.2. Reversed-phase HPLC

An aliquot of the pooled samples recovered from

the sensor surface was loaded at a flow-rate of 0.5 ml/min onto a Brownlee Aquapore RP 300 micropreparative RP-HPLC column (100×1 mm I.D.) (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA), equilibrated with the primary solvent, 0.15% (v/v) trifluoroacetic acid (TFA) in water. The flow-rate was then reduced to 50 μl/min and proteins were eluted with a linear 60-min gradient to 60% aqueous *n*-propanol–0.125% (v/v) TFA. The column temperature was 45°C. Protein detection was performed at 215 nm. The A33 antigen was specifically detected in eluent fractions using both biosensor and Western blot analysis.

2.4. Preparation and purification of humanised A33 monoclonal antibody and F(ab)$'_2$ fragment

Humanised A33 monoclonal antibody was purified from bioreactor supernatants using Protein-A affinity chromatography. F(ab)$'_2$ was obtained by pepsin digestion (1%, w/w) [27] of the humanised A33 monoclonal antibody in 0.1 *M* sodium acetate (pH 3.5). The resultant F(ab)$'_2$ was purified by size-exclusion chromatography (SEC) on a Sephacryl S-200 (60×2.8 cm) column (Amersham Pharmacia Biotech) which had been equilibrated with 50 m*M* sodium phosphate (pH 7.4) containing 0.15 m*M* NaCl. The elution was performed at a flow-rate of 0.5 ml/min. To ensure homogeneity, A33 IgG and F(ab)$'_2$ were re-purified by micropreparative SEC [Superose 12 HR 3.2/30 column connected to a SMART system (Amersham Pharmacia Biotech)] using the buffer system defined above, at a flow-rate of 100 μl/min prior to immobilisation onto the biosensor.

2.5. SDS–PAGE and Western blot analyses

Electrophoresis and Western blot analyses were performed on 10% SDS–PAGE precast gels (Gradipore, North Ryde, Australia). Chromatographic fractions were electrophoresed under non-reducing conditions and analysed using silver staining and Western blotting. For Western blot analysis, proteins were transferred onto poly(vinyl difluoride) (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and incubated with humanised mAb. IgG binding was probed with horseradish peroxidase-labelled goat anti-human IgG (Bio-Rad), and detected by electrochemiluminescence (ECL) (Amersham Pharmacia Biotech).

2.6. Biosensor analyses

2.6.1. Instrumental design

The IAsys Autoplus (Affinity Sensor, Cambridge, UK) is a fully automated optical biosensor that uses a resonance mirror device for detection [9,10]. A schematic of the instrument is shown in Fig. 1. It has dual cuvette surfaces with a reagent capacity of 10–80 μl which have simultaneous dual-channel monitoring and vibro-stirring. The cuvettes have integral sensors and optical coupling. Sample and reagent delivery and sample recovery are performed using a robotic arm connected to an auto sampler syringe pump, wash station and auto-aspirator which ensures accurate and reproducible low volume transfer (minimum volume 1 μl). Two racks allow the use of up to 96 samples or recovery positions.

The instrument detects small changes in refractive index within the evanescent field generated by the resonant mirror (extending approximately 200 nm from the sensor surface) [9,28]. Ligand binding to, or dissociating from, an immobilised binding partner will alter the refractive index profile close to the surface of the sensor and change the resonance angle. Data are presented as sensorgrams, which show the change in the resonance angle (arc seconds) versus time. For proteins a signal of 163 arc seconds is equivalent to a surface concentration of 1 ng/mm^2 using the CMD surface [29].

2.6.2. A33 antibody immobilisation

A33 IgG or F(ab)$'_2$ fragment were immobilised onto a CMD-SELECT (16 mm^2) preparative cuvette using a mixture of *N*-ethyl-*N'*-dimethylaminopropyl-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (Affinity Sensor). Briefly, the cuvette was washed with 80 μl water and equilibrated for 10 min with 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) (pH 7.4) containing 3.4 m*M* EDTA, 0.15 m*M* NaCl and 0.005% (v/v) Tween 20 (HBS buffer). The surface was activated using two injections of 50 μl of a mixture of 0.05 *M* NHS and 0.2 *M* EDC for 10 min. A33 F(ab)$'_2$ fragment was then injected (50 μl of a 100 μg/ml solution in 10 m*M* sodium acetate, pH 4.5) for 60 min. Residual

