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Synergies between micropreparative high-performance liquid chromatography and an instrumental optical biosensor

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ABSTRACT

The recent development of an automated surface plasmon resonance technology for the measurement of biomolecular interactions (Pharmacia BIAcore) has provided new opportunities for the detection and analysis of protein–protein interactions. In the BIAcore, detection is based on changes in surface plasmon resonance which are monitored optically. Changes in surface plasmon resonance correspond to changes in surface concentration of macromolecules and can be monitored in real time.

We have found that the detection sensitivity obtainable with this technology (ng/ml concentrations of specific ligands are readily detectable for many applications) is complementary "in a bidirectional manner" to micropreparative HPLC. Thus micropreparative HPLC may be used to purify and characterise reagents for the biosensor, whilst the biosensor may be used to define chromatographic parameters such as elution conditions for affinity chromatography or serve as an affinity detector for fractions obtained during chromatographic purification.

Examples of such applications, including the potential of the biosensor to search for and monitor the purification of unknown ligands for which the target molecule has been identified, are shown. In particular, the use of the biosensor to monitor the purification of soluble epidermal growth factor receptor from A431 cell conditioned media is demonstrated.

INTRODUCTION

The development of an instrumental optical biosensor (Pharmacia BIAcore) [1,2] to measure biomolecular interactions in real time has opened new perspectives for the detection of protein–protein interactions, including receptor ligand [3,4] and antibody–antigen [5–14] interactions and interactions between components of signal transduction pathways [15,16]. One of the interacting components is immobilised onto the carboxymethylated gold surface of the sensor [17] using conventional protein chemistry techniques (*e.g.* N-hydroxysuccinimide–N-ethyl-N'-(3-diethylaminopropyl)carbodiimide coupling [18]}. This coupling can be performed *in situ* using the integrated microfluidics of the instrument. Monoclonal antibodies, purified receptors, protein or peptide ligands can be readily coupled onto the surface, and real time binding studies performed by sequentially flowing reagents of interest over the derivatised sensor surface to which the target protein has been attached [1,2]. The automated injection system and sample loops are designed for injection volumes of 1–50 μl to be introduced at constant flow-rates typically between 1–5 μl/min. Detection is based on the phenomenon of surface plasmon resonance (SPR) [19,20], a technique which measures small changes in refractive index at, or near to, a metal surface. Such changes are directly proportional to the change in adsorbed mass [2], and have a potential lower limit of detection of 10 pg/mm^2

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[1]. This corresponds to the injection of samples at ng/ml concentration [1].

Fundamental to the success of the biosensor technology is the ability to produce, in a homogeneous form, the specific component for immobilisation onto the surface. We have previously shown [21–28] the potential of short (less than 10 cm) micropreparative (1–2 mm I.D.) columns for the purification, concentration and buffer exchange of samples prior to microsequence analysis. Such systems have been used for the purification of a number of growth factors [29–36] and related compounds [27,37], using not only reversed-phase, but also hydrophobic interaction [27,37], anion- and cation-exchange [27,37] and micro size-exclusion HPLC [37] to render the methods compatible with a wide range of proteins. With this methodology proteins and peptides are recovered in small eluent volumes (typically 50–100 μl). The resultant concentrations achieved permit detection (at 215 nm) of nanogram quantities [21,24, 27,28]. The sensitivity and sample/recovery volumes of micropreparative HPLC would therefore appear to be complementary with the biosensor. We describe the use of micropreparative HPLC to monitor sample purity, quantitation and heterogeneity, and to purify reagents suitable for application to the biosensor. We also demonstrate the use of micropreparative HPLC to desalt and buffer exchange proteins into solvent systems compatible with the BIAcore immobilisation chemistry.

Whilst material for immobilisation onto the sensor surface needs to be homogeneous, subsequent analyses can be performed on crude extracts, tissue culture media or bacterial broth without the need for prior purification [2], allowing the biosensor to be used for the rapid screening of potential sources of interacting ligands. This approach should be particularly useful in the search for "unknown ligands" of, for example, monoclonal antibodies raised against complex mixtures of proteins and carbohydrate, or for new members of receptor gene families (*e.g.* tyrosine kinases [38,39]) which have been elucidated from sequence homologies and polymerase chain reaction (PCR) based molecular biology techniques. In such cases the biosensor can then be used as a specific detector for monitoring fractions obtained during chromatographic purification of the ligand of interest. To demonstrate the feasibility of such an approach we describe the use of the biosensor, using immobilised recombinant human epidermal growth factor (hEGF), to monitor the purification of the soluble form of the epidermal growth factor receptor (sEGF-R) from A431 cells [40]. Purified sEGF-R, in a form suitable for immobilisation onto the sensor surface, is obtained by the sequential use of wheat germ lectin affinity chromatography, anion-exchange, size-exclusion and micropreparative anion-exchange HPLC.

Since, in the case of the search for new ligands, one of the interacting species (*e.g.* the receptor or antibody) will have already been purified, the use of affinity chromatography as part of the purification protocol would appear obvious. However, in many cases the specificity and/or the affinity of the interaction makes affinity chromatography quite difficult. Both binding and elution conditions must be defined if a reasonable yield of the active ligand is to be obtained. We illustrate how the biosensor can be used to define and optimise elution conditions for affinity chromatography, with the ability to check quantitatively the surface, or the purified ligand, for denaturation.

MATERIALS AND METHODS

Micropreparative HPLC

Separations were performed on a Pharmacia SMART system [26] equipped with a variable wavelength μPeak monitor, a built-in on-line conductivity meter and a microfraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). Columns used were (a) Pharmacia Mono Q PC 1.6/5 anion-exchange, (b) Pharmacia Superose 12 PC 3.2/30 micro size-exclusion, (c) Pharmacia PC 3.2/10 Fast Desalting and (d) Pharmacia μRPC C2/C18 PC 3.2/3 reversed-phase. Samples were loaded by means of a 1-ml or 100-μl external loop. Chromatographic conditions used for particular separations are given in the appropriate figure legends.

Matrix assisted laser desorption mass spectrometry (MALD-MS)

Mass spectra were obtained using a Lasermat mass spectrometer (Finnigan-MAT, San Jose, CA, USA) as described previously [41]. α-Cyano-4-hydroxy cinnamic acid [42] (10 mg/ml in 30% (v/v) acetonitrile, Aldrich, Milwaukee, WI, USA) was used as the matrix.

Biosensor measurements

All measurements were performed on the BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden) [1,2]. Sensor chip CM5, surfactant P20 (a 10% solution of a non-ionic detergent), N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (EDC) were also obtained from Pharmacia Biosensor. Tern neuraminidase, NC10 and NC41 Fab antibody fragments [43] were a kind gift from Dr. L.C. Gruen, CSIRO Division of Biomolecular Engineering, Melbourne, Australia. Peptide P20, corresponding to residues 306–328 of the C-terminus of the HA1 chain of influenza virus hemagglutinin subtype H3 [44] and the corresponding monoclonal antibody, 2/1 IgG, and 1/1 Fab' fragment were a kind gift from Dr. D. Jackson, Department of Microbiology, University of Melbourne, Australia. Recombinant hEGF was purchased from PeproTech (Rocky Hill, NJ, USA).

Immobilisation of proteins and peptides to the sensor surface

Proteins and peptides were immobilised onto the sensor surface essentially as described previously [8]. Briefly, immobilisation was performed at a constant flow-rate of 4 μl/min using HBS buffer [10 m*M* Hepes, pH 7.4 containing 0.15 *M* NaCl, 3.4 m*M* ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) surfactant P20]. The carboxymethylated surface of the sensor chip was first activated with 35 μl of a NHS–EDC mixture (0.05 *M* NHS, 0.2 *M* EDC in distilled water). Peptides or proteins (peptide P20, 50 μg/ml in 10 m*M* sodium acetate buffer, pH 6.0; recombinant hEGF, 100 μg/ml in 10 m*M* sodium acetate pH 4.5; NC10 and NC41 Fab fragments, 50 μg/ml in 10 m*M* sodium acetate pH 4.5) were then injected (50-μl aliquots) over the activated surface for covalent attachment. Following the coupling to the sensor surface, residual activated ester groups were blocked by the injection of 35 μl of 1 *M* ethanolamine hydrochloride, pH 8.5, followed by washing with 10 μl of 10 m*M* HCl in the case of peptide P20 and recombinant hEGF, or 10 m*M* sodium acetate pH 4.5 in the case of NC10 and NC41 Fab, to remove non-covalently bound material.

