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A synthetic Protein G adsorbent based on the multi-component Ugi reaction for the purification of mammalian immunoglobulins

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ABSTRACT

Numerous efforts have been devoted to develop synthetic affinity ligands mimicking natural immunoglobulin-binding proteins, such as Proteins A and L, in order to overcome intrinsic drawbacks involving their high cost and acidic pH elution. However, few reports have focused on a Protein G mimic. This work describes the use of the solid phase multi-component Ugi reaction to generate a low cost, rationally designed, affinity ligand to mimic Protein G for the purification of mammalian immunoglobulins, including the heavy-chain only camelid IgGs, with effective elution at neutral pH. An aldehyde-functionalised Sepharose™ resin constituted one component (aldehyde) of the four-component Ugi reaction, whilst the other three components (a primary or secondary amine, a carboxylic acid and an isonitrile) were varied to generate a tri-substituted Ugi scaffold, with a wide range of functionality, suitable for mimicking peptides for immunoglobulin purification. Ligand A2C11I1 was designed to mimic Asn35 and Trp43 of Protein G (PDB: 1FCC) and *in silico* docking into the Fc domain showed a key binding interface closely resembling native Protein G. This candidate ligand demonstrated affinity towards IgGs derived from human, cow, goat, mouse, sheep, pig, rabbit and rat serum, chicken IgY and recombinant camelid Fc domain, out of which cow and sheep IgG demonstrated 100% binding under the conditions selected. Preparative chromatography of IgG from human serum under a standardised buffer regime eluted IgG of ~65% purity, compared to ~62% with Protein G. This adsorbent achieved highest elution of IgG at neutral pH (0.1 M sodium phosphate pH 7.0, 30%, v/v, ethylene glycol), an advantage for purifying antibodies sensitive to extremes of pH. The ligand demonstrated a static binding capacity of 24.6 mg IgG ml^{-1} resin and a dissociation constant (K_d) of 4.78×10^{-6} M. The solid phase Ugi scaffold provides a strategy to develop pseudo-biospecific ligands to purify immunoglobulins and other potentially high-value biotherapeutic proteins.

1. Introduction

Monoclonal antibodies (mAbs) constitute the most rapidly growing category of biopharmaceuticals with more than 25 antibodies approved for human therapy by the FDA and 240 currently in clinical trials [1,2]. Advances in technology have greatly expanded the variety of mAbs available for study [3,4]; however, the purification process needs to be reliable and predictable to produce products suitable for human use [4] and must follow GMP guidelines [5]. As the upstream mAbs titres increased several thousand-fold from low milligrams to 5 g l^{-1} [6,7], the major cost incurred has shifted from the cell culture to the downstream processing steps [8], which typically accounts for 50–80% of total production cost [9]. The most popular techniques for antibody purification are the highly specific immunoglobulin-binding proteins isolated from the surface of bacteria, such as Protein A from *Staphylococcus aureus*, Protein G from group C and G *Streptococci* and Protein L from *Peptostreptococcus magnus*. The majority of early stage purification processes for mAbs are based on Protein A affinity chromatography, which results in a high degree of purity and recovery in a single step [10]. However, this high affinity also results in stability issues because extremely low pH is often required for antibody elution. Such a denaturing condition often causes certain antibodies to degrade and aggregate [11–13]. Another major disadvantage of these adsorbents is their inherently high cost

Abbreviations: ApA, artificial protein A; C_H1, C_H2 and C_H3, heavy chain constant domains 1, 2 and 3; DMF, dimethylformamide; EtOH, ethanol; Fc, crystallisable fragment; GMP, good manufacturing practice; h, height; i.d., inner diameter; IgG, immunoglobulin G; mAbs, monoclonal antibodies; MCR, multi-component reaction; MeOH, methanol; VH, heavy chain variable domain; VHH, camelid heavy chain only variable domain; VL, light chain variable domain.

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(approximately £4000–6000 l^{-1} resin) [14], which is almost an order of magnitude more expensive than traditional chromatographic media with non-proteinaceous ligands [15]. Therefore, various approaches have been developed to enhance usability by achieving milder elution conditions via protein engineering [16], using lower cost affinity matrices or novel chemically synthesised affinity ligands with improved properties and lower cost [17–21]. In particular, pseudo-biospecific ligands, such as the tailored triazine scaffold ligands [19], designed with the assistance of new computational tools, have demonstrated several advantages over their biological templates.

The triazine scaffold has been well defined and studied to mimic Protein A [18,22] and Protein L [5,23]. However, although triazine scaffolds can display functional groups to mimic specific peptide and non-peptide templates, the synthetic route comprises a multi-step procedure requiring temperature changes between 0 and 90 °C. This harsh reaction condition may restrict the scalability of this technology. An alternative approach is to apply the solid phase Ugi multi-component reaction (MCR) [24], which is conducted at a constant temperature (50 °C) in a facile one-pot synthesis. This reaction is versatile and generates higher yields of the final product compared to the usual multi-step syntheses [25], especially at an industrial scale. Ugi et al. [24] first described this four-component reaction incorporating aldehydes (or ketones), amino compounds, various acids and isonitriles to generate a scaffold able to mimic peptide and peptoid bonds. The Ugi reaction has become one of the most commonly used methods in the chemical industry to search for new desirable products using libraries formed by the Ugi-MCR along with other related one-pot reactions [26]. As an alternative to solution phase synthesis, solid phase Ugi synthesis has also emerged [27], which uses an aldehyde-functionalised chromatographic matrix support, such as Sepharose™ CL-6B resin, as the oxo-component (aldehyde or ketone), and the product is synthesised directly on the surface of the beaded matrix, with three locations available for substitution [28]. The Ugi scaffold can also adopt more structural flexibility by possessing a less planar structure than the triazine scaffold.