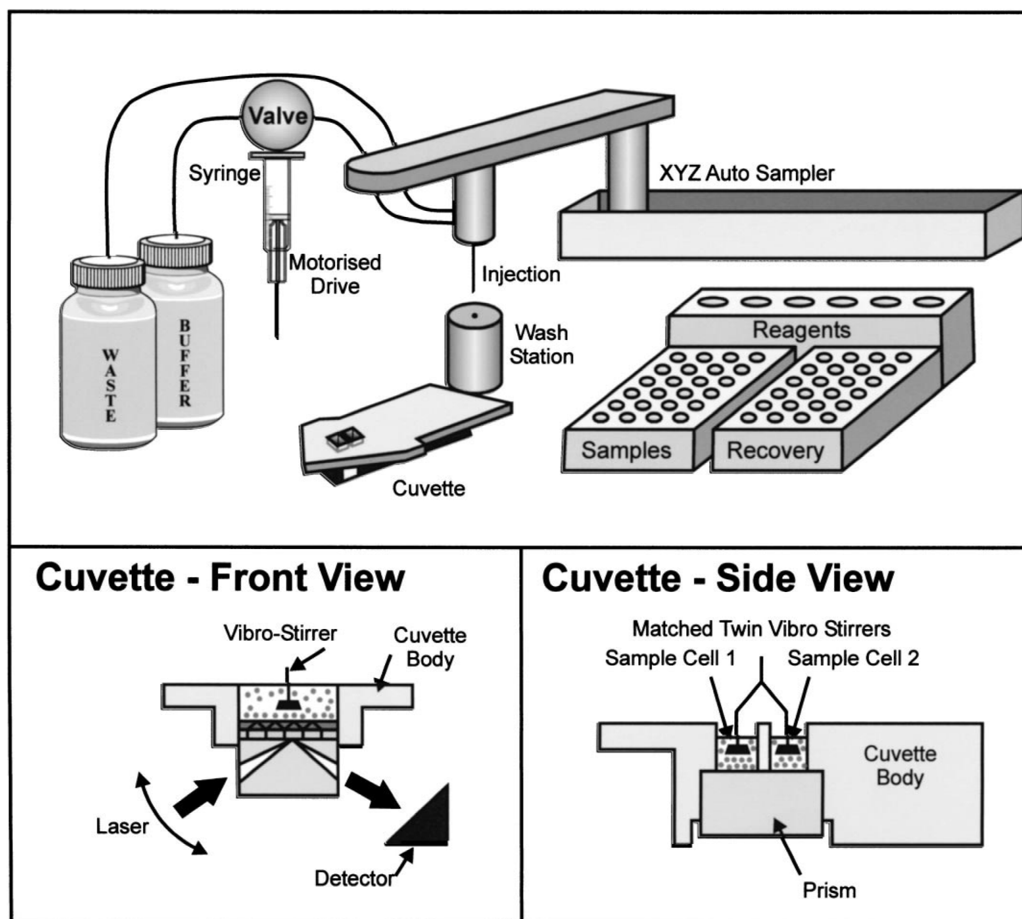

Fig. 1. Schematic of the IAsys Auto+ cuvette-based optical mirror resonance biosensor.

active ester sites were blocked with 1 *M* ethanolamine, pH 8.5 (2×50 μl for 10 min) and the surface washed with 2-min pulses of 10 m*M* HCl (50 μl) in order to obtain a stable baseline. For the screening of the Mono Q fractions, a control channel was derivatised with NHS/EDC as described above and then blocked immediately with ethanolamine. Residual bound antigen was eluted and the surface regenerated between injections using 40 μl of 10 m*M* NaOH or 10 m*M* HCl. This treatment did not significantly denature the protein immobilised onto the sensor surface as shown by virtually equivalent signals on re-injection of a sample containing the A33 antigen.

2.6.3. Screening of Mono Q fractions

Aliquots of individual Mono Q fractions of Triton X-114 cell extracts (10 μl from Sf9 cell fractions, 20 μl from LIM1215 fractions) were injected into the cuvettes [A33 surfaces and control cuvette] containing 40 μl of HBS buffer with a stirrer setting of 50. Association was performed for 10 min followed by 5 min of dissociation in HBS buffer alone. Regeneration was performed for 2 min using 10 m*M* HCl and was followed by 5 min equilibration with HBS buffer before injection of the next sample.

2.6.4. Preparative ligand fishing

Mono Q fractions which had given a positive biosensor response for the A33 antigen were pooled for the preparative ligand fishing. A 30-μl volume of the pooled fractions was injected into the A33 cuvette containing 40 μl of HBS buffer with a stirrer

setting of 50. The threshold for cycle termination if no binding was observed was set at 20 arc seconds whilst ligand fishing was automatically triggered if a response greater than 50 arc seconds was obtained. Binding was performed for 30 min. After aspirating residual sample and briefly washing the cuvette surface with 60 μl of buffer, the bound material was eluted with 10 m*M* HCl or 10 m*M* NaOH (25 μl) for 2.5 min and the eluate then transferred into a recovery vial and neutralised with 10 μl of 1*M* Tris–HCl, pH 8.0 or 10 μl of 1 *M* glycine, pH 2.5, respectively.

To assess the surface reactivity and stability during the cycles of binding/elution, the molar binding activities [8,30] were calculated from the equation:

$$\text{Molar binding activity} = \frac{(\text{antigen response}) \times (\text{antibody molecular mass})}{(\text{amount of immobilised antibody}) \times (\text{antigen molecular mass})}$$

The repetitive yield of recovery was calculated by least squares fitting using ORIGIN (Micro Cal Software, MA, USA) using the formula $y = AB^n$, where y is the repetitive yield, A is the initial yield, n is a number of cycles and B is the fractional yield.

