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Affinity chromatography on immobilized ‘‘biomimetic’’ ligands Synthesis, immobilization and chromatographic assessment of an immunoglobulin G-binding ligand

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Abstract

A synthetic bifunctional ligand (22/8) comprising a triazine scaffold substituted with 3-aminophenol (22) and 4-amino-1-naphthol (8) has been designed, synthesised, characterised and immobilized on agarose beads to create a robust, highly selective affinity adsorbent for human immunoglobulin G (IgG). Scatchard analysis of the binding isotherm for IgG on immobilized 22/8 (90 μmol 22/8/g moist weight gel) indicated an affinity constant (K_a) of $1.4 \cdot 10^5\ M^{-1}$ and a theoretical maximum capacity of 151.9 mg IgG/g moist weight gel. The adsorbent shows similar selectivity to immobilized protein A and binds IgG from a number of species. An apparent capacity of 51.9 mg human IgG/g moist weight gel was observed under the experimental conditions selected for adsorption. Human IgG was eluted with glycine–HCl buffer with a recovery of 67–69% and a purity of 97.3–99.2%, depending on the pH value of the buffer used for elution. Preparative chromatography of IgG from human plasma showed that under the specified conditions, 94.4% of plasma IgG was adsorbed and 60% subsequently eluted with a purity of 92.5%. The immobilized ligand was able to withstand incubation in 1 *M* NaOH for 7 days without loss of binding capacity for IgG.

Keywords: Biomimetic ligands; Combinatorial chemistry; Immunoglobulin

1. Introduction

Synthetic affinity ligands are ideally suited to the purification of high value biopharmaceutical proteins since they are inexpensive, chemically defined, non-toxic and contain no fissile bonds. They are resistant to both chemical and biological degradation, are sterilizable and cleanable in situ, and are readily immobilised to generate highly selective affinity adsorbents which display workable capacities for their complementary proteins [1–4]. The design and synthesis of such synthetic ligands has been greatly assisted by increased access to structural data, recent advances in computer-aided molecular design and the proliferation of novel combinatorial chemical techniques [5,6]. These approaches have recently been used to design and synthesise a ligand that mimics a key dipeptide motif on fragment B of

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protein A and which is known to play a pivotal role in the interaction with the Fc fragment of immunoglobulin G (IgG) [7]. This synthetic protein A mimic was shown to interact with human IgG and to be able to purify IgG selectively from diluted human plasma, ascites fluid and fetal calf serum. A subsequent study showed that this structure could be further refined and optimised by constructing combinatorial libraries of near-neighbour ligands based on the original lead compound [8]. This IgG-binding ligand library was synthesised by a solid phase assembly procedure on an agarose matrix using a modified ''mix-and-split'' procedure [9] with immobilized cyanuric chloride as the bifunctional scaffold. The triazine scaffold serves as the spatial framework for the display of attached functional groups and has been shown to deliver effective protein binding ligands [3,4,7,8]. The library was screened for the binding of pure human IgG, whence successful ligands were further assessed for their IgG specificity by determining the purity and recovery of IgG from human plasma. It was found that ligands comprising 3-aminophenol and aminonaphthol moieties substituted on the triazine nucleus were highly effective in binding human IgG; in particular, the lead immobilized ligand (22/8) displayed a high capacity for IgG from diluted human plasma and was able to purify the protein in high yield (>95%) and purity (>99%) [8].

The solid phase assembly procedure for synthesising affinity adsorbents is both facile and rapid, although it does suffer the disadvantage of introducing uncertainties in the chemical composition of the immobilized ligands. This is because it is not possible to purify intermediates and the final product may contain impurities created by side or incomplete reactions on the solid state matrix. It was thus considered essential to determine and confirm the structure of the lead ligand identified to bind IgG. The synthesis of the lead ligand in solution allows validation of each synthetic step, chemical characterisation of intermediates, confirmation of the final structure and immobilization onto the solid phase of a defined chemical entity with specified stability, toxicity and efficacy. This paper describes the solution synthesis, characterisation, immobilization and chromatographic evaluation of a fully characterised IgG-binding ligand comprising a triazine scaffold substituted with 3-aminophenol and 4-amino-1-naphthol.

2. Experimental

2.1. Materials

All chemicals were of the highest purity available unless otherwise stated. Butan-1-ol, hydrochloric acid (analytical grade), cyanuric chloride, hexane-1,6-diamine and 4-amino-1-naphthol hydrochloride were purchased from Aldrich (Gillingham, UK). Sodium carbonate, sodium bicarbonate, Coomassie Blue R-250, ammonium persulphate, *N*,*N*,*N*′*N*′-tetraethylenediamine (TEMED) and sodium dodecyl sulphate were purchased from Sigma (Poole, UK). Sodium hydroxide, ethanol, ethyl acetate, hexane, methanol, acetone, acetic acid, dimethyl formamide (DMF) and all other common solvents were provided by Fisons (Loughborough, UK). Immunoglobulin G from mouse, sheep, goat, rat, horse, pig, rabbit, cow and chicken were supplied by Sigma. Immobilized protein A was obtained from ProMetic BioSciences (Ballasala, Isle of Man, UK). Fresh frozen plasma was obtained from Baxter Healthcare (Thetford, UK). Sepharose 6B was purchased from Pharmacia Biotechnology (Milton Keynes, UK). Silica gel (40–63 μm) was supplied by Sigma. Analytical thin-layer chromatography plates coated with a 0.25 mm layer of silica gel containing a UV 254 nm fluorescent indicator were purchased from Aldrich. The reagent was applied using a spray unit purchased from Sigma.

2.2. Instrumentation

^{1}H nuclear magnetic resonance (NMR) spectra were performed either on a Bruker WP 250 MHz machine or on a Jeol JNM Lambda LA400 FT NMR spectrometer. Mass spectra were recorded on AEI MS30 or AEI MS50 mass spectrometers in electron impact mode in the Chemical Laboratory, University of Cambridge, Cambridge, UK. Fourier transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR instrument. Analytical thin-layer chromatography (TLC) was performed on commercial Merck plates coated with kieselgel 60 F_{254} (0.25 mm thick). Molecular modelling, molecular design and all calculations were performed on a Silicon Graphics 4D/35 personal Iris workstation from Silicon Graphics, with a Quanta 4.0 Software package from Molecular Simulations (Burlington,

MA, USA). The ultrasonic bath was a Decon FS200 from Decon Labs. (Hove, UK). The rotary evaporator was a Buchi RE111 with a Buchi 461 water bath supplied by Orme Scientific (Middleton, UK). The freeze drier was an Edwards Modulyo, operating at below 0.1 Torr and −40°C, Model Pitani 10 from Edwards High Vacuum (Crawley, UK) (1 Torr=133.322 Pa). The UV spectrometer was a Perkin-Elmer Lambda 16 UV–Vis spectrometer. The centrifuge was MSE MicroCentaur supplied by MSE (Crawley, UK); and the preparative centrifuge was a Sorvall RT 6000B refrigerated centrifuge supplied by Du Pont (UK) (Stevenage, UK). The dialysis tubing was purchased from Medicell International (London, UK).