Previous work conducted by Haigh et al. demonstrated the use of the Ugi solid phase scaffold to synthesise an immobilised ligand (A3C1I1) to mimic Protein L that binds preferentially to the Fab domain over the Fc domain [28]. This study supported the notion that the Ugi scaffold could be used as a credible alternative to triazines for affinity ligand synthesis. In contrast to the wide range of ligands mimicking Protein A and Protein L, there are no reports on Protein G mimics, despite the fact that Protein G binds to a wider range of IgGs and subclasses, and with higher affinity than Protein A [29,30]. Native Protein G binds to IgGs as well as albumin, but the albumin-binding domain is located at the N-terminal while the IgG binding regions are at the C-terminal [31] and most immobilised Protein G products exclusively employ recombinant versions from which these additional binding sites have been deleted [32]. Protein G includes three IgG-binding domains (C1, C2 and C3), each with 55 amino acid residues. The repeats are similar in their primary sequence, with two differences between C1 and C2, and six differences between C1 and C3 [33]. Sauer-Eriksson et al. [34] have reported the X-ray crystallographic structure of the complex between the C2 fragment of Protein G and the Fc domain of human IgG (PDB: 1FCC). Although the binding site of Protein G is similar to that of Protein A and both bind to the interface between the C_H2 and C_H3 domains of the Fc domain of IgG (Fig. 1A), they share neither sequence nor structural homology and the binding sites of Protein A and G are not super-imposable [20,34]. The relative binding of Protein A and Protein G to different immunoglobulins was compared under physiological conditions [35] and the results suggested that they show a complementary binding pattern: Protein G [29,30,36,37] binds stronger than Protein A [38–40] to polyclonal IgGs from cow, horse, and sheep, while the reverse was observed for polyclonal IgGs from guinea pig and dog.

Besides conventional mammalian antibodies, Protein A and Protein G also bind to camelid IgGs. These antibodies in the serum of *Camelus dromedarius* were first discovered by Hamers-Casterman et al. (1993) to contain subclasses that naturally lack light chains; they are termed "heavy-chain" antibodies or HCAb and display a molecular weight of ~95 kDa instead of 160 kDa for conventional mammalian antibodies [41]. cDNA sequence analysis revealed that the heavy chain also lacks the C_H1 domain, which appears to be spliced out during mRNA processing due to loss of a splice consensus signal [42,43], so that the heavy chain only variable domain (VHH) is connected directly to the constant domain (C_H2 and C_H3) via the hinge region. VHH has been reported to have four conserved originally hydrophobic amino acid residues substituted in the second framework region (FR2) [44,45]. These substitutions have a few implications: they play a key role in the binding to the light chain of the variable domain (VL) and their mutations to more hydrophilic residues can explain the lack of light chain in camelid antibodies. Secondly, these mutations could also explain why VHH has higher solubility than the VH domain isolated from conventional mammalian antibodies, which generally show a sticky behaviour [46], due to exposure of the hydrophobic interface with VL to an aqueous environment. Furthermore, the small size of these VHH domains (~15 kDa) allow greater access to buried epitopes and recognition of antigenic sites in clefts that generally could not be reached by larger conventional antibodies and hence they are effective enzyme inhibitors [47]. Besides serum, Dromedary camel milk also contains such heavy-chain only antibodies (IgG_2 and IgG_3) and the concentrations and heat resistance of camel milk antibodies were reported to be significantly higher than that in bovine and buffalo milk, which has shown to provide a stronger inhibitory system [48–50]. Therefore, the objective of this work is to design a Protein G mimic based on the solid phase Ugi-MCR and to investigate its ability to purify mammalian immunoglobulins including the non-conventional camelid IgGs.

2. Materials and methods

2.1. Chemicals and biologicals

All chemicals and biologicals were of at least reagent grade unless otherwise stated. 4-Aminobenzamide, epichlorohydrin, ethylene glycol, isopropyl isocyanide, sodium bicarbonate, sodium chloride, sodium hydroxide, sodium phosphate dibasic, sodium phosphate monobasic monohydrate, sodium periodate, sodium thiosulphate pentahydrate, bovine, human, goat, mouse, pig, rabbit, rat and sheep IgG (≥95% pure derived from serum), human serum (from human male AB plasma, sterile-filtered), Protein A – Sepharose® 4B Fast Flow and recombinant Protein G-Sepharose® Fast Flow were all purchased from Sigma–Aldrich (UK). Acetone, dimethylformamide (DMF), ethanol (EtOH), isopropanol (IPA) and methanol (MeOH) were purchased from Fisher Scientific (UK). Indole-3-acetic acid was purchased from Alfa Aesar (Germany). NuPAGE® MOPS SDS Running Buffer (20×), NuPAGE® LDS Sample Buffer (4×), NuPAGE® Antioxidant, Sample Reducing agent (10×), SimplyBlue™ SafeStain and Novex® Sharp Pre-stained Protein Standards (LC5800) were all purchased from Invitrogen Ltd. (UK). Chicken IgY was purchased from Newmarket Scientific (UK). Bovine Serum Albumin (BSA), Coomassie Plus (Bradford) Protein Assay reagent, disposable polystyrene columns (bed volume: 2 ml, 0.7 × 6.0 cm) and frits were purchased from Pierce (UK).