3. Results and discussion

The monoclonal antibody A33 (mAbA33) detects a tissue specific cell surface antigen expressed by both normal and transformed intestinal epithelium [31]. The A33 antigen has recently been identified as a novel member of the immunoglobulin superfamily [32]. The A33 antigenic system is the focus of several clinical studies on patients with colon cancer. Phase I/II clinical studies have shown that the monoclonal A33 antibody (i) localises with high specificity to colon cancer, (ii) is retained for prolonged period (up to 10 weeks) at tumour sites but is cleared rapidly from normal colon (5–6 days), (iii) has anti-tumour activity as a carrier of ^{125}I or ^{131}I [33,34]. Preclinical evaluation of mAb A33 has been performed in a nude mouse model using xenografts of the colorectal carcinoma cell line SW1222 [35–37]. A humanised antibody (huA33) has been developed [38] and is currently in clinical trials.

The A33 antigen was initially purified from Triton X-114 extracts of LIM1215 cells under non-denaturing conditions using a multidimensional micropreparative chromatographic protocol [15]. The cell extracts were applied sequentially to Green-Sepharose HE-4BD ligand dye, Mono Q HR 10/10 anion-exchange, Superose 12 HR 10/30 SEC and micropreparative (100×1 mm I.D.) Brownlee Aquapore reversed-phase RP 300 columns. The purification was monitored by both biosensor analysis using surface plasmon resonance detection (BIAcore 2000) with a $F(ab)'_2$ fragment of the humanised A33 monoclonal antibody immobilised on the sensor surface and Western blot analysis using humanised A33 IgG following SDS–PAGE under non-reducing conditions. Final purification to homogeneity was achieved using RP-HPLC, but this procedure was shown by biosensor analysis to cause loss and/or partial denaturation of the protein. Therefore, a modification of this purification protocol was subsequently designed [30] involving affinity chromatography [39] followed by micropreparative anion-exchange and SEC, in order to purify the A33 antigen under non-denaturing conditions for structure function studies. In this protocol, RP-HPLC was only used analytically to monitor sample purity.

The purification was initially complicated by an in vitro interaction between actin and the Fc domain of the A33 IgG immobilised onto the sensor surface, chromatographic affinity support or Western blot membranes [15]. This was overcome by using the A33 $F(ab)'_2$ fragment in biosensor analysis and by separating actin from the A33 antigen using ligand dye chromatography on Green-Sepharose HE-4BD. Furthermore, only low levels of antigen (1–2 μg/10^9 cells using the initial protocol, up to 5 μg/10^9 cells using the revised affinity chromatography protocol) can be purified from biological sources. Recombinant forms of the A33 antigen have therefore now been produced using recombinant DNA technology in order to pursue functional studies.

Because of our previous experience, the A33 antigenic system appeared to be an excellent model to evaluate the potential of a cuvette based biosensor for ligand fishing from biological sources. Before using a native biological source of A33 antigen, a pilot experiment was performed using a recombinant source of the antigen (A33 antigen expressing Sf9 cells using recombinant baculovirus) to optimise the

instrumental parameters for binding and recovery. The expression level of this cell line is 250 to 500 μg A33 antigen/10^9 cells.

3.1. Preparative ligand fishing of A33 antigen from A33 antigen transfected Sf9 cells using A33 IgG immobilised onto a preparative CMD-SELECT cuvette

3.1.1. Immobilisation of A33 IgG onto CMD-SELECT cuvette

The homogeneity of the antibody preparation used for immobilisation onto the sensor cuvette was confirmed, and the sample buffer exchanged into immobilisation buffer, by micropreparative SEC using Superose 12 HR 3.2/30 immediately prior to use (results not shown). A33 IgG was then immobilised onto the cuvette sensor surface using the NHS/EDC chemistry as described in Experimental (Fig. 2A, upper trace). A signal of 3525 arc seconds, corresponding to approximately 21.6 ng/mm^2 [29] of IgG, equivalent to 345 ng of IgG immobilised onto the sensor surface, was obtained. A control cuvette was derivatised with NHS/ECD and blocked with ethanolamine for the screening of the Mono Q fractions (Fig. 2A, lower trace).