Gel electrophoresis was carried out on Bio-Rad Mini-Protean Dual Slab Cell using the Model 200/2.0 power supply, obtained from Bio-Rad Labs. (Richmond, USA). Preparative fast protein liquid chromatography (FPLC) was performed on a Pharmacia-LKB FPLC system at 20–25°C, comprising of an LCC500 plus controller, two P-500 pumps, a single-path monitor UV detector and an LKB 2212 helical fraction collector. All spectrophotometric readings were performed on a Perkin-Elmer Lambda 16 UV–Vis recording spectrometer.

2.3. Methods

2.3.1. Synthesis of 2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-s-triazine (22/8) (route 1; Fig. 1)

A solution of 4-amino-1-naphthol hydrochloride (1.06 g, 5 mmol) in acetone (10 ml) and deionised water (10 ml) was added dropwise to a cyanuric chloride suspension made by pouring cyanuric chloride (1 g, 5 mmol) in acetone (10 ml) into cold deionised water (40 ml), with stirring, in an ice bath. $NaHCO_3$ (0.91 g, 10 mmol) in water (10 ml) was added to maintain the pH between 6 and 7 during the reaction. After 1.5 h, when no cyanuric chloride could be detected by TLC (solvent system: hexane–ethyl acetate, 2:3, v/v), the acetone was removed in vacuo. The crude product, 2-(4-amino-1-naphthol)-4,6-dichloro-*s*-triazine, was purified by flash chromatography (solvent system: hexane–ethyl acetate, 1:4, v/v) to afford a brown powder that decomposes at 360°C in a yield of 50% (0.76 g); R_F: (hexane–ethyl acetate, 2:3, v/v) 0.65. δ_H (400 MHz, DMSO): 6.88 (1H, d, J 7.6 Hz, Hd), 7.3 (1H, d, J 8.8 Hz, Ha), 7.47–7.50 (2H, m, Hb & Hc), 7.78 (1H, d, J 6.8 Hz, He), 8.16 (1H, d, J 4.8 Hz, Hf), 10.41 (1H, s, Hg), 10.88 (1H, s, Hh)~ppm.

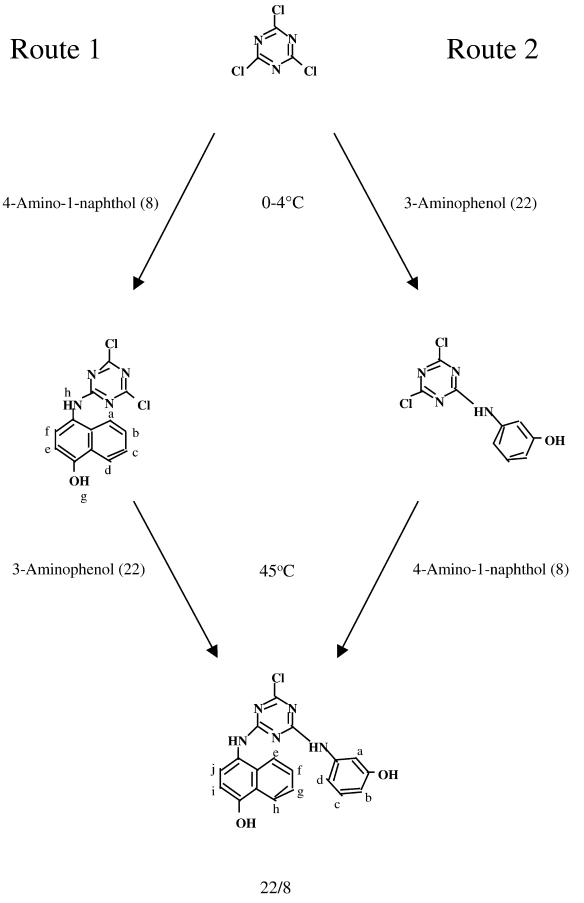

Fig. 1. Synthetic routes to ligand 22/8, 2-amino-3-phenol-6-(4-amino-1-naphthol)-4-chloro-*sym*-triazine.

A solution of purified 2-(4-amino-1-naphthol)-4,6-dichloro-*s*-triazine (0.68 g, 2.2 mmol) dissolved in acetone (12 ml) was added to 3-aminophenol (0.24 g, 2.2 mmol) dissolved in water (10 ml) and acetone (10 ml) stirred at 45°C in an oil bath. $NaHCO_3$ (0.185 g, 2.2 mmol) dissolved in deionised water (10 ml) was added. The reaction was stopped after 25 h when no 2-(4-amino-1-naphthol)-4,6-dichloro-*s*-triazine could be detected by TLC (solvent system: hexane–ethyl acetate, 3:2, v/v). The reaction mixture was concentrated to dryness on a rotary evaporator and final purification performed by flash chromatography (solvent system: hexane–ethyl acetate, 3:2,

v/v) to afford a brown powder in a yield of 50% (0.42 g); R_F: (hexane–ethyl acetate, 3:2, v/v) 0.23; δ_H (400 MHz, DMSO): 6.41 (1H, d, J 6.1 Hz, Hb or Hd), 6.84–6.98 (4H, m, Hi, Ha, Hc, Hb or Hd), 7.34 (1H, d, J 8.0, Hj), 7.42–7.5 (2H, m, Hf & Hg), 7.85 (1H, d, J 5.1Hz, Hh), 8.21 (1H, d, J 4.8Hz, He).

2.3.2. Synthesis of 2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-s-triazine (22/8) (route 2)

A solution of 3-aminophenol (2.96 g, 27.1 mmol) in acetone (40 ml) was added dropwise to a cyanuric chloride suspension made by pouring cyanuric chloride (5 g, 27.1 mmol) in acetone (40 ml) into cold deionised water (80 ml) under stirring in an ice bath. $NaHCO_3$ (2.28 g, 27.1 mmol) in water (26 ml) was added to maintain the pH between 6 and 7 during the reaction. After 1.5 h, when no 3-aminophenol could be detected by silica TLC (solvent system: hexane–ethyl acetate, 3:2, v/v) with Ehrlich's reagent, acetone was removed in vacuo. The product, 2-(3-aminophenol)-4,6-dichloro-*s*-triazine, a white precipitate, remained in aqueous solution, and was filtered and washed with cold deionsed water in order to remove salts to afford a white powder in a yield of 98.4% (6.85 g) and a melting point in the range 201–203°C. R_F: (hexane–ethyl acetate, 3:2, v/v) 0.5; δ_H (400 MHz, DMSO): 6.56 (1H, d, J 6.5 Hz, *o*-H or *p*-H), 7.03 (1H, d, J 6.5 Hz, *o*-H or *p*-H), 7.05–7.20 (2H, m, *o*-H and *m*-H); M^+ found 257, $C_9H_6N_4Cl_2O$ requires M^+: 257; *m/z*.