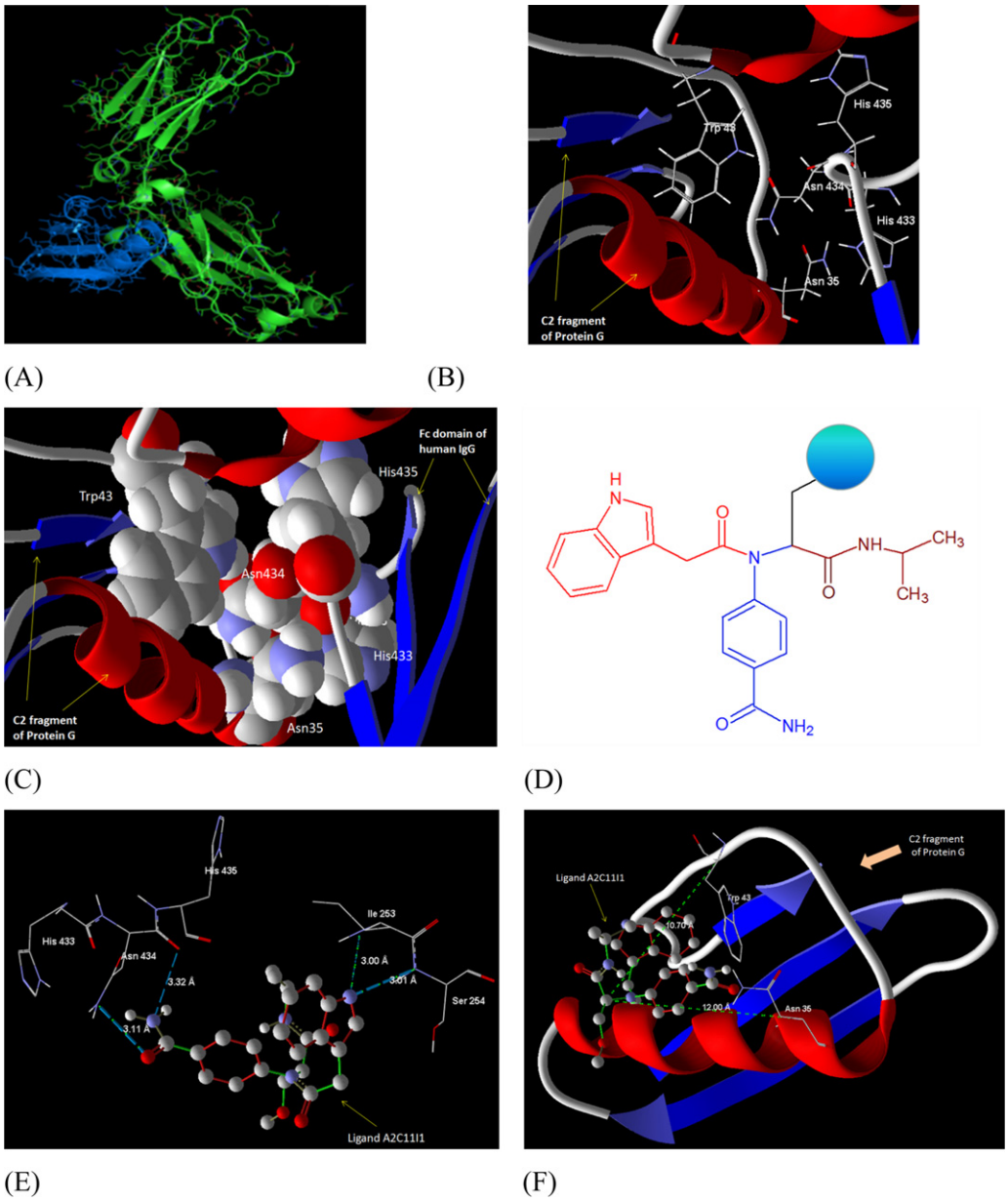

Fig. 1. (A) X-ray crystallographic structure of the C2 fragment of Protein G (blue) interacting with the Fc domain of human IgG (green) (PDB: 1FCC). The Fc domain is shown as a monomer in secondary structures for simplicity. Image created using PyMol v 2008.1.1 (Delano Scientific LLC, USA). (B) and (C): Asn35 and Trp43 residues on the C2 fragment of Protein G and the His433–Asn434–His435 triad on the Fc domain of human IgG in (B) wire form and (C) space-filling model. The protein complex (PDB: 1FCC) is shown in secondary structure (red: α-helices and blue: β-pleated sheets) with the relevant amino acid residues labelled. (D) Immobilised ligand A2C11I1 mimicking Asn35 and Trp43 on Protein G employing the solid-phase Ugi reaction comprised of an aldehyde-functionalised matrix (blue sphere: Sepharose™ CL-6B resin) to which the other three components, namely a primary amine (A2: 4-aminobenzamide, in blue), a carboxylic acid (C11: indole-3-acetic acid, in red) and an isonitrile group (I1: isopropyl isocyanide, in brown) are condensed to yield the ligand scaffold in a "one-pot" reaction. The diagram shows only one ligand per bead for clarity and the relative size is not to scale. (E) Ligand A2C11I1 docked into human Fc domain with putative H-bonds (blue dashed lines) distances between the docking interface of the ligand and relevant amino acid residues measured: Ile253 (3.00 Å), Ser254 (3.01 Å) and Asn434 (3.11 and 3.32 Å). (F) Distances (green dotted lines) between the main chain carbon of ligand A2C11I1 and the α-carbon of Asn35 (12.00 Å) and Trp43 (10.70 Å) on the C2 fragment of Protein G are labelled. Images created using Molegro Virtual Docker v 3.0.0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Sepharose™ CL-6B resin was purchased from G.E. Healthcare (Sweden).

2.2. Equipment and instrumentation

The 96-well microtitre plates were purchased from Corning Incorporated (Fisher Scientific, UK). Total protein concentration was determined using Coomassie Plus™ protein assay reagent by measuring the absorbance of samples at a wavelength of 600 nm using an EnVision 2104 Multilabel Reader (Perkin Elmer, USA). SDS-PAGE reducing gels conducted at Cambridge, UK were run using a Novex Mini-Cell gel tank (Invitrogen, USA) and scanned using a HP Scanjet G4050. SDS-PAGE reducing gels conducted at MonoJo, Jordan employed Mini-Protean® II Electrophoresis Cell (Bio-Rad, USA) and were photographed using a Canon camera model IXUS 8015.