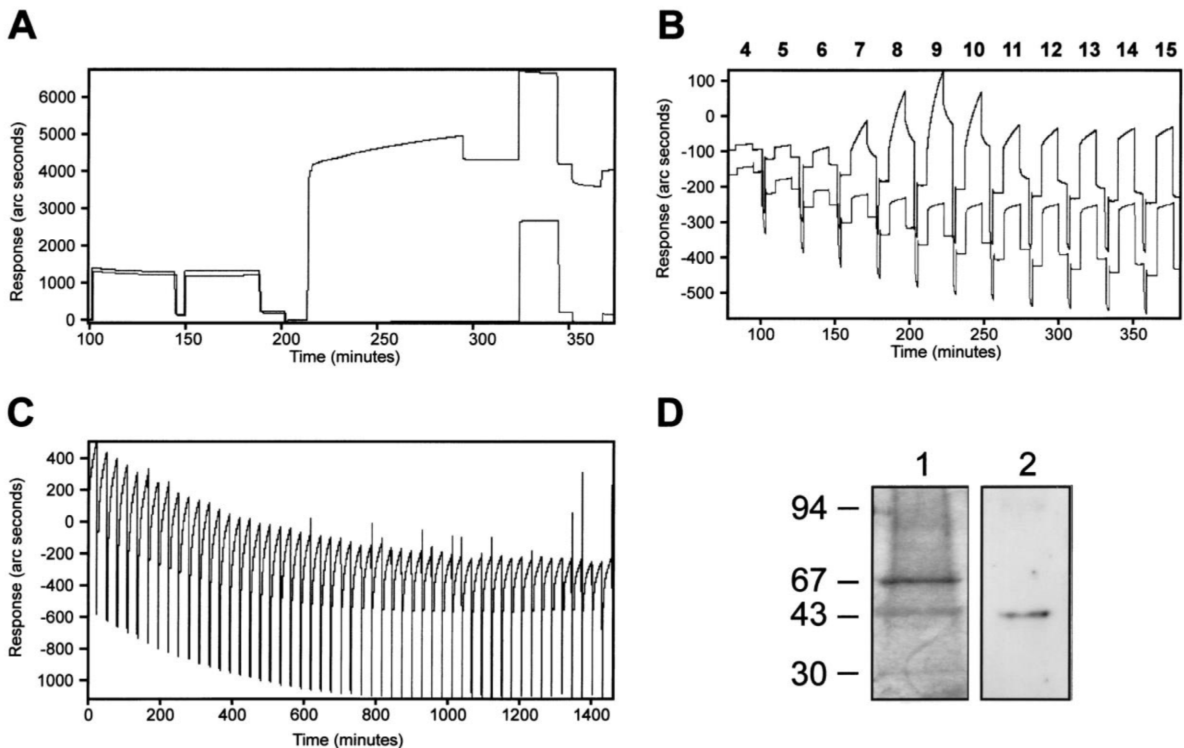

Fig. 2. Micropreparative ligand fishing for the A33 antigen from A33 expressing Sf9 cells lysate using A33 IgG immobilised onto IAsys CMD-SELECT cuvette. A33 IgG was immobilised onto a high-capacity dextran biosensor surface (CMD-SELECT) using the NHS/EDC chemistry as described in Experimental (A, upper trace). A signal of approximately 3525 arc seconds was obtained, corresponding to approximately 21.6 ng/mm^2 of IgG, representing a total amount of 345 ng of IgG immobilised. A control cuvette was derivatised with NHS/ECD and blocked with ethanolamine. (A, lower trace) Triton X-114 extracts of A33 expressing Sf9 cells were separated by Mono Q (HR 10/10) anion-exchange chromatography using the chromatographic conditions described in Experimental. An aliquot of each fraction (10 μl) was taken for biosensor analysis (B). A positive biosensor signal (>40 arc seconds) was registered when aliquots of fractions 7 to 12 were injected over immobilised A33 (a blank-derivatised channel was used as control) (C). Mono Q active fractions (8 to 10) were then pooled (4.5 ml) and repetitively injected (50 cycles) over immobilised IgG (C). Bound antigen was desorbed using 10 m*M* HCl, recovered from the cuvette and immediately neutralised using 1 *M* Tris–HCl, pH 8.0. The A33 antigen protein (M_r 43 000) in pooled eluates was identified using 8–25% silver stained SDS–PAGE gel (D, lane 1) and Western blot analysis (D, lane 2). The recovered sample was not homogeneous as shown by the silver stained gel (contaminating band with $M_r \approx 67\,000$).

3.1.2. Biosensor analysis of fractions from anion-exchange HPLC of a Triton X-114 extract of A33 antigen transfected Sf9 cells

The detergent phase of the Triton X-114 Sf9 cell extracts was diluted 10 times in 10 m*M* Tris–HCl (pH 7.4) containing 0.1% (w/v) CHAPS and loaded onto a Mono-Q anion-exchange HPLC column. Triton X-114 was exchanged with the zwitterionic detergent CHAPS after absorption of the detergent–membrane complex to the chromatographic support. CHAPS was previously found to greatly improve resolution on both the anion-exchange and SEC and did not affect the binding of the A33 antigen onto the immobilised antibody [15]. Aliquots of each fraction (10 μl added to 40 μl biosensor buffer) were injected over both the immobilised IgG (Fig. 2B, upper trace) and the blank derivatised channel (Fig. 2B, lower trace). A positive biosensor signal (>40 arc seconds) was registered when aliquots of fractions 7 to 12 were injected over the biosensor surface (Fig. 2B). After deduction of the responses obtained on the blank channel, the specific biosensor responses for fractions 7 to 12 were 96, 154, 206, 137, 90 and 85 arc seconds, respectively. The signal for fractions 13 to 15 remained constant at approximately 80 arc seconds. Fractions 8 to 10 were pooled and used for the ligand fishing experiment.

3.1.3. Ligand fishing of A33 recombinant antigen from A33 positive Mono Q fractions

Fifty cycles of injection/elution were performed using the pooled Mono Q active fractions (fractions 8–10, 4.5 ml) (Fig. 2C). In this experiment, the bound protein was eluted using 10 m*M* NaOH (25 μl), collected and transferred into recovery vials. The recovered material was immediately neutralised with 10 μl 1 *M* glycine, pH 2.5. At the beginning of the experiment, injection of the sample gave a surface molar binding activity of 0.3, allowing the recovery of approximately 30 ng of protein/cycle (Fig. 3). At the end of the 50 cycles, the surface was still active but showed a reduced molar binding