Preparation of membrane extracts containing EGF receptor (M_r 170 000 form) from A431 cells

A431 cells were grown in 600-ml Nunclon flasks (Nunc, Kamstrup, Denmark) in Dulbecco's modified Eagle's medium (DME) containing 10% foetal calf serum. Confluent cell monolayers (approximately 10^7 cells) were washed and then scraped from the flask in 10 ml PBS, and the cells collected by centrifugation at 5000 rpm (1700 *g*) for 5 min at 4°C in an Hereaus minifuge (Heraeus Sepatech, Osterode, Germany). The cells were then lysed in 1 ml PBS containing 1% (v/v) Triton X-100, 10 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma, St Louis, MO, USA) and 100 units/ml Trasylol (Bayer, Leverkusen, Germany). After leaving on ice for 30 min the sample was centrifuged at 5000 rpm (1125 *g*) for 5 min at 4°C in an Eppendorf microfuge (Eppendorf, Hamburg, Germany) and the supernatant, containing the membrane extract, retained for further studies.

Preparation of soluble secreted EGF receptor (sEGF-R, M_r 105 000 form) from A431 cells

sEGF-R was obtained from conditioned media from A431 cells using a modification of the method of Basu *et al.* [45]. Briefly, A431 cells were grown to confluence as described above and then changed into serum free DME for a further 24 h. The medium was collected and centrifuged at 2000 rpm (270 *g*) for 5 min at 4°C in an Hereaus Minifuge to remove cell debris. EDTA, PMSF, Trasylol, Leupeptin (Sigma) and Pepstatin (Sigma) were added to the clarified supernatant at final concentrations of 10 m*M*, 1 m*M*, 100 units/ml, 10 μ*M* and 1 μ*M*, respectively.

Chromatographic purification of sEGF-R

Lectin affinity chromatography. The combined conditioned supernatants of $3–4 \cdot 10^8$ A431 cells (320–360 ml) were concentrated 8-fold by ultrafiltration on YM-30 membranes (45 mm diameter, Amicon, Beverly, MA, USA). The concentrated medium was then incubated, at 4°C for 10 h on an end-over-end rotator, with 1 ml of wheat germ lectin (WGL)–Sepharose (Pharmacia). Unabsorbed material was removed by centrifugation [1200 rpm (65 *g*) for 5 min at 4°C in an Heraeus Minifuge] and the gel washed sequentially, for 2 h at 4°C, with 50-ml volumes of HG buffer (20 m*M* Hepes, pH 7.5, 10% glycerol, 10 μg/ml leupeptin, 2 m*M* PMSF), HG buffer containing 500 m*M* NaCl and finally by a further wash with HG buffer. Bound protein was eluted by incubation, for 2 h at 4°C, with 2 × 2 ml HG buffer containing 600 m*M* N-acetyl-D-glucosamine (Sigma-Aldrich, Castle Hill, NSW, Australia). Eluted material was recovered after "pelleting" the WGL–Sepharose by centrifugation. At each stage of the purification protocol aliquots (40 μl) were tested for EGF-binding activity using the biosensor.

Anion-exchange HPLC on Mono Q HR 5/5. The combined eluates from the WGL–Sepharose were adjusted to pH 8.5, using 20 m*M* Tris-base (pH 9.2) containing 0.02% Tween 20 to promote trace enrichment, before loading onto a Mono Q HR 5/5 anion-exchange column (Pharmacia), which had previously been equilibrated with 20 m*M* Tris–HCl, pH 8.5 containing 0.02% Tween 20. Proteins were eluted from the column, at a flow-rate of 1 ml/min, with a linear 40-min gradient of 0–600 m*M* NaCl in the equilibration buffer. Fractions were collected at 1-min intervals. Aliquots of each fraction (40 μl) were tested for EGF-binding activity using the biosensor.

Size-exclusion HPLC on Superose 12 HR 10/30. Fractions from the Mono Q column with maximum EGF binding activity were pooled and the volume reduced to 800–900 μl by concentration in a SpeedVac centrifuge (Savant Instruments, Hicksville, NY, USA). The concentrate was loaded onto a Superose 12 HR 10/30 column (Pharmacia) and proteins eluted at a flow-rate of 0.5 ml/min with HBS buffer. Fractions were collected at 1-min intervals. Aliquots (75 μl) from each fraction were diluted to 125 μl with HBS buffer for assay on the biosensor.

Micropreparative HPLC on Mono Q PC 1.6/5. Fractions from the Superose 12 size-exclusion column with EGF binding activity were pooled (1.5 ml total) and diluted 4-fold with 20 m*M* Tris-base (pH 9.2) containing 0.02% Tween 20 for trace enrichment by multiple injection onto a micropreparative anion-exchange HPLC column (Mono Q PC 1.6/5) as described previously [27,37]. Retained proteins were eluted, at a flow-rate of 100 μl/min and a column temperature of 25°C, with a linear 40-min aqueous sodium chloride gradient between 0 and 600 m*M* NaCl. The gradient solutions were adjusted to pH 8.5 with 10 m*M* sodium phosphate buffer (final concentration less than 1 m*M*, *i.e.* they were essentially buffer free [27,37]) immediately before use. Fractions were collected at 1-min intervals using the microfraction collector of the SMART system. Aliquots from each fraction (16 μl) were assayed for EGF binding activity following dilution (1:2.5) with the HBS biosensor buffer. Proteins were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on 4–15% gradient Phastgels using a PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden), and visualised by silver staining using a semi-automated protocol for the PhastSystem described previously [41].

Biosensor assay of EGF binding activity in chromatographic fractions

Aliquots of fractions obtained during the purification were assayed directly, or following dilution in HBS, as indicated above. Samples were loaded into the autosampler of the BIAcore and analysed automatically at a constant HBS flow-rate of 5 μl/min using a sensor chip which had previously been derivatised with recombinant hEGF (as described above). Injection volumes were 40 μl. The operating temperature was 25°C. Between measurements, material non-covalently bound to the sensor surface during sample assay was desorbed by injection of 15 μl of a buffer containing 6 *M* guanidine hydrochloride, 0.1 *M* Tris–HCl (pH 8.0), 5 m*M* EDTA and 5 m*M* ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Following desorption, the surface was re-equili-

brated with HBS buffer (25 μl) prior to injection of the next sample. The ability of immobilised EGF to bind sEGF-R was not affected by the regeneration procedure as shown by equivalent binding if duplicate samples were injected sequentially.

RESULTS AND DISCUSSION

Sample compatibility between micropreparative HPLC and the biosensor

We have shown previously the ability of micropreparative HPLC to generate samples in small eluent volumes (at concomitant high concentrations) suitable for microsequence analysis [21,23–28], or subsequent structure–function studies [33,35]. Using short (less than 10 cm) small diameter (1–3 mm I.D.) columns it is routinely possible to elute samples with high recovery in peak volumes of 100 μl or less [21,24,27]. Limits of sensitivity are in the low nanogram range using UV-detection at 214 nm (Fig. 1A). Thus samples can be readily separated, detected and recovered at concentrations of 100 ng/ml or greater. The signal response is linear at least over the range 12–500 ng (Fig. 1B) indicating that no band broadening (and hence eluent sample dilution) occurred over this range. Indeed, in previous studies on reversed-phase micropreparative columns [24,28] we had noted no apparent band broadening even with loads of 5 μg.

For the micropreparative HPLC to be compatible with the biosensor then, ideally, it should be possible to load samples from one instrument to the other without further manipulation. The autoinjection system of the biosensor is capable of injecting accurately samples of between 1 and 50 μl volume. In the case of the SMART system samples can be directly transferred from the microfraction collector of the HPLC to the autosampler racks of the biosensor.