$NaHCO_3$ (1.32 g, 15.7 mmol, 20 ml) was used to bring the pH of a 4-amino-1-naphthol hydrochloride (3.08 g, 15.7 mmol) solution to neutral in a mixture of acetone (20 ml) and water (20 ml) before addition to a solution of 2-(3-aminophenol)-4,6-dichloro-*s*-triazine (3.68 g, 14.3 mmol) in acetone (50 ml) and incubation at 45°C in an oil bath. $NaHCO_3$ (1.2 g, 14.3 mmol) in water (10 ml) was added to maintain the pH between 6 and 7. After 5 h, when no 4-amino-1-naphthol was detected by TLC (solvent system: hexane–ethyl acetate, 3:2, v/v) with ninhydrin reagent, the reaction mixture was concentrated to dryness on a rotary evaporator. Salt was removed by partitioning between ethyl acetate and water, whence the ethyl acetate phase was evaporated to dryness on a rotary evaporator. Final purification was performed by flash chromatography (solvent system: hexane–ethyl acetate, 3:2, v/v) to afford a brown powder which decomposed at 180°C in a yield of 50% (2.98 g). ν_{max}(KBr)/cm^{-1} 3357.6(OH) cm^{-1}; R_F: (hexane–ethyl acetate, 3:2, v/v) 0.23; δ_H (400 MHz, DMSO): 6.41 (1H, d, J 6.1 Hz, Hb or Hd), 6.84–6.98 (4H, m, Hi, Ha, Hc, Hb or Hd), 7.34 (1H, d, J 8.0 Hz, Hj), 7.42–7.5 (2H, m, Hf & Hg), 7.85 (1H, d, J 5.1 Hz, Hh), 8.21 (1H, d, J 4.8 Hz, He); M^+ found 380, $C_{19}H_{14}N_5O_2Cl$ requires M^+: 380, *m/z*.

2.3.3. Immobilization of ligand 22/8

Sepharose 6B (100 g moist weight gel) was transferred to a glass filter funnel, thoroughly washed with distilled water, and drained. After suspension in NaOH (1 *M*, 80 ml), epichlorohydrin (1 *M*, 80 ml) was added and the mixture was incubated on a rotary shaker (140 rpm) for 2 h at 30°C. Excess epichlorohydrin was removed by extensive washing with distilled water until no odour could be detected. The epoxy density was determined by titrating the pH from alkali to pH 7.0 or below with 0.1 *M* HCl after the addition of sodium thiosulphate (1.3 *M*, 3 ml) to 1 g of moist weight gel. The amount of epoxy groups present is then calculated from the amount of hydrochloric acid needed in order to maintain neutrality which corresponds to: volume (ml) HCl added/10. The epoxy-activated gel (50 μmol epoxy groups/g moist weight gel) was converted into 6-aminohexyl-modified agarose with the addition of hexane-1,6-diamine. Hexane-1,6-diamine (10 molar excess to free epoxy groups on the gel) dissolved in water was added to the epoxy-activated agarose and shaken on a rotary shaker (140 rpm, 30°C) for 18 h to give 6-aminohexyl-activated agarose. The gels were left to settle, whence, their supernatants were decanted and the gels were washed generously with water. Ninhydrin reagent was used to determine the amine ligand density of the 6-aminohexyl-activated gel.

6-Aminohexyl-activated agarose was thoroughly washed with water (3×gel volumes) and transferred to bottles. Ligand 22/8 (two molar excess compared to amino concentration on gel, 90 μmol/g moist weight gel) dissolved in DMF–water (1:1, v/v, 16 ml) was added to 5-aminohexyl-activated gel (8 g). NaOH (1 *M*) was added to adjust the pH to neutral. The bottles were incubated under rotation (30 rpm) in an oven for 72 h at 85°C. Once the gels had

settled, their supernatants were decanted and the gels washed with 3×gel volumes DMF–water (1:1, 3:1, 1:0, 3:1, 1:1, 0:1, v/v) to remove unreacted ligand 22/8.

2.3.4. Affinity chromatography

Columns (6.2×0.9 cm) containing immobilized ligand 22/8 (1 ml; 90 μmol 22/8/g moist weight gel) were equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0) at 20°C. Human plasma from anticoagulated blood was centrifuged at 200 *g* for 10 min, whence a sample (2.5 ml), diluted four-fold with sodium phosphate buffer (0.05 *M*, pH 8.0), was applied and the columns washed with sodium phosphate buffer (0.05 *M*, pH 8.0) until the absorbance at 280 nm was <0.002. Bound proteins were eluted with selected buffers and the purity was determined by 12.5% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3.5. Purification of IgG from human plasma with immobilized protein A

This was performed on automated chromatography equipment with a fraction collector (FPLC, Pharmacia, Sweden). Human plasma diluted four-fold with sodium phosphate buffer (0.05 *M*, pH 8.0) was applied to affinity columns (6.2×0.9 cm) packed with affinity matrices (1 ml) equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0) and washed with the same buffer until the absorbance at 280 nm was <0.002. Finally, IgG was eluted with sodium citrate buffer (0.05 *M*, pH 3.0) at a flow-rate of 0.6 ml/min. Elution peaks were collected, dialysed with Tris–HCl buffer (0.01 *M*, pH 7.0), and stored at 0°C prior to further use.

2.3.6. Determination of the apparent capacity of immobilized 22/8 for IgG

A column (5.5×0.6 cm) containing immobilized 22/8 (0.1 g; 90 μmol 22/8 per g moist gel) was equilibrated with phosphate-buffered saline (PBS) buffer, pH 7.4, whence pure human IgG (4 mg/ml, 1.5 ml) was applied. The flow through was collected, continuously recirculated through the column until the absorbance at 280 nm was constant, whence the bound IgG was eluted with citric acid buffer (0.025 *M*, pH 2.4; 10 ml).

2.3.7. Measurement of the affinity constant between IgG and immobilized 22/8

Moist immobilized ligand 22/8 (0.1 g; 90 μmol 22/8 per g moist gel) was suspended in PBS (1 ml) with 0.5–8.5 mg pure IgG and incubated on a roller mixer at room temperature for 3 h; the proportion of unbound protein was deduced by measuring the absorbance of the supernatant at 280 nm (assuming $A^{1\%}_{280\ nm}=14$ for IgG) [10]. Bound protein was calculated by subtracting the unbound from the total added. The data was plotted as a Scatchard plot [11] in order to deduce the affinity constants and the number of binding sites from the equation $q/C=K_aQ_{max}-K_aq$, where q/C is the ratio of bound protein to free protein; q is the bound proteins and C is the free protein. K_a is the association constant and Q_{max} is the binding capacity [11].

2.3.8. Determination of the selectivity of immobilized ligand 22/8 for antibodies from different species

Columns (6.2×0.9 cm) packed with immobilized 22/8 (1 ml; 90 μmol/g moist weight gel) were equilibrated with PBS, whence pure IgG from different sources (1–2 mg/ml; 1 ml) was applied and washed with PBS (5 ml) at room temperature. Finally, the IgG was eluted with glycine–HCl buffer (0.1 *M*, pH 2.9, 3 ml) followed by citric acid buffer (0.025 *M*, pH 2.4, 3 ml) at a flow-rate of 0.6 ml/min. Protein concentrations were estimated from the absorbance at 280 nm (1 cm pathlength) using the extinction coefficient for IgG from all sources: $E^{1\%}_{280\ nm}=14$ [10].

2.3.9. Stability of immobilized ligand 22/8 in 1 M NaOH

A column (6.2×0.9 cm) packed with immobilized ligand 22/8 adsorbent (1 ml; 90 μmol 22/8 per g moist gel) was equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0), whence four-fold diluted human plasma, pH 8.0) was applied, the column washed with sodium phosphate buffer (0.05 *M*, pH 8.0) until the absorbance at 280 nm was <0.002, whence IgG was eluted with glycine–HCl buffer (0.1 *M*, pH 2.9) at a flow-rate of 0.6 ml/min. Eluted IgG was quantified by measuring its absorbance at 280 nm. The column was incubated in 1 *M* NaOH for 18 h at 20°C, whence the column was re-equilibrated

with sodium phosphate buffer (0.05 *M*, pH 8.0) and the IgG capacity re-measured.