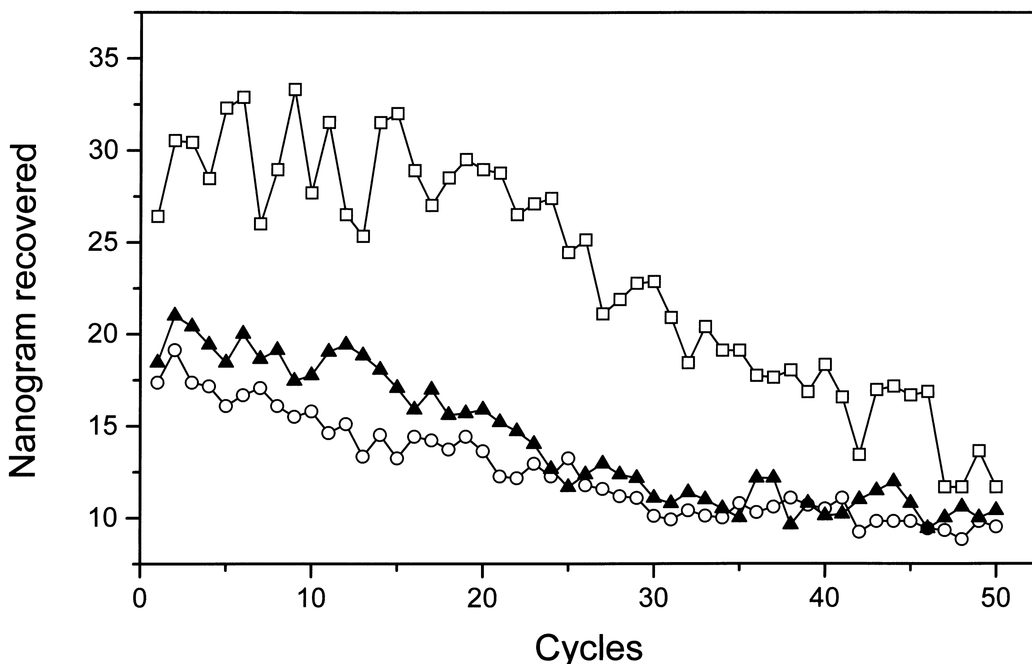

Fig. 3. Protein recovery during micropreparative ligand fishing for the A33 antigen. The amount of protein recovered at each cycle was calculated from the level of the binding signal obtained before elution, assuming that 163 arc seconds correspond to 1 ng/mm^2 [29]. The 50 cycles of injection/elution of the pooled Mono Q fractions from A33 antigen expressing Sf9 cells onto immobilised A33 IgG (□) gave a total recovery of 1.160 μg of protein. The two parallel runs of 50 cycles of injection/elution of the pooled LIM1215 Mono Q active fractions onto immobilised A33 F(ab)$'_2$ (○, ▲) gave a total recovery of approximately 710 ng and 630 ng, respectively.

activity of 0.12, giving a recovery value of 11 ng of protein/cycle (Fig. 3). The 50 cycles displayed a repetitive yield of approximately 98.4% allowed the recovery of approximately 1.2 μg of protein, giving an average recovery of 24 ng/cycle (Fig. 3). It should be noted that the apparent dissociation rate constant ($4.6 \cdot 10^{-3}$ s^{-1}) of the A33 antigen–antibody interaction did not result in significant losses of bound protein. Such losses are also minimised by the short time interval (1 min) between the end of binding and beginning of elution, low wash volumes (60 μl) and the absence of continuous flow.

The recovered fractions were pooled and dried using a Savant Speed Vac concentrator. The dry fractions were either resuspended in sample buffer for analysis by silver stained SDS–PAGE (Fig. 2D, lane 1) and Western blotting using A33 IgG (Fig. 2D, lane 2) or in 0.15% (v/v) TFA in water for RP-HPLC analysis (result not shown). The A33 antigen (apparent molecular mass of 43 000) could be identified in the recovered fractions using SDS–PAGE and Western blot (Fig. 2D). However, in this case, the sample recovered from the cuvette surface was not homogeneous as shown by both RP-HPLC (result not shown) and the silver stained gel (Fig. 2D, lane 1) (there was a contaminating band of approximate M_r 67 000 on SDS–PAGE, possibly due to serum albumin, which was present in large excess in the culture medium). It has frequently been our experience that, particularly when working with trace components at low levels, non-specific adsorption onto affinity supports may be significant. However, this pilot experiment clearly shows the potential to fish the A33 antigen from a complex biological starting material in sufficient yield for subsequent analytical studies. The A33 antigen could be purified to homogeneity by further micropreparative HPLC (data not shown).

In a subsequent experiment, A33 $F(ab)'_2$ fragment was immobilised onto the CMD-SELECT cuvette instead of the IgG in an attempt to reduce the heterogeneity of the eluted sample due to binding with the Fc region of the antibody, as we had previously observed with actin. It was also of interest to investigate whether it was possible to fish the A33 antigen from a non-recombinant source containing a significantly lower concentration of ligand. We therefore used the LIM1215 colonic carcinoma cell line, which we had used in our original four-step chromatographic protocol [15], as source of the antigen.