It has been shown [1] that the potential lower limit of SPR detection of the BIAcore is 10 pg/mm^2, which corresponds to a signal of 10 refractance units (RU). Since the detection is based on changes in refractive index at, or close to, the surface, and since the refractive index increment for proteins is constant up to high

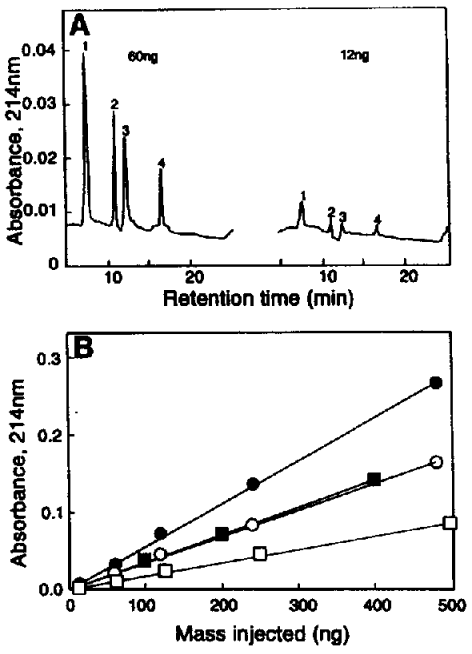
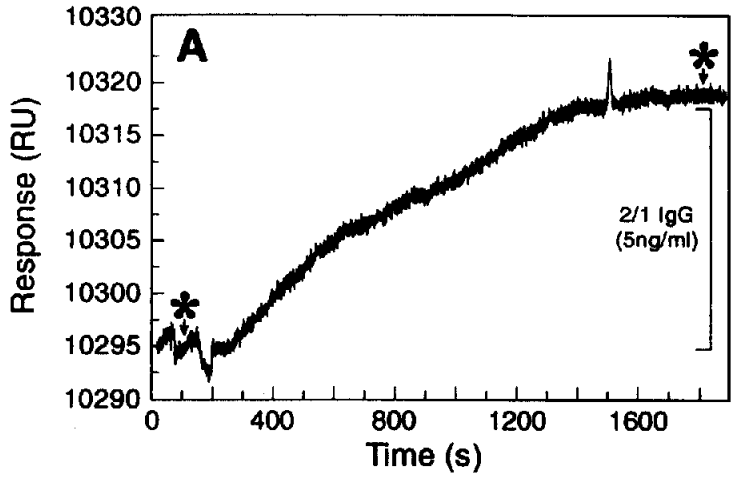


Fig. 1. (A) Sensitivity of detection with micropreparative anion exchange HPLC. Protein standards were separated, at a flow-rate of 100 μl/min, on a Mono Q PC 1.6/5 column (50 × 1.6 mm I.D.) using a linear 50-min gradient between 0 and 1 *M* NaCl in 20 m*M* Tris buffer (pH 7.5). The column temperature was 25°C. Protein standards used were (1) mouse EGF, (2) α-lactalbumin, (3) hEGF and (4) trypsin inhibitor. Chromatograms obtained with loadings of 60 and 12 ng/protein are shown. The absorbance is expressed in absorbance units (AU). (B) Linearity of detector response. The observed peak height (AU, 214 nm) for protein standards, separated on a Mono Q PC 1.6/5 column using the chromatographic conditions described in Fig. 1A, is plotted against the mass of protein standard injected (data from Fig. 1A is included). ● = mEGF, ○ = α-lactalbumin, ■ = rhEGF and □ = trypsin inhibitor.

bulk concentrations [46], the detection limit should be independent of the sample injected, although glycoproteins and lipoproteins have a slightly lower refractive index increment and hence give a slightly lower (approximately 8%) SPR signal [1]. To estimate the sensitivity of detection in terms of bulk analyte concentration these authors [1] calculated, assuming that 40% of the total analyte in the sample could bind to the surface uniformly in a univalent manner, that concentrations of 1 ng/ml would be required for a measurable response.

That such sample concentrations do indeed result in a measurable signal is shown in Fig. 2A.



Time	AbsResp	Baseline	RelResp	Id
103.50	10295	YES	0	INJ_2/1IgG_(0.005µg/ml)
1791.00	10319	NO	24	2/1IgG_(0.005µg/ml)

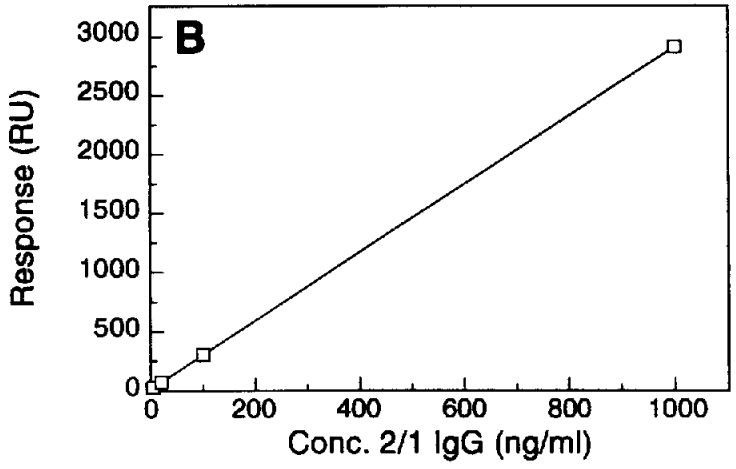


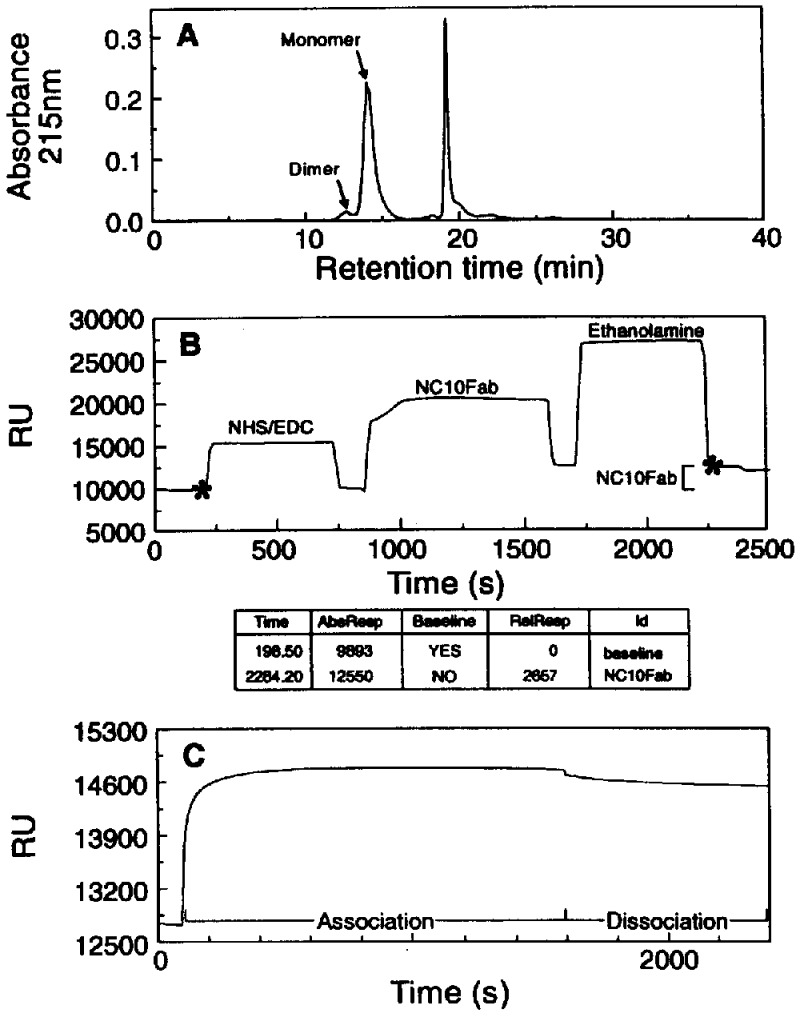
Fig. 2. Low level detection with the BIAcore biosensor. Aliquots (50 μl) of a monoclonal antibody (2/1 IgG) were injected at a flow-rate of 2 μl/min over a sensor surface which had been immobilised with the corresponding peptide antigen (P20). Immobilisation conditions are given in Materials and Methods. The change in detector response (resonance units, RU) with time was recorded as a sensorgram. (A) The sensorgram obtained with 2/1 IgG at a concentration of 5 ng/ml. The corresponding report table is shown below. (B) Linearity of detector response. 2/1 IgG was injected (50-μl aliquots) over the P20 sensor surface at concentrations of 5, 20, 100 and 1000 ng/ml as described above. The relative response (RU) between the initial baseline and the signal at the end of the injection pulse is plotted as a function of the concentration injected.