2.3.10. Large scale purification of IgG with immobilized ligand 22/8

A column (7×1.2 cm; 5 ml) with immobilised ligand 22/8 (90 μmol/g moist gel) was equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0) at 20°C. Centrifuged human plasma (12.5 ml), diluted four-fold with sodium phosphate buffer (0.05 *M*, pH 8.0), was applied and the column was washed with sodium phosphate (0.05 *M*, pH 8.0) at a flow-rate of 0.6 ml/min until the absorbance at 280 nm was <0.002. Bound proteins were eluted with glycine–HCl buffer (0.1 *M*, pH 2.9) until the absorbance at 280 nm was <0.002, whence the column was washed with 1 *M* NaOH. The purity of the eluted protein was deduced by reducing 12.5% (w/v) SDS–PAGE [7]. Protein content was determined using the Coomassie dye binding method [12] according to the protocol provided by the reagent supplier. Bovine serum albumin (BSA) was used to construct the calibration curve (0–25 μg/ml).

3. Results and discussion

3.1. Molecular modelling

Molecular modelling of the interactions between ligand 22/8 and the putative binding site on the Fc fragment of IgG was performed. The structure of ligand 22/8 was imported into QUANTA software and moved to the binding site whence functional groups were adjusted to the most likely orientations. The structure was energy minimised with the CHARMm program. The total CHARMm energy, minimised in situ, was −125.98 kcal/mol; the energy of constraint was zero and the individual bond, angle, dihedral, improper and Lennard–Jones energies were less than the thermal dynamic energy (~10 kcal/mol), suggesting that this conformation was practical. The position and orientation of ligand 22/8 was adjusted to maximise the docking energy. The optimum van der Waal's and electrostatic interaction between ligand 22/8 and IgG in vacuo, ΔH, was −222.93 kcal/mol. In this position, ligand 22/8 interacts with 270 Å^2 hydrophobic and 130 Å^2 hydrophilic solvent accessible surface on the Fc region of IgG. There appears to be nine pairs of non-hydrogen atoms having direct contact between ligand 22/8 and the Fc region of IgG and nine freely rotatable bonds in the ligand. Fig. 2 shows the putative binding position of ligand 22/8 with the CH_2–CH_3 inter-domain region of the Fc moiety of IgG.

Two hydrogen bonds are formed in the putative complex between the Fc fragment of IgG and ligand 22/8. Both involve the ring nitrogens of the imidazole side chain of His-435 on the Fc domain of IgG: one hydrogen bond is formed between the ring imino group and the hydroxyl group of the 3-aminophenol substituent, whilst the second is formed with the oxygen atom of 4-amino-1-naphthol. It is worth noting that His-435 plays a key role in the interaction between protein A and the Fc fragment of IgG [13]. The only sub-class of human IgG, IgG_3, which does not bind to protein A, features a substitution of this amino acid with an arginine residue. These modelling studies suggest that the protein A mimic should interact with human IgG in a similar manner to native protein A.

3.2. Synthesis of 2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-sym-triazine (22/8) (Fig. 1)

The usual strategy employed for the synthesis of ligands based on 1,3,5-trichloro-*sym*-triazine or cyanuric chloride entails reacting the least reactive amine with the first chlorine atom and the most reactive amine with the last chlorine atom, since the chlorine atoms on cyanuric chloride become progressively deactivated as substitution of the triazine ring with amines ensues [14]. However, since the pK_a values of the aromatic amines on the two reactants used here, 3-aminophenol and 4-amino-1-naphthol, were comparable, 4.17 and 4.0, respectively, the order of addition is less significant and both routes were investigated in order to ascertain the optimal route to synthesise ligand 22/8 (Fig. 1). Initially, route 1 was followed as it was the route adopted when ligand 22/8 was synthesised on the solid phase [8]. The intermediate, 2-(4-amino-1-naphthol)-4,6-dichloro-*sym*-triazine was prepared by nucleophilic substitution of the first chlorine on cyanuric chloride by the amino group on 4-amino-1-

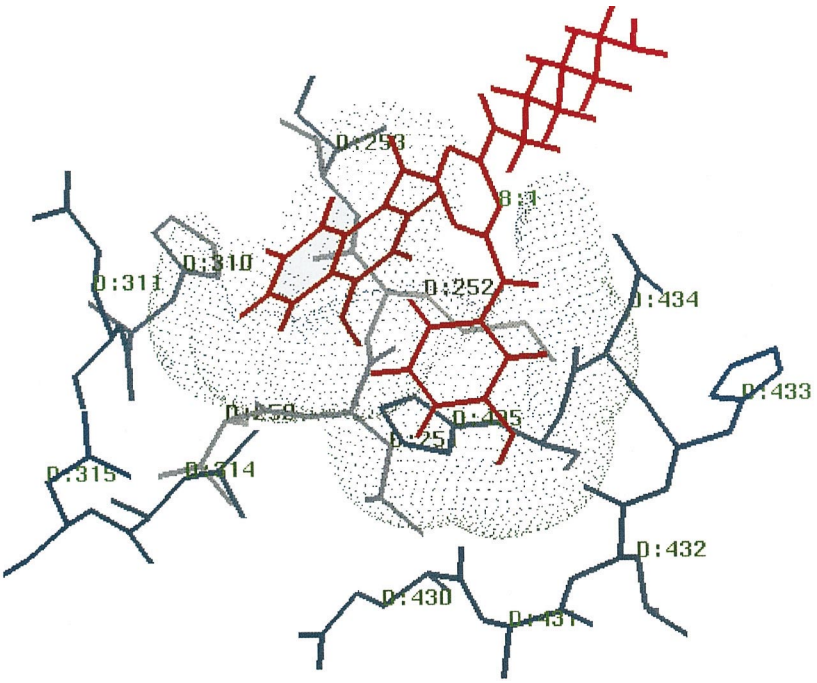

Fig. 2. Putative binding site of ligand 22/8 (red) on the CH_2–CH_3 interdomain region of human IgG (blue) showing the close proximity of the hydroxyl group of the 3-aminophenol substituent and the oxygen atom of 4-amino-1-naphthol to the ring nitrogens of the imidazole side chain of His-435 (D-435). Amino acid residues of IgG (blue; D) involved in binding are: Glu-430, Leu-314, Gln-311, His-310, Thr-250, His-435, Asn-434, His-433, Ile-253, Leu-251, Asp-315, Leu-432, Ala-431 and Met-252.

naphthol at 0–4°C. Final purification by flash chromatography afforded the product in ~50% yield. The identity of the product was confirmed by ^{1}H-NMR and mass spectrometry (MS) and the purity estimated to be >95% by ^{1}H integration. The second substitution was performed by reacting 2-(4-amino-1-naphthol)-4,6-dichloro-*sym*-triazine with 3-aminophenol at 45°C for 25 h. After completion of the reaction, solvent was removed in vacuo and the product was subjected to flash chromatography to yield a pure compound in ~50% yield. The identity of the product, 2-amino-3-phenol-6-(4-amino-1-naphthol)-4-chloro-*sym*-triazine (22/8) was confirmed by MS, ^{1}H-NMR and by synthesis of the same compound by route 2 (Fig. 1). It was anticipated that this alternative route might offer a more facile synthesis of 22/8.