2.3. Softwares

Ligand docking was conducted using Molegro Virtual Docker 2008 software MVD v 3.0.0 (Molegro Bioinformatics Solutions, Denmark) run on a standard Windows XP. Protein images were

visualised using Molegro Virtual Docker 2008 and PyMol v 2008.1.1 (Delano Scientific LLC, USA) Gel images were analysed using Image J 1.44 (National Institute of Health, USA).

2.4. Methods

2.4.1. Solid-phase Ugi synthesis

The experimental protocol and ligand nomenclature are identical to that described in Haigh et al. [28]. The Ugi ligand A2C11I1 was synthesised with the three reactants: 4-aminobenzamide (A2), indole-3-acetic acid (C11) and isopropyl isocyanide (I1).

2.4.2. In silico modeling and docking of ligand A2C11I1

The preparation and docking of the ligand (A2C11I1) followed the protocol described in Haigh et al. [28] except that human Fc domain (PDB accession code: 1FCC [34]) was imported into Molegro with the removal of the C2 fragment of Protein G, all water molecules and co-factors.

2.4.3. Chromatographic analysis and Bradford assay

Synthesised adsorbents (50%, w/v, slurry in 20%, v/v, ethanol) were gravity-packed into polystyrene columns, each with a column volume (c.v.) of 0.5 ml (0.7 × 1.3 cm). The packed resin was washed (D.I. H_2O, 10 c.v.) and equilibrated (20 mM Na_2HPO_4, pH 7.0, 10 c.v.) prior to loading the sample at a flow rate of 0.4 ml min^{-1} at 23 °C. After loading the protein sample (0.16 mg, 0.4 mg ml^{-1}, 0.4 ml, reconstituted in equilibration buffer), the column was washed (20 mM Na_2HPO_4, pH 7.0, 10 c.v.), and eluted with 0.1 M $NaHCO_3$, 30% (v/v) ethylene glycol, pH 10.0, 10 c.v. unless otherwise specified. Protein A and Protein G columns (c.v.: 0.5 ml) were eluted with 0.1 M Glycine–HCl, pH 2.7, 10 c.v. without regeneration. All the columns were rinsed (D.I. H_2O then 20%, v/v, ethanol, 10 c.v. each) and stored in 20% (v/v) ethanol (50%, w/v, slurry) at 4 °C for future use. Fractions (~0.4 ml) were collected at a flow rate of 0.2–0.5 ml min^{-1} dependent on the buffer and analysed using a standard Bradford assay protocol [51].

Chromatographic analysis of camel milk followed the identical protocol above, except that the elution buffers for immobilised Protein G were 0.15 M NaCl, 0.58% (v/v) acetic acid, pH 3.5 followed by 0.1 M Glycine–HCl, pH 2.7.

Total protein concentrations were measured using Bradford assay [51]. BSA and bovine IgG Bradford assay standard curves in various buffers were constructed within the linear range, from which the percentages of wash and elution of the total applied protein were calculated.

2.4.4. SDS-PAGE analysis

Reduced protein samples (excluding the molecular weight markers) were prepared by adding 6.5 μl sample to 2.5 μl NuPAGE® LDS Sample Buffer (4×) and 1 μl NuPAGE® sample reducing agent (10×) and incubated at 70 °C for 10 min following the electrophoresis procedure previously described [28]. The molecular weight marker was Novex® Sharp Protein Standard LC5800 (Invitrogen, UK). Additionally, BioRad Mini-Protean® II Electrophoresis Cell Instruction manual was followed for the gel electrophoresis conducted on the peak fractions of the preparative chromatography of camel milk, using Promega V8491 as the protein marker. Gel images were analysed using Image J 1.44 to determine the purity and the relative band intensities based on SDS-PAGE lane densitometry.

2.4.5. Partition equilibrium

Sepharose™ CL-6B resin and immobilised ligand A2C11I1 (0.02 g; ~24 μmol epoxy-ring g^{-1} moist weight gel) was equilibrated (20 mM sodium phosphate, pH 7.0) and incubated with 0.1 ml of bovine IgG solution (0–6 mg ml^{-1}) in equilibration buffer, under agitation at 23 °C for 24 h. The amount of unbound protein was measured using Coomassie Plus Bradford assay and the amount of bound protein was calculated by subtracting the free solute from the total solute added. The data was plotted as a Scatchard plot [52] to deduce the dissociation constant (K_d) and the binding capacity (Q_{max}) from the equation [22,53]:

$$\frac{C}{q} = \frac{1}{Q_{max}} \times C + \frac{K_d}{Q_{max}} \quad (1)$$

The control experiment comprised two sets: (1) incubating the respective concentrations of bovine IgG with unmodified Sepharose™ CL-6B resin and (2) incubating all the resins only with the equilibration buffer.

2.4.6. Synthesis and chromatography of Controls 1–10 of Ugi solid-phase ligand

At each stage of the Ugi solid-phase synthesis, samples (Controls 1–10) were examined for their respective binding capacity towards a fixed amount of bovine IgG (0.12 mg, 0.3 mg ml^{-1}, 0.4 ml). 5 g (moist weight) of each control sample was washed and stored in 20% (v/v) ethanol at 4 °C for chromatographic analysis. All incubation was carried out with agitation at160 rpm.