3.2. Preparative ligand fishing of A33 antigen from LIM1215 colonic carcinoma cells using A33 $F(ab)'_2$ fragment immobilised onto a preparative CMD-SELECT cuvette

3.2.1. Immobilisation of A33 $F(ab)'_2$ fragment onto CMD-SELECT cuvette

The homogeneity of the $F(ab)'_2$ fragment preparation was analysed by micropreparative SEC using Superose 12 HR 3.2/30 prior to immobilisation. Two $F(ab)'_2$ sensor surfaces were prepared, using the NHS/EDC chemistry, to enable parallel recovery at each cycle. The level of $F(ab)'_2$ fragment immobilised on each surface was approximately 22 ng/mm^2, corresponding to 350 ng of $F(ab)'_2$ fragment on each sensor surface (data not shown).

3.2.2. Analysis of fractions from anion-exchange HPLC of a Triton X-114 extract of LIM1215 cells

The detergent phase of the Triton X-114 LIM1215 cell extract was diluted 10 times in 10 m*M* Tris–HCl (pH 7.4) containing 0.1% (w/v) CHAPS and loaded onto a Mono Q anion-exchange HPLC column. Aliquots of each fraction (20 μl added to 40 μl buffer) were injected over the immobilised $F(ab)'_2$ on the cuvette sensor surface (Fig. 4A). A strong positive biosensor signal (>40 arc seconds) was registered when aliquots of fractions 7 to 12 were injected over the biosensor surface (Fig. 4A). After subtraction of the responses obtained on the blank channel, the specific biosensor responses for fractions 7 to 12 were 58, 299, 390, 296, 207 and 73 arc seconds, respectively. The presence of the A33 antigen was confirmed by Western blot analysis using A33 IgG (Fig. 4B). To investigate the complexity of the sample, aliquots (10 μl) of each fraction were analysed by silver stained SDS–PAGE (Fig. 4C). It is of interest to note that the A33 antigen (M_r 43 000) is not readily identifiable on the silver stained gel at this stage of the purification. Fractions 8 to 11 were pooled for the capture experiment.