In this example 50 μl of a high-affinity monoclonal antibody (2/1 IgG, 5 ng/ml), raised against a synthetic peptide (P20) representing the C-terminal 24 residues of the HA1 chain of influenza virus hemmagglutinin [44], were injected, at a flow-rate of 2 μl/min, over the sensor surface onto which had been immobilised the corresponding peptide antigen (P20). A steady increase in the SPR signal with time resulted, with a final relative response of 25 RU being obtained. A linear response was observed for injections of the 2/1 IgG antibody over the concentration range 5–1000 ng/ml (Fig. 2B).

The use of micropreparative HPLC to generate reagents for the biosensor

The specificity of the sensor surface necessarily depends critically on the homogeneity of the

material used for immobilisation, particularly if kinetic studies are to be undertaken. Heterogeneity of the immobilised material frequently gives rise to non-linear plots when analysing data for association or dissociation rate constants [47]. Additionally the presence of any amine containing buffers in the sample "poisons" the N-hydroxysuccinimide coupling via primary amino groups. Amine containing buffers therefore need to be removed before immobilisation. In the example shown (Fig. 3) a Fab fragment of a monoclonal antibody to influenza N9 neuraminidase (NC10 Fab), of which the three-dimensional structure of the antibody/ligand

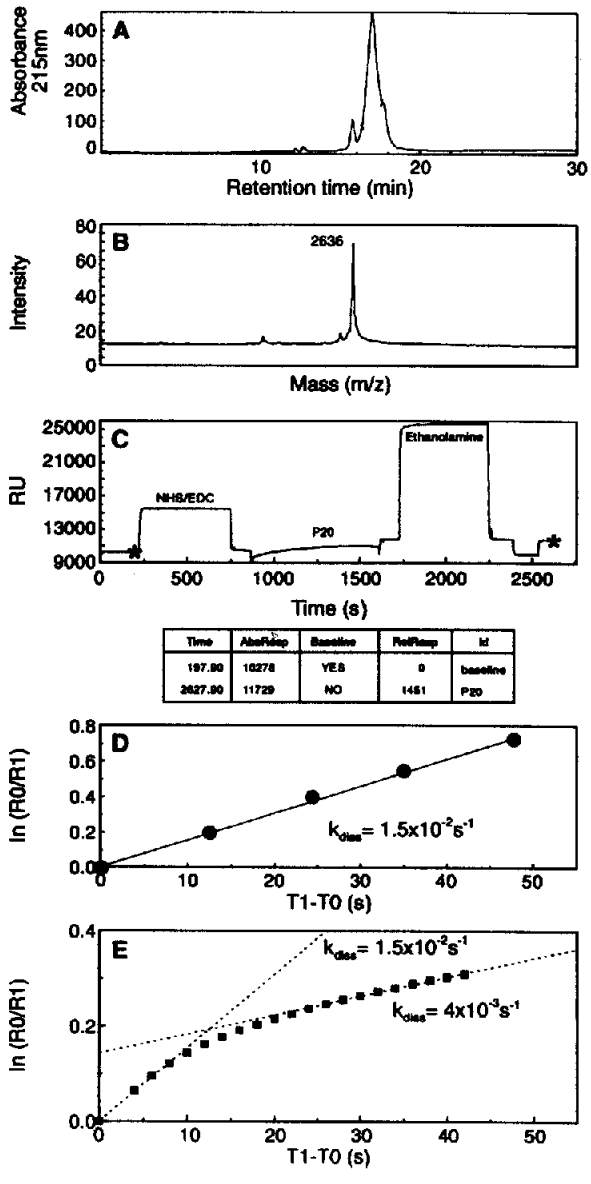


Time	AbsResp	Baseline	RelResp	Id
196.50	9893	YES	0	baseline
2284.20	12550	NO	2657	NC10Fab

Fig. 3. The use of micropreparative size-exclusion HPLC to purify and buffer exchange a Fab fragment for immobilisation onto the BIAcore. (A) NC1O Fab (100 μg/ml, 100 μl injection volume) in 50 m*M* Tris buffer (pH 8.0) was purified and buffer exchanged into a buffer compatible with NHS–EDC immobilisation using a Superose 12 PC 3.2/30 column. The mobile phase was 20 m*M* sodium acetate (pH 4.5) and the flow-rate 100 μl/min. Column temperature was 25°C. Note the presence of a small impurity with the apparent molecular mass of the dimer. (B) The HPLC-purified NC10 Fab was adjusted to a concentration of 50 μg/ml with 20 m*M* sodium acetate buffer (pH 4.5) and immobilised onto the sensor surface as described in Materials and Methods. The resulting sensorgram and associated report table are shown. The regions of the sensorgram corresponding to the surface activation with NHS–EDC, the covalent binding of the NC10 Fab fragment and subsequent blocking of residual activated ester groups with ethanolamine are indicated. The net relative response (2657 RU) corresponds to a surface concentration of approximately 2.6 ng/mm^2 [1]. (C) Binding of tern neuraminidase to the immobilised NC10 Fab. To confirm the viability of the sensor surface, tern neuraminidase (100 μg/ml, 50-μl injection) was passed, at a flow-rate of 2 μl/min, over the sensor surface. The association and dissociation phases of the resultant sensorgram are indicated.

complex has been determined by X-ray crystallography [48], was required for immobilisation onto the sensor surface. An aliquot of the NC10 Fab (100 μl, 100 μg/ml in 50 m*M* Tris buffer, pH 8.0) was purified and buffer exchanged into a buffer compatible with NHS/EDC immobilisation (10 m*M* sodium acetate, pH 4.5) using a Superose 12 PC 3.2/30 micro size-exclusion column (Fig. 3A). The NC10 Fab fragment was effectively buffer exchanged and at the same time separated from a small impurity with an apparent molecular mass of the dimer. The purified NC10 Fab fragment, which was recovered in a volume of 150 μl, was made up to 200 μl (final concentration 50 μg/ml) with the 10 m*M* sodium acetate buffer, and used for immobilisation onto the sensor chip (Fig. 3B). The net increase in signal following the immobilisation (2657 RU) corresponds to a NC10 Fab surface concentration of approximately 2.6 ng/mm^2 [1,49]. The ability of the immobilised antibody to bind neuraminidase is shown in Fig. 3C. A sample (50-μl injection) of tern neuraminidase (100 μg/ml) was passed over the immobilised NC10 Fab (see Fig. 3B) at a flow-rate of 2 μl/min. A positive response of 2187 RU was observed, demonstrating the integrity of the immobilised HPLC purified NC10 Fab.