The control samples consisted of: *Control 1*: Unmodified Sepharose™ CL-6B resin. *Control 2*: Epoxy-activated Sepharose™ CL-6B created by incubating Sepharose™ CL-6B resin with epichlorohydrin under basic conditions [22,28] and washing with UHP H_2O. *Control 3*: *Cis*-diol compound generated after incubating the epoxy-activated resin in NaOH (5 M) overnight, and washing the sample with UHP H_2O. *Control 4*: Aldehyde-activated resin obtained after oxidation of the *cis*-diol compound [28], the aldehyde-derivatised Sepharose™ resin was washed thoroughly with UHP H_2O. *Control 5*: Schiff base formation by adding the amine component (A_2) to the aldehyde-derivatised Sepharose™ resin. Aldehyde-activated resin was washed with increasing concentrations of methanol (MeOH). The MeOH saturated resin was incubated with the selected amine, dissolved in 100% (v/v) MeOH (5-fold molar excess of the aldehyde-activated resin) for 1 h, and the resultant resin was split into two batches: the first batch was for Controls 6–8 which followed the Ugi MCR and the second batch was used for the synthesis of Controls 9 and 10, which involved reduction of the Schiff base. *Control 6*: Schiff base with the addition of the carboxylic acid component (C11). After formation of the Schiff base (Control 5), the carboxylic acid component (C11) dissolved in 100% (v/v) MeOH was added in 5-fold molar excess and incubated for 48 h at 50 °C. After incubation, the resin was washed in sequential post-synthesis wash steps [28]. *Control 7*: Schiff base with the addition of the isonitrile component (I1). After formation of the Schiff base (Control 5), the isonitrile component (generally isopropyl isocyanide, liquid) was added with 5-fold molar excess and incubated for 48 h at 50 °C. The resin was then washed following post-synthesis washes. *Control 8*: Complete Ugi MCR product: The Schiff base was mixed with both the carboxylic acid (C11) and isonitrile (I1) components and incubated for 48 h at 50 °C with agitation at160 rpm. Post-synthesis serial wash steps were conducted. *Control 9*: Reduced Schiff base. Schiff base (Control 5, 10 g) was reduced by incubation with $NaBH_3CN$ (10 ml, 50 mM dissolved in 1 M NaOH) overnight at 23 °C with agitation. After incubation, the reduced resin (A2′) was washed with UHP H_2O and stored in 20% (v/v) EtOH at 4 °C. *Control 10*: Reduced Schiff base (A2′, 5 g) with the addition of both the carboxylic acid (C11) and isonitrile (I1) components. Reduced Schiff base (A2′) was washed with UHP H_2O followed by washes (20 ml each) of increasing concentrations of MeOH until 100% (v/v) MeOH with 10% (v/v) increments. The carboxylic acid component (C11) dissolved in 100% (v/v) MeOH along with isonitrile (I1) were added to the MeOH saturated reduced resin and incubated for 48 h at 50 °C, followed by post-synthesis washes.

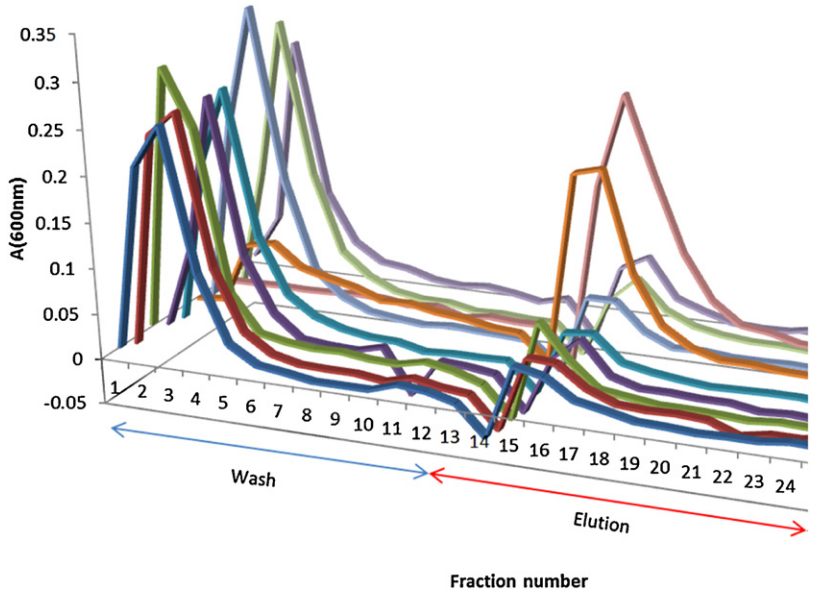

Fig. 2. Chromatography of bovine IgG on Controls 1–10 of immobilised A2C11I1 ligand. Controls 1–10 (from front to back) of the immobilised A2C11I1 Ugi ligand were synthesised (Section 2.4.6) and gravity packed into 2 ml disposable polystyrene columns (c.v. = 0.5 ml, *h*: 1.3 cm, i.d.:0.7 cm) for chromatographic analysis (Section 2.4.3). Bovine IgG (0.12 mg, 0.3 mg ml^{-1}, 0.4 ml) constituted in equilibration buffer (20 mM sodium phosphate, pH 7.0) was loaded onto each control adsorbent, washed with the equilibration buffer (10 c.v.) and eluted with the standard elution buffer (0.1 M $NaHCO_3$, 30%, v/v, ethylene glycol, pH 10.0, 10.c.v.). Each fraction was ~0.4 ml and the *x*-axis represents the fraction number, while the *y*-axis represents the absorbance at 600 nm in the Bradford assay.

Chromatographic analysis of each control was conducted identical to the protocol described in Section 2.4.3, except that a lower amount of bovine IgG (0.12 mg, 0.3 mg ml^{-1}, 0.4 ml) constituted in equilibration buffer was applied to each column.

3. Results and discussion

3.1. In silico design of the Protein G mimic

The C2 fragment of Protein G comprises one α-helix lying diagonally across a four-stranded β-sheet, with the amino acid residues involved in the IgG binding located at the C-terminal of the α-helix (Asn35), the N-terminal of the third β-strand (Glu42 and Trp43) and the loop (Asp40) connecting these two structures [34]. Furthermore, the following amino acid residues of Protein G: Glu27, Lys28, Lys31, Gln32, Asn35, Asp40, Glu42 and Trp43 [34] have been identified to interact with the Fc domain of human IgG, involving mainly three residues (Ile253–Ser254 and Gln311) in the C_H2 domain, and two areas (Glu380, Glu382 and residues His433–Gln438) in the C_H3 domain [34]. His435 on the Fc domain has been shown to play an important role in the binding to Protein A [18], whereas for Protein G binding, His433 and Asn434 demonstrate a more critical role [34]. Asn434 on the Fc domain forms hydrogen bonds (H-bonds) with Asn35 and Trp43 on the C2 fragment of Protein G, and also possibly forms H-bonds with the main-chain nitrogen atom of Gly41 and the carboxyl group of Asp40 [34]. The involvement of Asn434 in many interactions suggests that it is vital for the protein–protein interaction [34]; therefore, Asn35 and Trp43 from Protein G forming H-bonds with Asn434 were selected as the template for designing the Ugi ligand mimicking Protein G (Fig. 1B and C).