The amino group of 3-aminophenol readily displaced a chlorine atom on cyanuric chloride within 1.5 h and in good yield. The product was easily recovered by removal of acetone by rotary evaporation followed by filtration to remove the aqueous medium. A small amount (<1%) of a more soluble polar contaminant formed during the reaction was readily removed after filtration. No further purification was required in order to obtain a 98.4% yield of the intermediate 2-amino-3-phenol-4,6-dichloro-*sym*-triazine. The presence of a phenolic hydroxyl group in the compound was confirmed with the Folin–Ciocalteau reagent, whilst the use of Ehrlich's reagent confirmed the absence of an aromatic amine. The observations suggest that the 3-aminophenol moiety is substituted on the cyanuric chloride via the aromatic amine rather than the phenolic hydroxyl group.

The intermediate 2-amino-3-phenol-4,6-dichloro-*sym*-triazine was reacted with 4-amino-1-naphthol over a period of 5 h, whence the final product was obtained in ~50% yield and >95% purity by flash chromatography. The identity of the product was confirmed by the use of Folin–Ciocalteau and Ehrlich's reagents, and by IR, MS and ^{1}H-NMR

spectroscopy. IR spectroscopy confirmed the presence of aromatic hydroxyl groups as a broad band at 3357 cm^{-1}. This observation suggested that both chlorine atoms on the triazine scaffold were substituted by the amino groups rather than the hydroxyl groups on 3-aminophenol and 4-amino-1-naphthol.

Ligand 22/8 could be synthesised by either routes shown in Fig. 1 to yield identical compounds; however, route 2 is preferred owing to its higher yield, ease of product purification and shorter reaction times.

3.3. Chromatographic characterisation of immobilised ligand 22/8

Synthesised, purified and characterised ligand 22/8 was coupled to 6-aminohexyl-agarose to yield immobilised 22/8 with a ligand density equivalent to 90 μmol/g moist weight gel (Fig. 3). The apparent capacity of the adsorbent for IgG was deduced by overloading matrices (0.1 g moist weight gel) equilibrated in PBS with pure human IgG (4 mg/ml; 1.5 ml). The flow through fraction was continuously reapplied to the column until the absorbance at 280 nm of the applied and flow through fractions were identical, whence bound IgG was eluted with citric acid buffer (0.025 *M*, pH 2.4; 10 ml). Under the specified experimental conditions, immobilised ligand 22/8 displayed an apparent capacity of 51.9 mg IgG/g moist weight gel, equivalent to approximately 0.004 mol IgG/mol ligand immobilised or ~0.4% molar ligand occupation. The yield of IgG recovered from the column was 98%.

The affinity constant of human IgG for immobilised 22/8 was measured by suspending a known amount of moist gel (0.1 g; 90 μmol ligand 22/8/g moist weight gel) in PBS buffer (1 ml) containing increasing amounts of purified human IgG (0–8.5 mg). After incubation for a period of 3 h on a roller mixer at 20°C, the concentration of free IgG was calculated by subtracting the bound IgG from the total added immunoglobulin. The binding isotherms and corresponding Scatchard plots are shown in Fig. 4. The isotherms are of the Langmuir type and the Scatchard plots are linear in the experimental range, indicating that a single IgG molecule binds to each binding site on the adsorbent.

The equation for the Langmuir adsorption isotherm, $q=(Q_{max}K_aC)/(1+K_aC)$, can be rearranged to form the Scatchard plot, $q/C=K_aQ_{max}-K_aq$, where q/C is the ratio of bound to free protein, K_a is the association constant and Q_{max} is the binding capacity [11]. Table 1 shows the calculated association and dissociation constants and the equilibrium capacities for the binding of human IgG to immobilised ligand 22/8 and compares these values with those calculated for protein A and the lead protein A mimetic [7]. Although the association constant of immobilised 22/8 for IgG is comparable to that of the lead mimetic, and slightly less than that of native protein A, the equilibrium binding capacity (Q_{max}) for immobilised 22/8 (151.9 mg IgG/g moist weight gel) is approximately 5.5- and 9-times higher than the protein A mimetic and native protein A, respectively. This observation is most likely attributable to the higher immobilised ligand concentration used

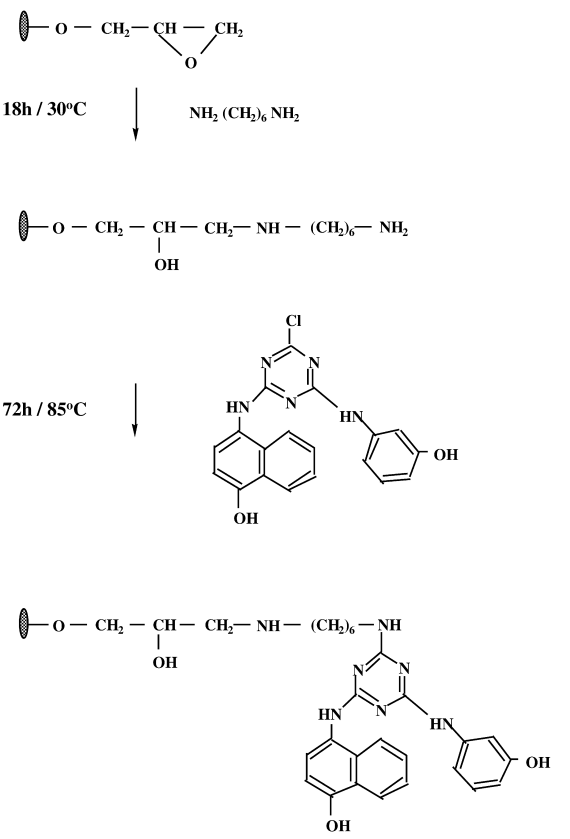

Fig. 3. Synthesis of the immobilized ligand 22/8 affinity adsorbent.