A further example of the use of micropreparative HPLC to generate suitable reagents for immobilisation is given in Fig. 4. In this case a synthetic peptide, P20, corresponding to residues 306–328 of the C-terminus of the HA1 chain of the influenza virus hemmagglutinin subtype H3 [44] was purified by RP-HPLC on a Pharmacia μRPC C2/C18 3.2/3 column prior to immobilisation. A major species was resolved from an earlier eluting contaminant, as well as a later eluting shoulder (Fig. 4A). MALD-MS of the RP-HPLC purified P20, using 1 μl of the eluent



Time	AbsRdap	Baseline	RelRdap	Id
197.90	16278	YES	0	baseline
2627.90	11729	NO	1451	P20

Fig. 4. The use of micropreparative RP-HPLC to purify a synthetic hemagglutinin peptide prior to immobilisation on the BIAcore. (A) A sample (7 μg) of a synthetic peptide (P20) corresponding to residues 306–328 of the C-terminus of the HA1 chain of influenza virus hemagglutinin subtype H3 [44] was purified by RP-HPLC on a Pharmacia μRPC PC 3.2/3 column (30 × 3.2 mm I.D.). Proteins were eluted with a linear 60-min gradient between 0.15% (v/v) trifluoroacetic acid (TFA) and 60% aqueous acetonitrile containing 0.125% (v/v) TFA. The flow-rate was 240 μl/min and the column temperature 45°C. The sample was recovered manually as indicated by the dashes. Absorbance is expressed in mAU. (B) An aliquot (1 μl) of the RP-HPLC purified P20 was analysed by MALD-MS as described in Materials and Methods. (C) The remainder of the RP-HPLC purified P20 was dried using a Speed Vac concentrator, and redissolved in 10 m*M* sodium acetate buffer (pH 6.0) for immobilisation onto the sensor surface using NHS–EDC chemistry as described in Materials and Methods. (D) Following binding of a Fab' fraction of a monoclonal antibody raised against peptide P20 (1/1 Fab') to the sensor surface, dissociation was monitored under conditions of constant HBS buffer flow. R_0 and R_1 refer to the resonance values at times T_0 and T_1 respectively. A linear first order dissociation plot was obtained. (E) If peptide P20 was immobilised without prior RP-HPLC purification a markedly biphasic dissociation plot was obtained.

fraction, yielded the anticipated mass of the synthetic peptide. As we have noted previously [41] the concentration of proteins or peptides recovered from micropreparative columns is ideally suited to direct analysis by MALD-MS. The recovered peptide was dried using a SpeedVac concentrator and redissolved in 10 m*M* sodium acetate buffer, pH 6.0, (final concentration 50 μg/ml) for immobilisation onto the sensor surface using the NHS–EDC chemistry (Fig. 4C). A surface concentration of approximately 1.5 ng/mm^2 was achieved. When the surface was exposed to a Fab' fragment of a monoclonal antibody raised against this peptide (1/1 Fab') and, following association, the dissociation monitored at constant buffer flow, linear first order kinetics were observed (Fig. 4D) with an apparent k_{diss} of $1.5 \cdot 10^{-2}$ s^{-1}. When the peptide was immobilised without prior RP-HPLC purification a markedly biphasic dissociation plot was observed (Fig. 4E).

Since the SPR detector is sensitive to refractive index changes at, or near to, the surface, the system is sensitive to differences in sample or buffer components (note the rapid changes in signal at the beginning and end of the injection cycles during immobilisation, Figs. 3B and 4C). Whilst measurements can be made at "steady state" conditions when buffer alone is flowing between injections, buffer-related refractive index changes confuse interpretation of the data when analysing equilibrium binding, with rapid dissociation kinetics, or when studying weak or low level interactions. In order to minimise such refractive index effects, we have used microdesalting columns (Pharmacia Fast Desalting PC 3.2/10) to buffer-exchange samples into the BIAcore running buffer. In the example shown (Fig. 5A) an aliquot (50 μl) of the Triton X-100 membrane extract of A431 cells, containing the M_r 170 000 form of the EGF-R [50], was injected at a flow-rate of 5 μl/min over a sensor chip onto which recombinant hEGF had been immobilised. Because of refractive index differences between the Triton X-100 membrane extract and the HBS buffer, a buffer-related decrease of 2450 RU was observed at the end of the injection cycle. The overall relative response due to the specific interaction between the EGF-R and the hEGF on the sensor surface was 1479 RU. An aliquot of the membrane extract (100 μl) was then desalted in the HBS running buffer used on the BIAcore (Fig. 5B). The protein fraction was recovered, as indicated, in an eluent volume of 140 μl. Injection of this fraction (50 μl, flow-rate 5 μl/min) over the hEGF sensor surface (Fig. 5C) gave rise to a final relative response of 1064 RU, with a refractive index change of only 94 RU at the end on the injection cycle (*cf.* Fig. 5A). It should be noted that the Triton X-100 membrane extract contains proteins which will not bind to hEGF which account for the small negative refractive index change observed at the end of the injection cycle. Since the desalted sample has been diluted during the chromatographic procedure (100–140 μl), the relative EGF-R related binding responses observed (1479 and 1064 RU, Fig. 5A and C, respectively) suggest that recovery from the micro-desalting column was essentially quantitative (a dose–response curve of the A431 Triton X-100 membrane extract in HBS was linear over the range 50–1500 RU (correlation coefficient 0.999), data not shown).

The use of the biosensor to monitor fractions during chromatographic purification

Having immobilised a specific reagent onto the sensor surface, the BIAcore can be used to screen rapidly a wide range of biological extracts for possible sources of interacting proteins. Once a source has been identified, then the biosensor can be used to monitor the purification of the protein of interest. To demonstrate the potential of such co-operativity we have used the biosensor, with recombinant hEGF immobilised onto the surface, to develop and monitor a purification protocol for sEGF-R (M_r 105 000 form) secreted into tissue culture medium by A431 cells [40].

Highly purified sEGF-R, in a form suitable for immobilisation onto the sensor surface, was obtained from concentrated A431 cell conditioned supernatants by sequential use of WGL affinity chromatography, anion-exchange, size-exclusion and micropreparative HPLC.

BIAcore-measurements of crude supernatant, wash buffers (see Materials and Methods) and eluates from the WGL–Sepharose indicated that approximately 65% of the EGF binding activity