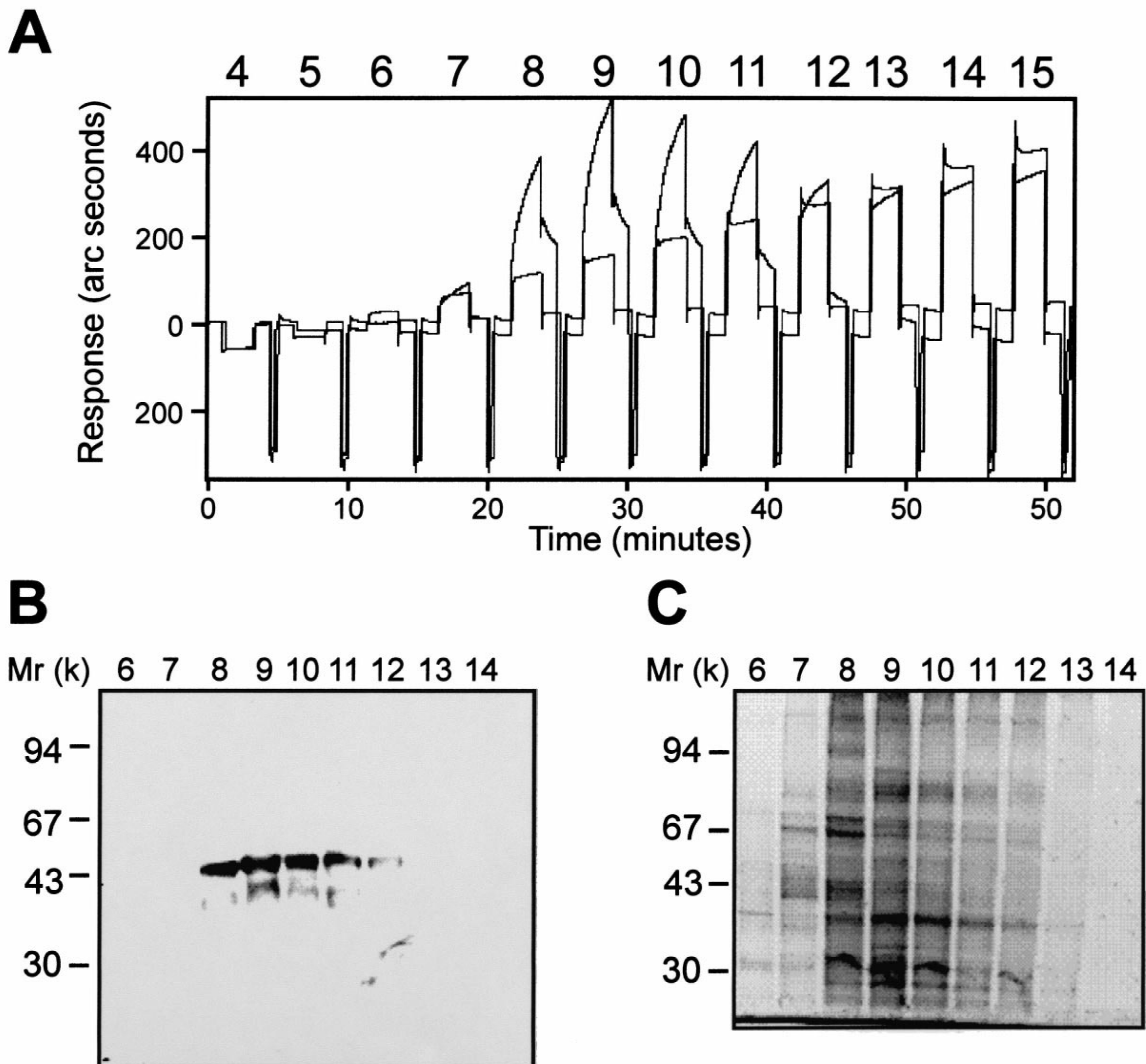

Fig. 4. Analysis of fractions from anion-exchange HPLC of a Triton X-114 extract of LIM1215 colonic carcinoma cells. Triton X-114 extracts of LIM1215 cells were separated by Mono Q (HR 10/10) anion-exchange chromatography using the chromatographic conditions described in Experimental. An aliquot of each fraction (20 μl) was taken for biosensor analysis using A33 F(ab)$'_2$ immobilised onto a CMD cuvette (a blank-derivatised channel was used as control) (A). A biosensor signal (>40 arc seconds) was registered in fractions 7 to 12. The presence of A33 antigen was confirmed using Western blot analysis under non-reducing conditions using A33 IgG (B) whilst the complexity of the sample was shown by silver-stained SDS–PAGE (C).

3.2.3. Ligand fishing of A33 antigen from LIM1215 using immobilised F(ab)$'_2$ fragment

Two parallel runs of 50 cycles of injection/elution were performed with the pooled LIM1215 Mono Q active fractions (fractions 8–11, 6 ml) using both sensor surfaces (Fig. 5A). An expanded view of two consecutive cycles of injection/recovery is shown in (Fig. 5B). Again, the apparent dissociation rate constant for the interaction between the A33 antigen and the A33 F(ab)$'_2$ fragment ($3.8 \cdot 10^{-3}$ s^{-1}) did not result in significant losses of bound protein.

In this experiment, the bound protein was eluted using 10 m*M* HCl instead of 10 m*M* NaOH. The HCl buffer facilitated low level analysis by mi-

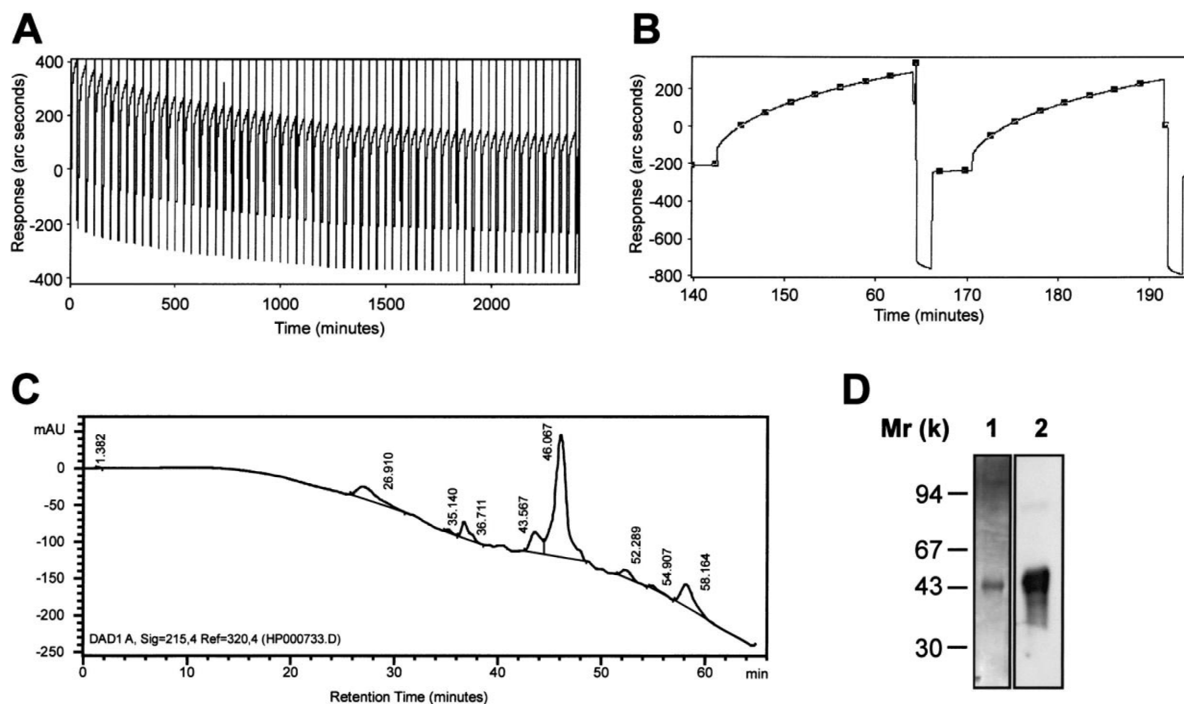

Fig. 5. Micropreparative ligand fishing for the A33 antigen from LIM1215 colonic cell lysate using A33 $F(ab)'_2$ fragment immobilised onto IAsys CMD-SELECT cuvette. Mono Q active fractions (8 to 11, 6 ml) were pooled and repetitively injected over an A33 $F(ab)'_2$ fragment immobilised onto the CMD-SELECT cuvette (two parallel runs of 50 cycles) (A). Bound antigen was eluted using 10 m*M* HCl, recovered and neutralised using 1*M* Tris–HCl, pH 8.0. An enlarged view of two consecutive cycles of injection/recovery is shown (B). Analysis of the recovered sample by micropreparative RP-HPLC onto a Brownlee Aquapore RP 300 column (10×1 mm I.D.) gave a major symmetrical peak eluting around 46 min (C). Minor peaks eluting at approximately 27, 36, 43, 52, 54 and 58 min were present in the corresponding blank run. The biosensor-recovered fraction was also shown to be essentially homogeneous by silver stained gel SDS–PAGE analysis, revealing a M_r 43 000 protein (D, lane 1) which was recognised by the A33 mAb using Western blot analysis under non-denaturing conditions (D, lane 2).