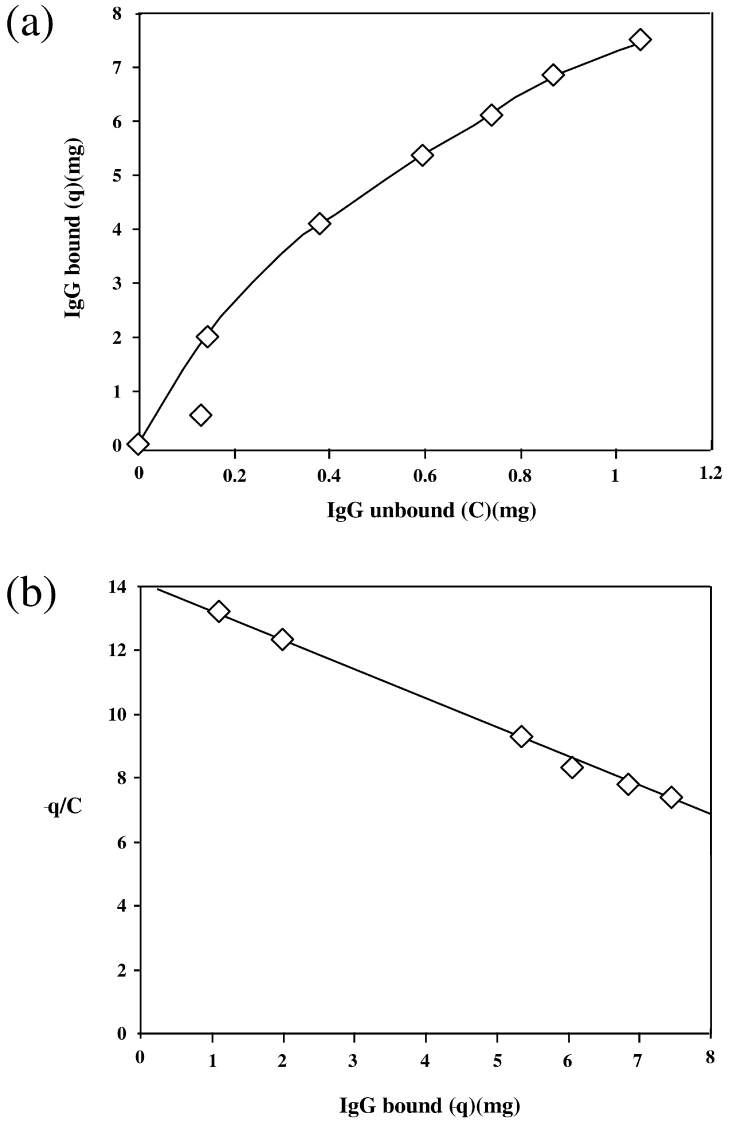

Fig. 4. The binding isotherm for human IgG to immobilized ligand 22/8: (a) the curve was fitted to the equation $q=(Q_{max}K_aC)(1+K_aC)$. Moist gel-bound ligand 22/8 (0.1 g; 90 μmol/g moist weight gel) was suspended in PBS buffer (1 ml) containing pure human IgG (0.5–8.5 mg) and incubated on a roller mixer at 20°C for 3 h. The proportion of unbound protein was deduced by measuring the absorbance of the supernatant at 280 nm. Bound protein was calculated by subtraction of the unbound fraction from the total added. (b) Scatchard plot of IgG binding to immobilized ligand 22/8. The data were fitted to the linear equation for IgG binding: $q/C=14.205-0.935q$ ($R^2=0.996$).

Table 1
Comparison of binding isotherms of human IgG to immobilized protein A, protein A mimetic and ligand 22/8

	Protein A	Protein A mimetic [7]	22/8
K_a (M^{-1})	$3.65\cdot10^5$	$1.97\cdot10^5$	$1.4\cdot10^5$
K_d (μM)	2.74	5.06	7.1
Q_{max} (mg IgG/g moist weight gel)	17.0	26.8	151.9

here (90 μmol 22/8/g moist weight gel) compared to the mimic (15 μmol ligand/g moist weight gel) [7] and native protein A [2.5±0.5 mg (42±8 nmol) protein A/g moist weight gel].

3.4. Selectivity of immobilized ligand 22/8 for immunoglobulin classes and subclasses from different species

It has been suggested that one of the major drawbacks of protein A is its inability to bind all antibody classes and subclasses and a number of antibodies from other species [15]. For example, this limitation is exemplified by the fact that protein A binds only a fraction of the immunoglobulin G from chicken, which offers promise in the purification and detection of highly conserved mammalian proteins [16].

The binding of immobilized ligand 22/8 to antibodies from 10 different species was assessed. Evidence of interaction was found between immobilized 22/8 and all the antibodies tested. It was found that, under the conditions selected >60% of the antibody applied (1–2 mg) to the column (1 g) was adsorbed to immobilized 22/8. The adsorbent binds antibodies in the following order of percentage adsorption under the specified conditions: human> chicken > cow > rabbit > pig > horse > rat > goat > sheep>mouse. It is also known that protein A binds to antibodies from all the species listed above, although only a fraction of chicken IgG was adsorbed by immobilised protein A [17,18]. The percentage protein A reactivity with IgG from various species has been calculated from the amount of protein adsorbed onto the column [19]. A comparison of the percentage reactivities of immobilised protein A and 22/8 for immunoglobulins from various species is shown in Fig. 5. It is clear that the percentage reactivity of 22/8 for IgG from five species (human, cow, rabbit, pig, rat) is similar to that of protein A and >80%. However, it should be noted that the protein A league table was obtained by interacting immobilised protein A with immunoglobulins from normal sera from different species, whereas the % reactivity with immobilised 22/8 was deduced from studies with pure immunoglobulin fractions. The % protein A reactivity of horse, sheep and rat IgG given in Fig. 5 represents the mean value

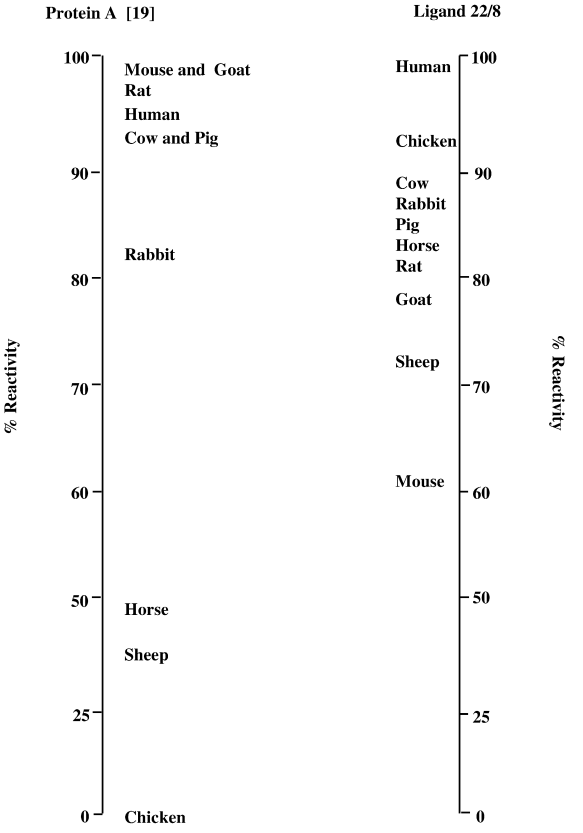

Fig. 5. Comparison of the % reactivity of immobilized protein A and immobilized ligand 22/8 with antibodies from different sources. The % reactivity was calculated from the amount of protein adsorbed on to the column [19].

taken from the individual subclasses since the % reactivity for polyclonal IgG was unavailable. Nevertheless, immobilized 22/8 appears to have a wider specificity than protein A and could have potential for the purification of antibodies from a number of sources. In addition, the % reactivities of immobilized ligand 22/8 for IgG from different species varied over a wider range than that of immobilized protein A. This observation suggests that there is a higher probability of resolving a mixture of antibodies from two species, such as human and cow, on immobilized 22/8 that on immobilised protein A.

Other human antibody classes and subclasses bound to immobilised 22/8. The percentage of human immunoglobulins adsorbed lay in the sequence IgG>IgM>IgA, with immobilized 22/8 displaying tight binding to IgA and IgM, since only

12% and 1%, respectively were eluted with 0.1 *M* glycine–HCl buffer, pH 2.9, compared to 68% of the adsorbed IgG. The higher avidity of both IgA and IgM is probably due to their oligomeric composition. Immobilised protein A is known to bind some IgM and IgA [20,21]. Immobilized 22/8 exhibits affinity for all human IgG subclasses, including IgG_3. Protein A does not complex with IgG_3 due to a single amino acid substitution of His for Arg at position 435 of the heavy chain [22,23], although it has been shown that a fraction of subclass IgG_3 was able to bind to protein A [24].