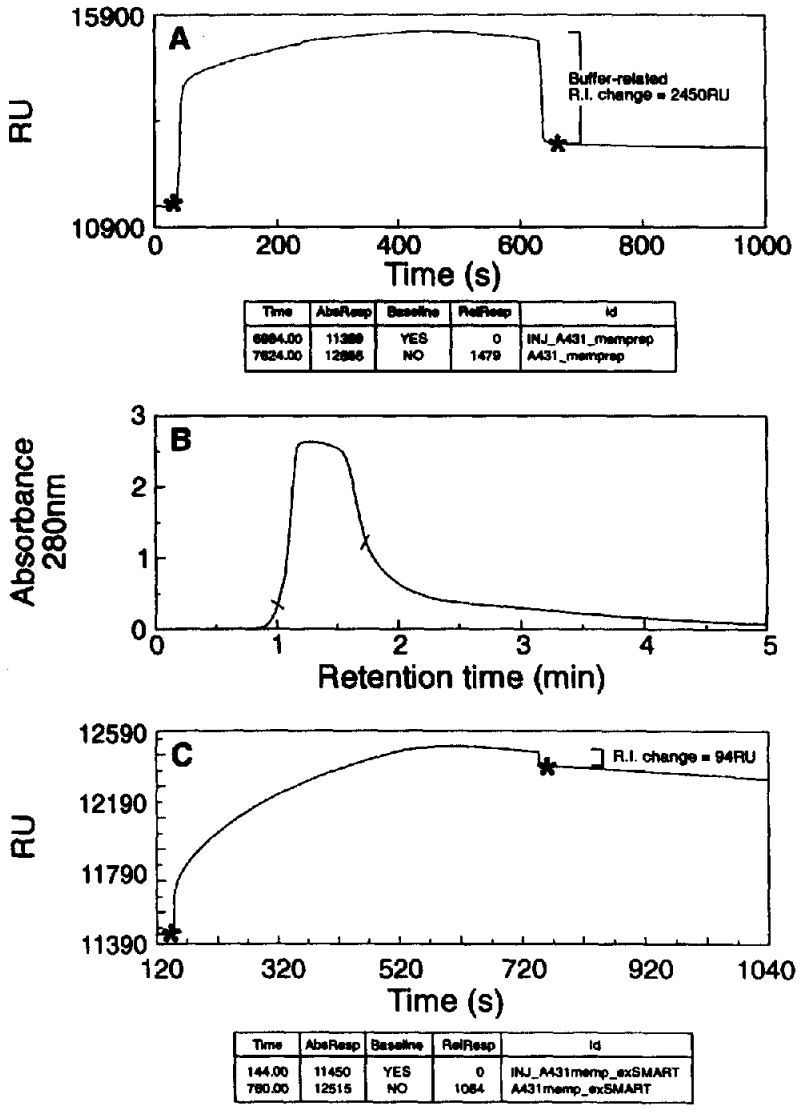


Fig. 5. The use of a micropreparative Fast Desalting column to buffer exchange samples into BIAcore buffer. (A) An aliquot (50 μl) of a Triton X-100 extract of A431 membranes containing hEGF receptor [50] was injected, at a flow-rate of 5 μl/min, over the sensor surface onto which hEGF had been immobilised. Note the rapid buffer related decrease in response (−2450 RU) at the end of the injection pulse. (B) An aliquot (50 μl) of the Triton X-100 extract was desalted into the BIAcore buffer (see Materials and Methods) using a Pharmacia Fast Desalting PC 3.2/10 column. The flow-rate was 180 μl/min and the column temperature 25°C. The desalted sample was recovered, as indicated by the dashes, in a volume of 140 μl. (C) The buffer exchanged sample from the Fast Desalting column (panel B) was injected over the hEGF sensor surface as described in (A). Note the greatly reduced buffer related signal decrease (−94 RU) at the end of the injection pulse compared with that observed with the same extract before buffer exchange (see panel A).

of the starting material was found in the unbound fraction, although the amount of lectin used in our protocol was in 10-fold excess compared to a previously reported method of Basu *et al.* [45]. Further incubation of the unbound fraction with an additional 2 ml of fresh WGL–Sepharose only reduced this value by a further 15% (data not shown), suggesting carbohydrate heterogeneity of the EGF-binding components of the starting material. Indeed, Hurwitz *et al.* [51] have shown that sEGF-R from A431 cells could be resolved into at least 24 discrete bands

TABLE I

RECOVERY DATA FOR THE PURIFICATION OF sEGF-R OBTAINED USING THE BIOSENSOR

Stage	RU loaded[a]	RU recovered[a]	Stage recovery (%)	Overall recovery (%)
WGL-MonoQ	63 938	45 100	71	71
Mono Q-Sup 12	30 450	14 196	49	22.5[b]
Sup12-μMono Q	9146	7525	82	12[b]
μMono Q No. 7	N/A[c]	1436	N/A	2.2
μMono Q No. 8	N/A	406	N/A	0.6
μMono Q No. 9	N/A	1244	N/A	1.9
μMono Q No. 10	N/A	1923	N/A	3.0
μMono Q No. 11	N/A	1931	N/A	3.0
μMono Q No. 12	N/A	581	N/A	0.9

[a] The total signal was obtained by extrapolating the signal observed with a given injection volume to the total sample volume.
[b] The difference between the overall recovery and the cumulative stage recovery is due to the fact that only the major active fractions from the Mono Q HR 5/5 (Nos. 9–12) and Superose 12 columns (Nos. 21–23) were used at the next stage of the purification.
[c] N/A = Calculation not applicable to these fractions.

by isoelectric focusing. Only the sEGF-R which binds to WGL–Sepharose [45] was taken for further purification, and EGF-binding activity in the eluate was given a relative value of 100% for subsequent recovery calculations (Table I). Anion-exchange HPLC of this preparation (Fig. 6A) yielded a complex elution profile, demonstrating further the heterogeneity of the preparation. SDS-PAGE of the WGL eluate demonstrated the presence of at least 10 protein components, ranging in M_r from 20 000–200 000 (Fig. 6A, inset). This finding is in marked contrast to the data of Basu *et al.* [45], who claimed to obtain homogeneous sEGF-R from A431 cell conditioned medium by the sole use of WGL chromatography.

Biosensor measurements revealed that fractions with EGF-binding activity eluted from the Mono Q column as a distinct peak between 8 and 13 min, corresponding to a salt concentration of 100–200 m*M*. This is in good agreement with the data of Lax *et al.* [52] who found that recombinant sEGF-R expressed in Chinese hampster ovary (CHO) cells also eluted from a Mono Q column between 150 and 200 m*M* NaCl. By contrast, sEGF-R produced using a baculovirus expression system eluted from a Mono Q column between 75 and 100 m*M* NaCl [53], presumably due to alternative patterns of glycosylation. SDS-PAGE of the peak fractions of EGF binding activity from the Mono Q HR 5/5 column (Fractions 9, 10, and 11, Lanes 4–6 in Fig. 6A) showed the presence of a major protein band with an apparent M_r of 110 000. The total activity recovered (expressed in response units, RU) for the peak fractions was 71% relative to the WGL-eluate (Table I).

Further separation of the sEGF-R from contaminating proteins was achieved by size-exclusion HPLC on Superose 12 HR 10/30 of concentrated active Mono Q fractions (Fig. 6B). A major peak of binding activity (fractions 21–24) corresponding to a protein with an apparent M_r of 100 000 was observed. SDS-PAGE of these fractions (inset) showed that fractions 21, 22 and 23 were highly enriched for the M_r 110 000 component. Fraction 24 also contained proteins of M_r 80 000, 65 000 and 55 000 (data not shown), and was therefore not taken for further purification. A total of 49% of the applied activity was recovered from the size-exclusion column (Table I).

Final micropreparative anion-exchange chromatography [27,37] performed on a Pharmacia SMART system (Fig. 6C) concentrated the sEGF-R preparation into a buffer suitable for immobilisation onto the sensor chip of the biosensor. The total recovery of activity from this column was 82% (Table I). This activity was associated with an early eluting sharp peak, and