The final product of the solid phase Ugi reaction mimics a dipeptide [28]; 4-aminobenzamide (A2) and indole-3-acetic acid (C11) were employed to mimic the amide (Asn35) and indole (Trp43) functional groups, respectively. Both the amine and carboxylic acid components are fairly bulky, and to avoid excessive steric hindrance, a small isocyanide, isopropyl isocyanide (I1) was selected for the third reactant (isonitrile) (Fig. 1D).

3.2. In silico docking of ligand A2C11I1

The designed A2C11I1 ligand was docked into the Fc domain of human IgG (PDB: 1FCC) and suggested a putative binding interface at the junction of the C_H2 and C_H3 domains through four defined H-bonds (Fig. 1E), similar to native Protein G . The putative docking pose is buried inside the protein complex and the amino acid residues on the Fc domain involved in H-bonding (Ile253–Ser254 and Asn434) were also reported to be critical in the binding to native Protein G [34]. The relative orientation of the putative pose and the peptide template, i.e. Asn35 and Trp43, on the C2 fragment of Protein G were compared (Fig. 1F), and interestingly, the amide group of 4-aminobenzamide (A2) and the indole ring of indole-3-acetic acid (C11) of the ligand are positioned in a very similar orientation to the side chains of Asn35 and Trp43, respectively.

3.3. Chromatography of Controls 1–10

Chromatograms of bovine IgG on all 10 controls (Fig. 2) showed that Control 8, which is the intact immobilised A2C11I1 ligand, demonstrated 100% binding, followed by a sharp elution peak (~95% elution, w/w, of total bovine IgG applied). Control 6, which has incorporated the main functional groups of the ligand after the addition of the amine (A2) and the carboxylic acid (C11), had a similar chromatogram as Control 8, demonstrating some degree of binding (~56%). The other controls demonstrated ≤25% (w/w) binding, probably due to non-specific binding to the solid phase matrix. This reinforced the notion that the complete structure of the solid phase Ugi ligand is required for the high binding capacity to IgG under this set of adsorption and elution conditions.

3.4. Chromatography of immunoglobulins derived from different sources

The binding of IgG samples from different species and chicken IgY to immobilised A2C11I1 was assessed. Under the conditions selected, cow and sheep IgG showed 100% binding, with pig IgG binding ~83%. Mouse, rabbit and goat IgGs showed >70% binding, followed by chicken IgY (~70%) and human IgG (~66%). Rat IgG demonstrated the lowest affinity (~56% binding). Based on the percentage of binding of each immunoglobulin sample on immobilised A2C11I1, in comparison with the binding specificity of Protein A [38–40] and Protein G [29,30,36,37] (Fig. 3A andB), the immunoglobulin samples located on the straight line ($y = x$) indicate that they show similar binding strength to the two affinity ligands on the x and y-axes. A comparison of Protein A and A2C11I1 adsorbents (Fig. 3A) shows that there is no IgG sample lying on the $y = x$ line, whereas Protein G (Fig. 3B) has four. This suggests that immobilised A2C11I1 binds to a range of IgGs with a similar profile as Protein G, and thus supporting the design of this ligand as a Protein G mimic.

Furthermore, the range of immunoglobulins that bound to immobilised A2C11I1 confirms its potential to purify IgGs from a wide range of species, particularly those that show no affinity to either Protein A or Protein G. This behaviour is similar to synthetic Protein A mimics such as the peptide mimic TG19318 (or PAM: protein A mimetic) [54,55] and the triazine-based synthetic ligand ApA (artificial Protein A) [18]. In contrast, 23-nucleotide RNA aptamer (Apt8-2) mimicking Protein A shows high specificity and affinity only to human IgG but not to other IgGs [56]. It is advantageous that the A2C11I1 adsorbent binds to a wider range of immunoglobulins.

3.5. Purification of IgG from human serum

A human serum sample diluted 5-fold in equilibration buffer was loaded onto immobilised A2C11I1, Protein A and Protein G adsorbents, and their respective purification efficiencies were compared. Fig. 4 shows that human serum albumin (HSA: 66 kDa) is the major component present in the applied sample and the wash fractions of the three adsorbents. More importantly, the elution fractions (Fig. 4; lanes 5, 7 and 9) from the three adsorbents showed similar protein profiles. Although the immobilised A2C11I1 eluted ~25% less human IgG (hIgG) than the Protein A and Protein G columns, the purity of the eluted hIgG (~65%) was similar to that eluted from the Protein A (~75%) and Protein G (~62%) adsorbents. HSA appeared to remain as a contaminant in the elution fractions of the immobilised A2C11I1 (~7%), Protein A (~3%) and Protein G (~3%) adsorbents. The other main contaminants in the elution fraction of the immobilised A2C11I1 (Fig. 4; lane 5) include α-2-macroglobulin (MW: 180 kDa, ~4%) and an unspecified protein (MW: ~28 kDa, ~10%). It is conceivable that the performance of immobilised A2C11I1 could be improved after extensive optimisation of the buffer conditions and some contaminants could be eliminated via step-wise elution.