cropreparative RP-HPLC since it resulted in a much cleaner background signal. Each surface displayed a similar initial molar reactivity of 0.12, which was reduced to 0.07 at the end of the 50 cycles (Fig. 3). The two individual runs each displayed a repetitive yield of approximately 99% resulting in a total recovery of 710 ng and 630 ng of protein (average of 14.1 and 12.6 ng/cycle) for each channel (Fig. 3). The eluted fractions were pooled and separated into two batches for micropreparative RP-HPLC using a Brownlee Aquapore RP300 micropreparative column (100×1 mm I.D.) and SDS–PAGE/Western blotting analyses. Using RP-HPLC analysis, a major symmetrical peak was recovered which eluted at around 46 min (Fig. 5C). This peak fraction was dried using a Savant Speed Vac concentrator, resuspended in 100 μl HBS buffer and re-injected over a sensor surface (BIAcore 2000) derivatised with A33 IgG. This fraction was specifically recognised by the immobilised IgG, confirming the identity of the eluted antigen (result not shown).

The biosensor recovered fraction was also shown to be essentially homogeneous by silver stained gel SDS–PAGE analysis (Fig. 5D, lane 1) which revealed a protein with a M_r of 43 000 corresponding to the band recognised by A33 mAb using Western blot analysis (Fig. 5D, lane 2).

In these experiments, microgram levels of A33 antigen were recovered using A33 IgG or $F(ab)'_2$ fragment immobilised onto the sensor surface with the conventional NHS/EDC chemistry. This procedure results in random orientation of the protein

due to attachment via both the amino terminus and lysine residues and may cause loss, or reduction, of biological activity by coupling near the antigen-binding site. Therefore, the molar binding activity and overall ligand recovery may be further improved by immobilising A33 IgG or $F(ab)'_2$ fragment in a defined surface orientation (Protein-A cross-linking with dimethyl pimelimidate for IgG, thiol conjugation via the hinge region for Fab′) [30]. These chemistries have previously been shown to give higher molar binding activities for both IgG (two- to three-fold higher) and Fab'_2 fragment (1.5- to 2-fold increase) compared with the NHS/EDC chemistry [30]. Although the dimethyl pimelimidate cross-linking may interfere with the kinetics of the A33 antigen–antibody interaction [30], a more active surface would be generated for the ligand fishing experiment. Overall recovery may also be further improved by optimising the level of antibody immobilisation in order to obtain the best binding stoichiometry.

4. Conclusions

The cuvette-based IAsys biosensor, which uses the resonant mirror optical detection principle, was used to perform micropreparative ligand fishing. The A33 antigenic system was used to validate the methodology, using both the recombinant A33 expressing Sf9 cells as well as the LIM1215 colonic carcinoma cell line, from which the A33 antigen was originally identified and purified to homogeneity [15,30], as sources of antigen.

Using a high capacity dextran biosensor surface (surface area 16 mm^2) with an immobilisation level of $F(ab)'_2$ fragment of approximately 350 ng, we were able to recover microgram quantities of A33 antigen from the sensor surface using automated repetitive injection and recovery (approximately 14 ng/cycle) and to perform post-recovery analysis using SDS–PAGE, Western blotting and micropreparative RP-HPLC. Compared to conventional affinity chromatography, the use of the biosensor as an ‘‘on-line affinity chromatograph’’ allows a large number of automated cycles to be performed with on-line monitoring of the surface viability throughout the experiment. In this experiment, the $F(ab)'_2$ sensor surface was found to retain 58% of the initial binding activity after 50 cycles of binding/elution. On-line monitoring also permits the direct quantitation of both bound and recovered material. Furthermore, the cuvette provides a constrained environment with low surface area, reducing non-specific binding, and enabling the recovery of nanogram quantities of protein in small volumes (25 μl) at concomitant high concentration. The sensitivity and sample recovery volumes are complementary with analytical techniques such as micropreparative HPLC, mass spectrometry or amino acid microsequence analysis. Indeed, even the use of only one or two preparative cycles using the conditions described herein would enable the recovery of sufficient material to perform further down stream analysis using sensitive analytical techniques such as bioassay or mass spectrometry where the lower level of detection is in the femtomole range [40]. Such sensitivity may make biosensor analysis a complimentary partner to other separation technologies in modern proteomic approaches [41]. The biosensor may be involved in the systematic study of protein–protein interaction through the automated ligand fishing of protein and protein complexes from biological sources and may provide defined material for 2D gels, MS and database analyses.

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