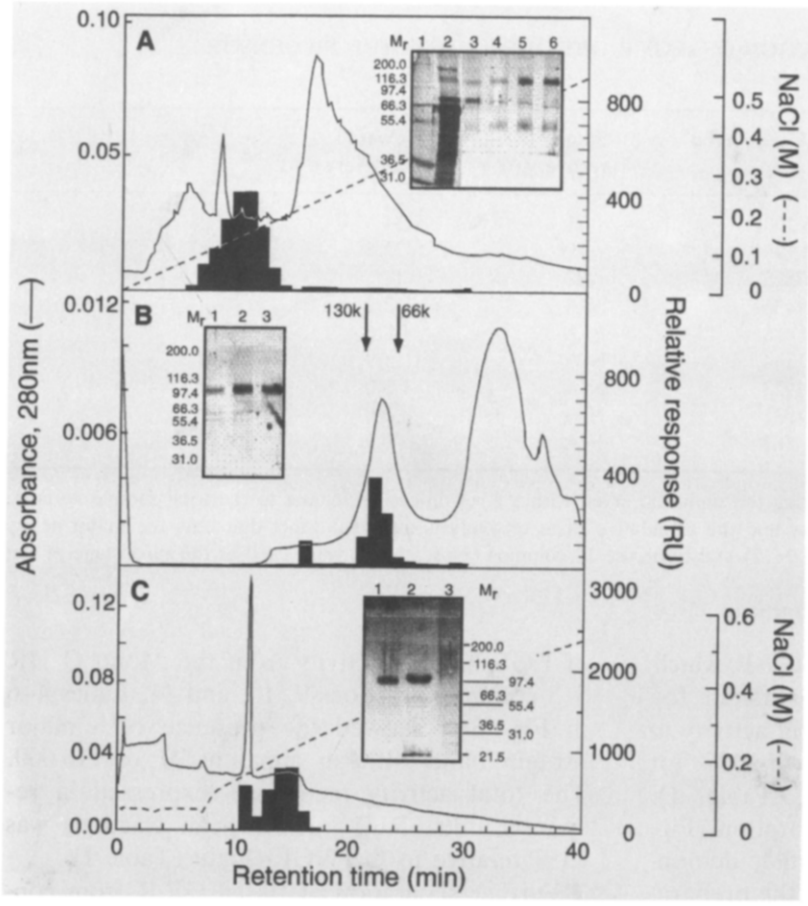


Fig. 6. The use of the biosensor to monitor the purification of sEGF-R. Conditioned medium from A431 cells, containing sEGF-R [40,45], was concentrated and purified on WGL–Sepharose as described in Materials and Methods. (A) The WGL eluates were adjusted to pH 8.5 and purified on a Mono Q HR 5/5 column. Chromatographic conditions are given in Materials and Methods. 1-min fractions were collected and aliquots (40 μl) tested for EGF-binding activity using the biosensor (■). Inset: SDS-PAGE analysis of (Lane 1) molecular mass standards, (Lane 2) A431 conditioned medium concentrate, (Lane 3) WGL–Sepharose eluent, (Lanes 4–6) fractions 9, 10, and 11 respectively from the Mono Q HR 5/5 column. (B) Size-exclusion HPLC of active sEGF-R fractions from Mono Q HR 5/5. Fractions 9–12 from the Mono Q 5/5 column (see panel A) were concentrated and loaded onto a Superose 12 HR 10/30 size-exclusion column. Experimental conditions are given in Materials and Methods. Fractions were collected at 1-min intervals. Aliquots (20 μl) from each fraction were taken for biosensor assay (■). Inset: SDS-PAGE analysis of (Lane 1) fraction 21, (Lane 2) fraction 22, (Lane 3) fraction 23. (C) Micropreparative HPLC on Mono Q PC 1.6/5. Fractions 21–23 from the Superose 12 column (see Panel B) were concentrated and buffer exchanged using a Mono Q PC 1.6/5 micropreparative anion-exchange column. Experimental details are given in Materials and Methods. Fractions were collected at 1-min intervals and aliquots (16 μl) were assayed for EGF binding activity (■) following dilution (1:2.5) with HBS biosensor buffer. Inset: SDS-PAGE analysis of (Lane 1) fraction 12, (Lane 2) fraction 15, (Lane 3) molecular mass standards.

a later eluting, more diffuse, component (Fig. 6C). SDS-PAGE analysis of these peak fractions (Fig. 6C, inset) demonstrated essentially homogeneous proteins of M_r 100 000–105 000, in agreement with the previously reported size of sEGF-R [40,45]. Careful investigation of the gel showed that the early eluting species had a slightly lower M_r than the later eluting form(s), suggesting that glycosylated variants had been resolved.

Biosensor analysis of EGF-binding ability provided a reliable and rapid means of monitoring the recovery from the various column matrices used during the purification. Since BIAcore measurements are compatible with the buffer systems used in the purification protocol, no sample preparation was necessary prior to the assay, therefore minimising both sample consumption and manipulation between purification

steps. In addition to the quantitative data obtained from the relative response observed at the completion of the biosensor injection cycle, we also noted that analysis of the dissociation phase of the sensorgram provided a very sensitive and specific distinction between "specific" and "non-specific" responses (Fig. 7). When crude A431 supernatant was applied to the BIAcore (Fig. 7A), at the end of the injection cycle, and following the buffer related refractive index change, a dissociation curve was obtained (Fig. 7B) which showed that, whilst a minor component of the preparation showed a rapid initial dissociation ($k_{diss1} = 1.4 \cdot 10^{-2}$ s^{-1}), the majority of the bound activity exhibited a much slower dissociation ($k_{diss2} = 7.6 \cdot 10^{-4}$ s^{-1}). Indeed, after

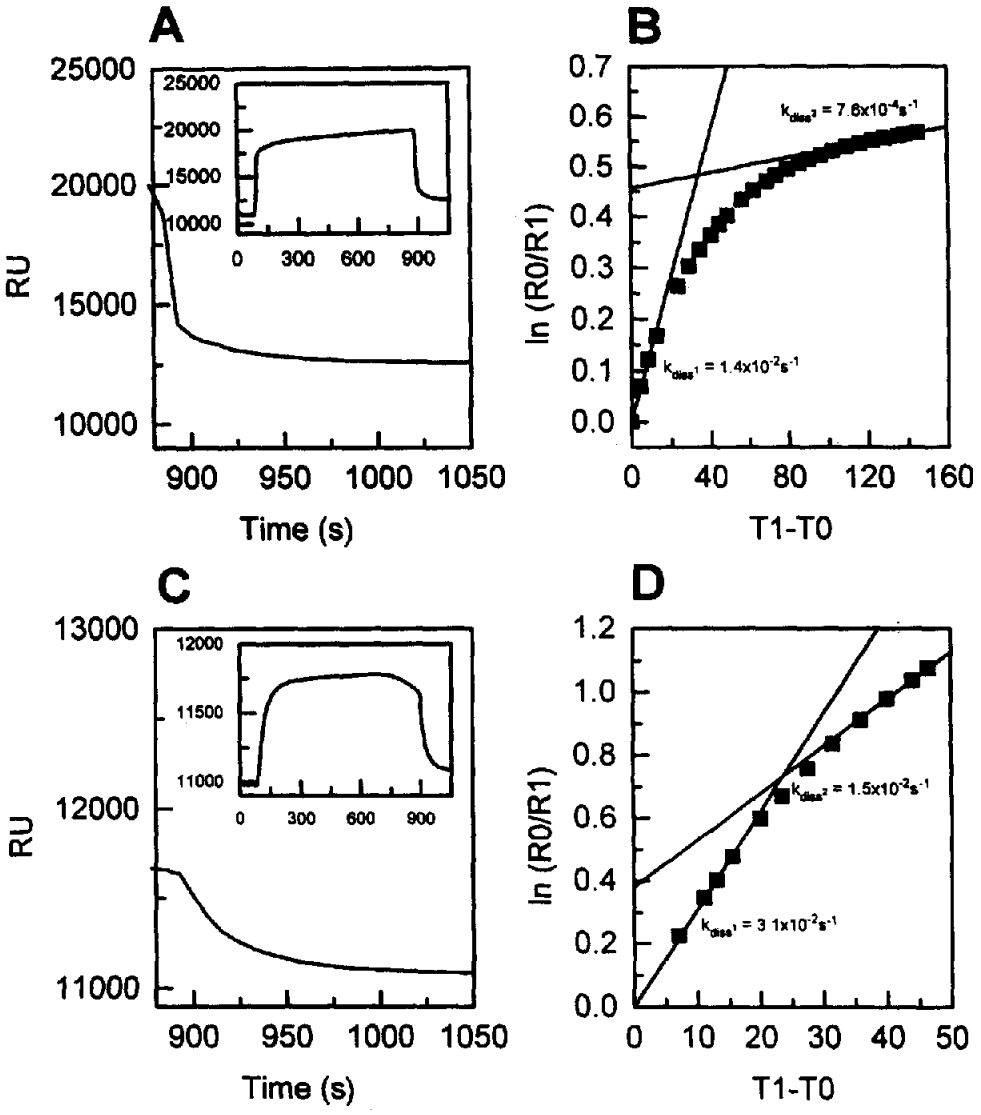


Fig. 7. Kinetic analysis of sEGF-R chromatographic fractions. (A) Sensorgram showing the dissociation of bound material in the A431 supernatant from hEGF under constant buffer flow (3 μl/min). Inset: sensorgram showing the total interaction curve for the injection of A431 supernatant (40 μl). (B) Dissociation plot obtained from the data in Panel A. (C) Sensorgram showing the dissociation of purified sEGF-R from hEGF under constant buffer flow (3 μl/min). Inset: sensorgram showing the total interaction curve for the injection of purified sEGF-R (40 μl). (D) Dissociation plot obtained from the data in panel C.