3.5. Optimisation of the chromatographic parameters for the purification of IgG from human plasma

The protocol for the purification of IgG from human plasma on immobilized 22/8 was similar to that described previously [7]. It was found that the majority (80–90%) of IgG present in human plasma was captured by immobilised 22/8 when up to 2 ml diluted plasma was loaded onto a column (1 ml) equilibrated with 0.05 *M* sodium phosphate buffer, pH 8.0, but that the more usual elution regimes, comprising a stepwise series of sodium citrate buffers (0.05 M; pH 4.7, 3.5, 3.0) terminating in citric acid (0.025 M; pH 2.4), were ineffectual at recovering bound IgG. Since IgG shows strong binding to immobilized 22/8, it was expected that some optimisation of the elution conditions would be required in order to establish a simple one-step procedure for the purification of IgG from human plasma.

It is known that elution of IgG from immobilized protein A can be achieved by altering the pH of the irrigant buffer [25]. A range of elution conditions were assessed, including 0.1 *M* glycine–HCl, pH 1.8–5.0, 0.05 *M* sodium carbonate, pH 9–13, glycerol (1–10%, v/v), propionic acid (1 *M*, 1–3%, v/v), acetonitrile (1–3%, v/v), acetonitrile (1–3%)–propionic acid (3–1%) (v/v), acetic acid (1 *M*), NaSCN (3 *M*) and $MgCl_2$ (4 *M*). Under the specified conditions, IgG was eluted with purities and recoveries in the range 0–99.2% and 2–94%, respectively. The highest recovery of eluted IgG was obtained with sodium carbonate buffer (0.05 M; pH 13), although the purity of the product was $<$50%. As a general rule, buffers with acidic pH values gave higher yields and purities of the eluted IgG. The optimum combination of yield and purity was obtained by elution with 0.1 *M* glycine–HCl buffer, pH 2.3–2.9. Fig. 6 shows the % of the applied IgG eluted and the % purity of the eluted IgG as a function of the pH of the 0.1 *M* glycine–HCl buffer. It is observed that 0.1 *M* glycine–HCl buffer, pH 2.7–2.9 gives optimum recovery (67–69%) and purity (99.2–97.3%) of the IgG recovered from human plasma. Furthermore, reducing the ionic strength of the glycine–HCl buffer from 0.1 to 0.05 *M*, whilst maintaining the pH at 2.9, decreased the amount of antibody eluted, as it did with further increases in ionic strength to 0.3 *M*. These optimised conditions are very similar to those used for the purification of IgG from immobilised protein A [25–28]. It is believed that ionisation of a residue found in all IgG subclasses, His 310, plays a major role in the dissociation of the complex between protein A and IgG at pH 5 [29] and is largely responsible for the elution capacity of acidic buffers.

3.6. Stability of the biomimetic adsorbent

One of the principal concerns in adapting affinity media to process scale is the stability of the adsorbent to the conditions used for cleaning- or sterilising-in-place. At this scale, adsorbent stability may be the most significant factor determining overall process costs [30,31]. In particular, resistance to 1 *M* NaOH used in depyrogenation is essential and was therefore used as the benchmark to assess the stability of the immobilized 22/8 adsorbent.

The stability of the immobilized 22/8 affinity adsorbent was monitored during incubation with 1 *M* NaOH over a period of 7 days at 20°C. The capacity of immobilized 22/8 for IgG was measured daily with human plasma and expressed as a change in capacity following each cycle of treatment. The capacity of the adsorbent remained unchanged following treatment with 1 *M* NaOH for six cycles and a total of $>$140 h. The stability of the adsorbent is comparable with that of the immobilized protein A mimetic [7] and suggests that immobilized 22/8 is able to withstand general procedures for sterilisation and cleaning-in-place. It is anticipated that the immobilized ligand will withstand $>$500 cycles of adsorption, desorption, cleaning-in-place and re-

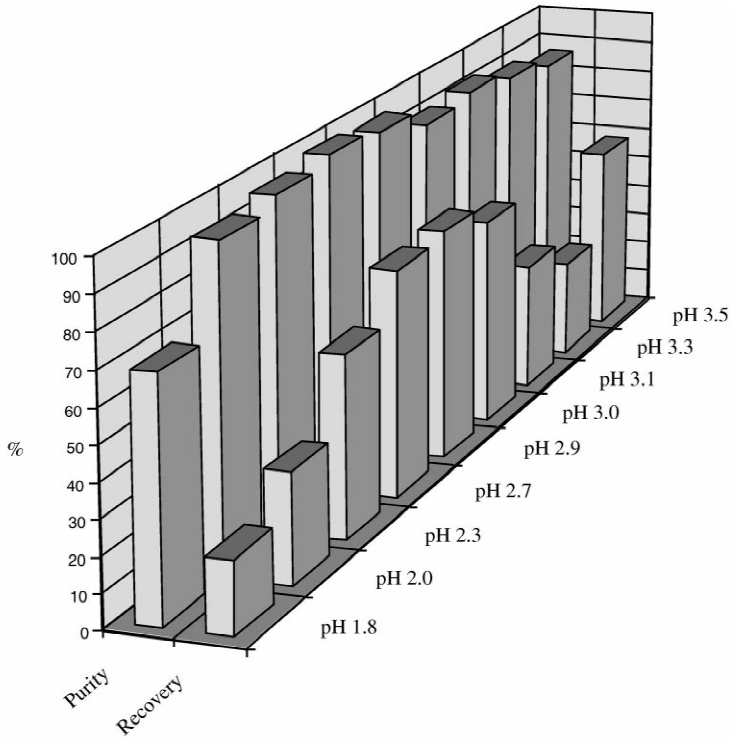

Fig. 6. Effect of the pH of 0.1 *M* glycine–HCl buffer on the % purity and recovery of human IgG from an immobilized 22/8 adsorbent. Columns (6.2×0.9 cm) packed with immobilized ligand 22/8 (1 ml; 90 μmol/g moist weight gel) were equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0), whence four-fold diluted human plasma (2.5 ml) was applied and columns were washed with sodium phosphate (0.05 *M*, pH 8.0) until the absorbance at 280 nm was <0.002. IgG was eluted with 0.1 *M* glycine–HCl buffers, pH 1.8–3.5.

equilibration without significant loss of chromatographic performance.

3.7. Large scale purification of IgG from human plasma with immobilized 22/8

A column (7×1.2 cm) packed with immobilised 22/8 (90 μmol 22/8/g moist weight gel; 5 ml) was equilibrated with 0.05 *M* sodium phosphate buffer, pH 8.0 and human plasma (12.5 ml; diluted four-fold with equilibration buffer) applied at a flow-rate of 0.6 ml/min. The column was washed with equilibration buffer until the absorbance at 280 nm was <0.002 before eluting the bound proteins with 0.1 *M* glycine–HCl buffer, pH 2.9. Residual proteins tightly bound to the adsorbent were removed by treatment with 1 *M* NaOH.

Fig. 7 shows the profile of human plasma proteins eluted from a column of immobilised 22/8, whilst Fig. 8 shows the SDS–electrophoretic analysis of the purity of the peaks eluted from the gel. The applied sample contained a total protein load of 222 mg, comprising albumin (155.4 mg), IgG (30.2 mg) and other unspecified proteins (36.4 mg). The majority of the albumin (103.5 mg, 66.7%) passed through the column in the void volume, whilst 94.4% of the IgG was adsorbed and 60% eluted with a purity of 92.5% with the acid glycine treatment. A proportion (34.4%) of the IgG remained on the column and was eluted, along with a small proportion of albumin and a number of unspecified proteins with the 1 *M* NaOH wash. The total recovery of human IgG was 100%.