3.6. Recombinant camelid Fc domain expressed in E. coli cells

Although the design of the ligand A2C11I1 was based on the Fc domain of human IgG, the primary sequence of the Fc domain of camelid IgG (GenBank accession number: Z48947) shares ~70% homology with human IgG (PDB: 1FC2) (supplementary data Fig. 1), with the C-terminus of the Fc domain being identical, including the key residues His433–Asn434–His435–Tyr436 in the protein sequence in one-letter code: VMHEALHNHYTQKS (supplementary data Fig. 1 underlined). Therefore, it was hypothesised that the immobilised A2C11I1 adsorbent should also bind to camelid Fc and thereby purify camelid IgGs. Due to the lack of commercial availability of camelid IgG, the cDNA of the Fc domain was extracted from camel lymphocytes, cloned into pET15(b) plasmid, encoding a His_6-tag at the N-terminus and expressed in *E. coli* cells in the form of inclusion bodies, which subsequently underwent solubilisation, purification (Ni-NTA affinity chromatography) and refolding.

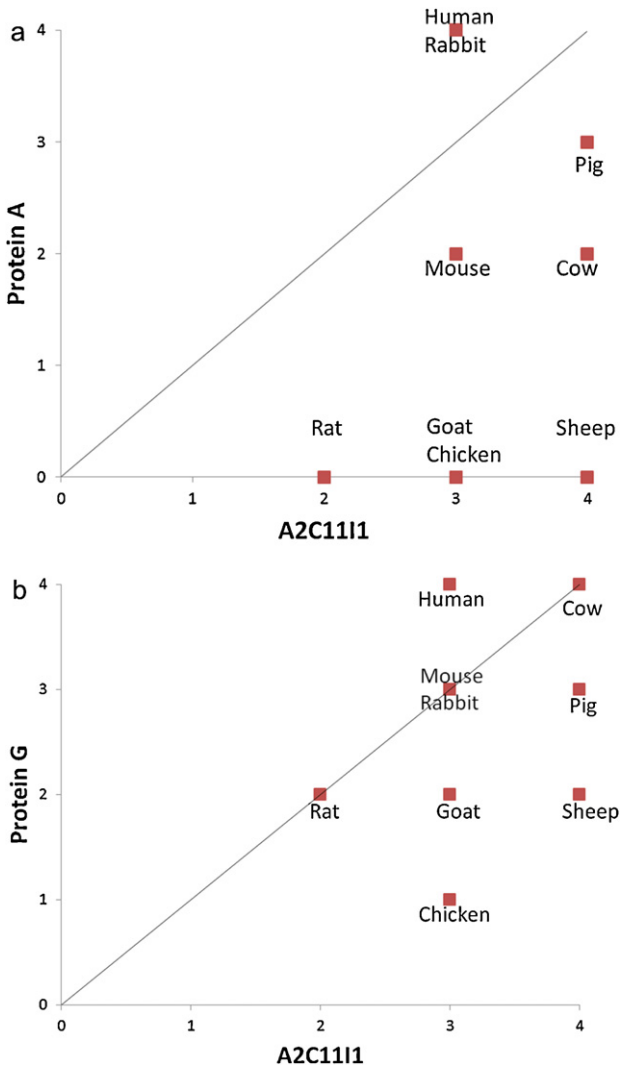

Fig. 3. Comparison of the binding strength of immunoglobulin samples between Protein A, Protein G and immobilised A2C11I1 adsorbents. Each immunoglobulin sample (0.16 mg, 0.4 mg ml^{-1}, 0.4 ml) was constituted in equilibration buffer (20 mM sodium phosphate, pH 7.0) and applied to each equilibrated A2C11I1 adsorbents for chromatographic analysis. The % binding of each immunoglobulin sample on immobilised A2C11I1 adsorbent was calculated. Based on the respective binding strength of immobilised A2C11I1 and that of Protein A and Protein G, with the number of "+"s indicating the binding strength, a comparison was conducted by plotting the different immunoglobulin samples belonging to each category of number of "+"s. The ranking of the binding strength, i.e. the number of + for Protein A and G is obtained from Sigma and Merck Product information with the relevant references indicated. For immobilised A2C11I1 adsorbent, it was arbitrarily categorised that based on the % (w/w) binding, ++++ for >80%, +++ for 60–80%, ++ for 40–60%, +for 20–40% and – for <20% (w/w) binding. The binding specificity of immobilised A2C11I1 adsorbent with (A) Protein A and (B) Protein G was compared and the number on the x- and y-axes represents the number of "+" signs for each immunoglobulin sample. The inclined line represents $y = x$.

Proteins or peptides required for biochemical and structural studies are usually produced via bacterial expression systems when post-translational modification, such as glycosylation, is not required [57,58]. Glycosylation of antibodies mainly impacts the Fc effector functions, such as binding to Fc receptors on effector cells [59,60] and complement activation [61] rather than affinity