160 s only 55% of the bound material had dissociated. Fractionation of the crude A431 supernatant on WGL–Sepharose resulted in the separation of EGF binding activity into a lectin-bound component characterised by a fast off-rate and a non-bound component with negligible dissociation from the immobilised EGF. Biosensor assays of fractions from size-exclusion HPLC of the non-bound component indicated no activity in the expected molecular mass range of sEGF-R, the activity eluting predominantly in the void volume of the column (data not shown). The biosensor analysis of the purified fraction obtained from the micropreparative anion-exchange column (Fig. 6C) also displayed an initial fast off rate ($k_{diss1} = 3.1 \cdot 10^{-2}$ s^{-1}, Fig. 6D). Although this dissociation plot also indicated a biphasic dissociation, the later dissociation was still rapid ($k_{diss2} = 1.5 \cdot 10^{-2}$ s^{-1}) In this case 70% of the bound material had dissociated after 50 s. It has been suggested [16] that biphasic dissociation plots may be due, in part, to rebinding of dissociated material before it can diffuse out of the matrix. However, similar dissociation rates (k_{diss} $6.2 \cdot 10^{-2}$ s^{-1} [54]) have been observed for biosensor measurements of the dissociation of sEGF-R, purified from CHO cell expression systems [52], from hEGF. By providing qualitative data on the interaction between purified protein and known (*i.e.*, immobilised) ligand during final purification stages the kinetic information derived from the biosensor detection system can deliver complimentary and very specific information on the characteristics of the purified molecule.

The use of the biosensor as a detector as described above would appear to be particularly valuable in the search for "unknown ligands" for, as an example, antibodies raised against complex mixtures of proteins and carbohydrate, or for new members of gene families (*e.g.* tyrosine kinases [38,39]) which have been elucidated from sequence homologies and PCR-based molecular biology techniques. In such cases the biosensor can firstly be used to screen for possible sources of the ligand (*e.g.* tissue extracts, conditioned media) and then subsequently be used as a specific detector for monitoring fractions obtained during chromatographic purification of the ligand of interest, provided that sufficient quantities of the purified recombinantly derived target protein (1–10 μg), at suitable concentration (typically 10–100 μg/ml), can be generated for the initial immobilisation. Additionally the use of affinity chromatographic procedures as part of the purification protocol would appear advantageous, providing that suitable elution conditions can be defined.

Optimisation of elution conditions for affinity chromatography

It is possible to consider the BIAcore as being analagous to an analytical affinity chromatography system, using step wise elution conditions with "on-column" detection. Additionally the NHS–EDC immobilisation chemistry used with the biosensor is similar to that offered with several currently available affinity chromatography supports [*e.g.* Affi-Gel 10 (Biorad), NHS–Superose (Pharmacia)], so that similar immobilised surfaces can be compared. By defining immobilisation and binding conditions for the sensor chip which can be directly translated to the corresponding affinity chromatography system, it is then possible to use the biosensor to rapidly investigate a series of elution/desorption conditions, and quantitatively check the surface (and the ligand treated under similar conditions) for denaturation following the analysis. The BIAcore can also be used to screen a range of antibodies to determine those which have suitable association/dissociation characteristics and stability for use as affinity matrices [14,55].

In the example presented herein we have investigated the use of the biosensor to define suitable conditions for the affinity purification of influenza virus N9 neuraminidase. A Fab fraction (NC41 Fab) of a monoclonal antibody raised against neuraminidase heads [43] was immobilised onto the sensor surface as described in Materials and Methods. Injection of tern neuraminidase (100 μg/ml, 50 μl injection, flow-rate 2 μl/min) over the NC41 Fab surface gave a positive response of 5213 RU (Fig. 8A), demonstrating the ability of the Fab fraction to bind the neuraminidase following immobilisation. Additionally, the shape of the sensorgram suggested rapid association and slow dissociation phases

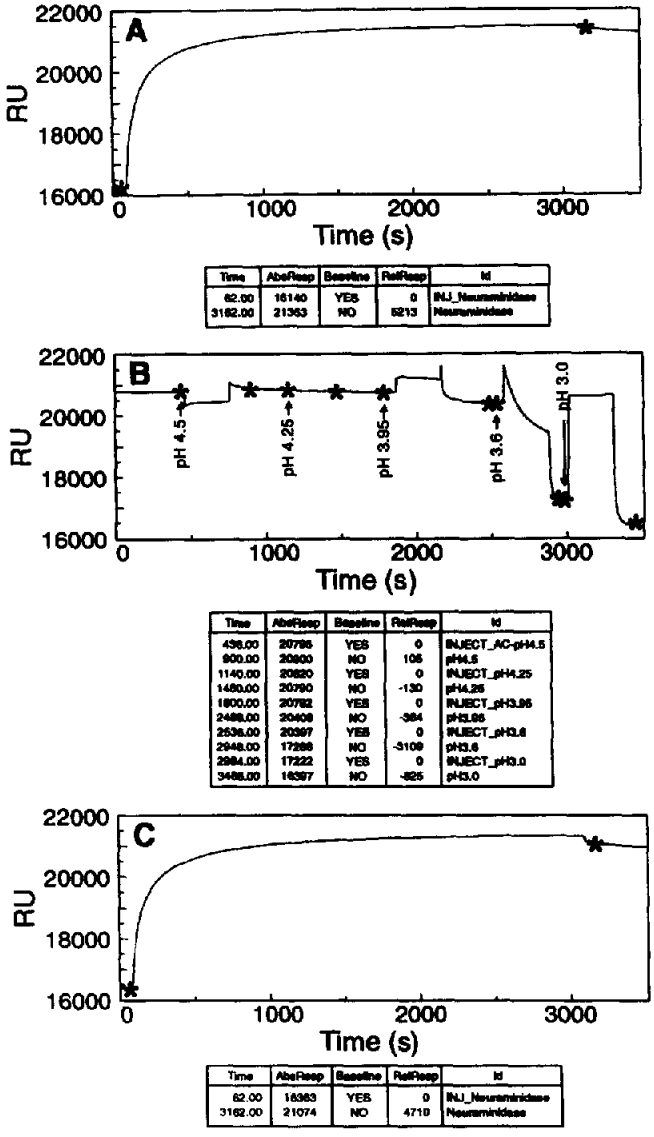


Fig. 8. The use of the biosensor to determine elution conditions for affinity chromatography. (A) Tern neuraminidase (100 μg/ml, 50-μl injection) was passed, at a flow-rate of 1 μl/min, over a sensor surface onto which NC41 Fab had been immobilised. (B) Following binding of the tern neuraminidase (Panel A), aliquots (25 μl) of buffers of decreasing pH were sequentially injected over the sensor surface. The flow was 5 μl/min. (C) Following desorption at pH 3.0 (Panel B) the NC41 Fab was rechallenged with tern neuraminidase using the conditions described in Panel A.

which are ideal characteristics for antibodies to be used in affinity supports, providing that suitable desorption conditions can be found. Following binding the flow-rate was increased to 5 μl/min and sequential 25-μl injections were performed using buffers of progressively reducing pH (Fig. 8B). Injection of pH 4.5 or pH 4.25 buffer caused no desorption. The use of pH 3.95 buffer caused desorption of 8.2% of the bound neuraminidase. Approximately 67% desorption was evident after lowering the pH to 3.6, with a further 18% eluted at pH 3.0.

When the sensor surface was rechallenged with tern neuraminidase (injection conditions as

for Fig. 8A), a response of 4710 RU was obtained (Fig. 8C), showing that the surface was at least 90% viable following the desorption at pH 3.0. The conditions derived using the BIAcore for the elution of tern neuraminidase from NC41 Fab can be used successfully for affinity purification (L.C. Gruen, personal communication).

CONCLUSIONS

We have presented a number of examples of the synergy between micropreparative HPLC and an optical biosensor (Pharmacia BIAcore). The eluent sample volumes and concentration achieved with the HPLC have been shown to be ideally suited for subsequent manipulation on the biosensor. We have also shown how the biosensor may be used as a detector to monitor the purification of fractions from the HPLC, and how it may be used to define elution conditions for affinity chromatography. Thus there is a "bidirectional" synergy between these two techniques which will facilitate the development of efficient purification schemes and permit quantitative kinetic analyses of the interactions.

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