4. Conclusions

The results described in this paper represent the final two phases of a four-part strategy designed to

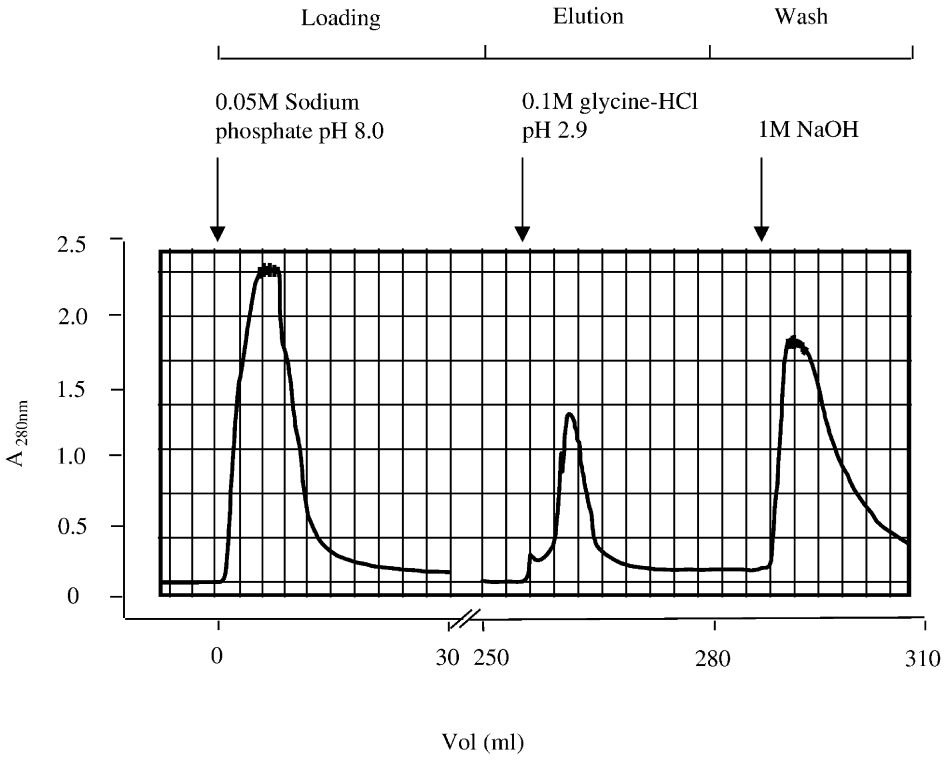

Fig. 7. Chromatography of human plasma on an immobilized 22/8 adsorbent. A column (7×1.2 cm) of immobilized 22/8 (5 ml; 90 μmol/g moist weight gel) was equilibrated with sodium phosphate buffer (0.05 *M*, pH 8.0) at 20°C. Four-fold diluted human plasma (12.5 ml) was applied and the column washed with sodium phosphate buffer (0.05 *M*, pH 8.0) until the absorbance at 280 nm was <0.002, whence bound proteins were eluted at a flow-rate of 0.6 ml/min with glycine–HCl buffer (0.1 *M*, pH 2.9), followed by a wash with NaOH (1 *M*).

create durable, sterilisable and highly selective affinity adsorbents for the biopharmaceutical industry [1,7,8]. The four distinct phases comprise: (i) identification of a target site and design of a complementary ligand with no fissile bonds, (ii) solid phase synthesis and evaluation of an intentionally biased combinatorial library of related ligands, (iii) solution phase synthesis and characterisation of the lead ligand, and (iv) immobilisation and optimisation of the adsorbent and chromatographic parameters for the purification of the target protein. Earlier work defined a putative lead IgG-binding ligand based on modelling a dipeptide motif in protein A [7]. This ligand was subsequently refined in a limited combinatorial array [8] to produce a second generation ligand, 22/8, which was synthesised, chemically characterised, immobilized and evaluated in the present work. The structure of the new ligand, 22/8, based on a triazine scaffold substituted with 3-aminophenol and 4-amino-1-naphthol, has been chemically confirmed and shown to interact selectively with immunoglobulins from various sources. The immobilised ligand is ultrastable and is able to withstand incubation in 1 *M* NaOH for seven days without loss of binding capacity for IgG and thus may find application in the large scale purification of human and other immunoglobulins. Current data suggests that IgG can be extracted with a purity of 92.5% from human plasma in a single step with 60% yield. It is anticipated that further improvements in the yield and purity may be achieved.

The nature of the interaction between ligand 22/8 and IgG has yet to be established; the ligand is analogous to the protein A mimic reported previously [7], which is known to bind to to IgG competitively with native protein A and thus presumably binds

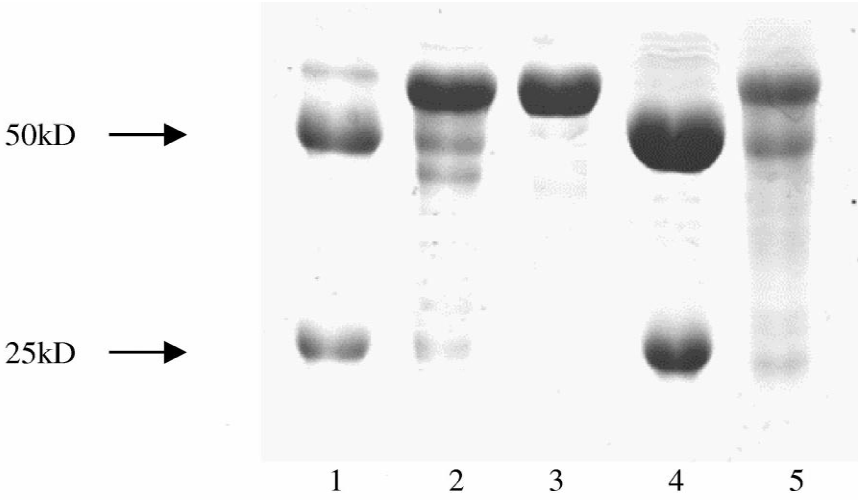

Fig. 8. Reducing 12.5% (w/v) SDS–PAGE analysis of the purity of each peak eluted from the immobilized 22/8 adsorbent and stained with Coomassie Brilliant Blue G-250. Lane 1: Immunoglobulin G eluted from an immobilized protein A column; lane 2: human plasma; lane 3: flow-through fraction; lane 4: fraction eluted with 0.1 *M* glycine–HCl buffer, pH 2.9; lane 5, 0.1 *M* NaOH wash fraction.

at or near the CH_2–CH_3 interdomain region on the heavy chain. Both immobilized ligand 22/8 and the protein A mimic bind to IgG with similar association constants (K_a), $1.4 \cdot 10^5$ M^{-1} and $1.97 \cdot 10^5$ M^{-1}, respectively. Furthermore, there is evidence that ligand 22/8 binds to both the Fc and Fab fragments in a manner reminiscent of both protein A [32] and the mimic [7]. These observations suggest that immobilized ligand 22/8 may retain all the advantages of selectivity and capacity of immobilized protein A, whilst obviating disadvantages such as sterilizability, leakage and toxicity.

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