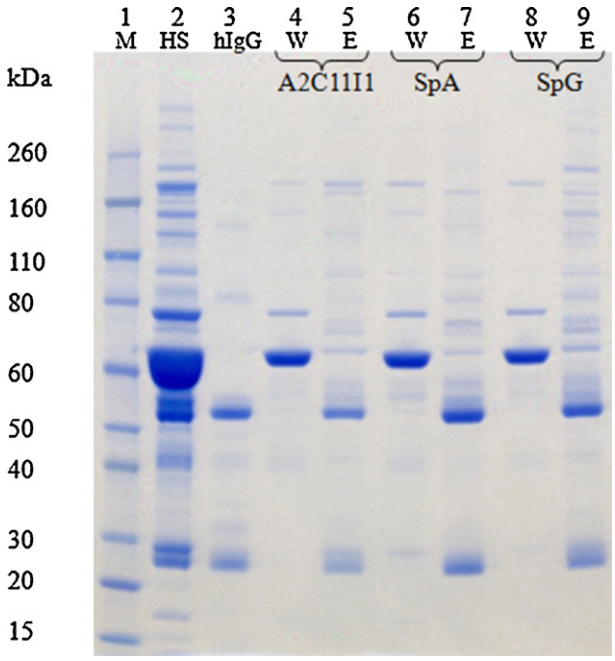

Fig. 4. SDS-PAGE of the peak fractions in the purification of IgG from human serum on immobilised A2C11I1, Protein A and Protein G adsorbents. Human serum (HS) diluted 5-fold in equilibration buffer (20 mM sodium phosphate, pH 7.0) was loaded onto the equilibrated immobilised A2C11I1, Protein A and Protein G adsorbents (c.v. = 0.5 ml (0.7 × 1.3 cm)) at a flow rate of ~0.4 ml min^{-1} at 23 °C (0.4 ml diluted sample applied to each column) for chromatographic analysis. Based on the chromatograms, peak fractions were analysed using reducing SDS-PAGE. Lane 2: Applied human serum (HS) sample diluted 10-fold. Lane 3: Human IgG (hIgG 0.2 mg ml^{-1}) to indicate the position of the heavy (~50 kDa) and light (25 kDa) chains. Lanes 4–5, 6–7, 8–9: Peak wash and elution fractions of immobilised A2C11I1, Protein A and Protein G adsorbents, respectively. SpA: Protein A column, SpG: Protein G column, W: peak wash fraction, E: peak elution fraction. All the wash fractions were diluted 5-fold prior to loading the gel.

to Protein A and G. Expression of the Fc domain of human IgG in *E. coli* produced a non-glycosylated protein which showed the same binding to Protein A as the whole immunoglobulin [62]. In addition, since the Fc domain contains disulphide bonds, reducing agents such as β-mercaptoethanol, dithiothreitol (DTT) or cysteine are often used to allow reduction of the disulphide bonds by thiol-disulphide exchange and thus increase solubility [63,64]. Inclusion bodies were first solubilised with chaotropic agents (7 M urea), purified and then refolded by gradually removing the denaturants in the presence of an oxido-shuffling system, which consists of a combination of reduced and oxidised low molecular weight thiol reagents [65], such as reduced and oxidised glutathione (GSH/GSSG) [65–67].

The successful renaturation of the camelid Fc domain was supported by the observation that ~99% of the loaded refolded recombinant protein bound to immobilised Protein A and Protein G columns and eluted in a sharp peak at pH 2.7. Furthermore, the immobilised A2C11I1 adsorbent also quantitatively (~100%) bound to the recombinant camelid Fc domain.

3.7. Purification of IgGs from camel milk

Since the recombinant camelid Fc domain bound to immobilised A2C11I1, it was desirable to establish if the adsorbent could purify IgGs directly from camel milk. The three IgG subclasses of camel serum [68] and milk [69] have been separated using a combination of Protein A and Protein G chromatography. Protein A binds to all three subclasses, but could not resolve them, whereas Protein G binds to only IgG_1 and IgG_3, with IgG_3 (43 kDa) eluted at pH 3.5

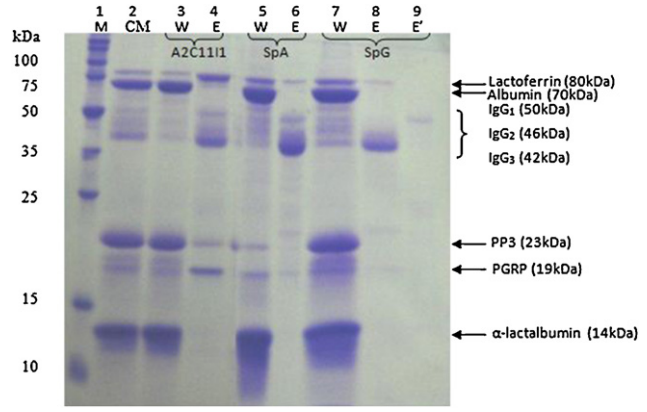

Fig. 5. Comparison between immobilised Ugi adsorbent A2C11I1, Protein A and Protein G resin in the purification of IgGs from camel milk. Gravity-packed immobilised Ugi ligand A2C11I1 (~24 μmol ligand g^{-1} moist weight gel), Protein A and Protein G columns (c.v. = 0.5 ml, h = 1.3 cm, i.d. = 0.7 cm) were washed with 5 ml dH_2O and equilibrated at 25 °C. De-fatted and de-caseinated camel milk (1 ml) was loaded onto each column at a flow rate of 0.4 ml min^{-1}. Lane 2: Camel milk diluted 5-fold, lanes 3–4: peak wash and elution fraction of immobilised A2C11I1, lanes 5–6: peak wash and elution (pH 2.7) fractions of Protein A, lanes 7–9: peak wash, elution (E: pH 3.5 and E': pH 2.7) fractions of Protein G. M: Molecular weight marker: Promega V8491, A2C11I1: immobilised A2C11I1, SpA: Protein A column, SpG: Protein G column, PP3: component 3 of proteose peptone and PGRP: peptidoglycan recognition protein.

(0.15 M NaCl, 0.58%, v/v, acetic acid) and IgG_1 (heavy chain: 50 kDa, light chain ~25 kDa [70]) at pH 2.7 (0.1 M Glycine–HCl). Preparative chromatography of camel milk was conducted as described in Section 2.4.3 and the electrophoresis result of the peak fractions for each adsorbent is shown in Fig. 5.

Under standardised adsorption and elution conditions, the purity of IgGs, including all three subclasses eluted from the immobilised A2C11I1 column (Fig. 5; lane 4) was ~50%, with the main contaminants being lactoferrin (80 kDa [69], ~25%), component 3 of proteose peptone (23 kDa [71], ~8%) and peptidoglycan recognition protein (19 kDa [72], ~16%). There is minimal contamination with albumin in the elution fraction, which is consistent with the results purifying IgGs from human serum (Fig. 4). In comparison, the purity of camelid IgGs eluted from Protein A (Fig. 5; lane 6) was ~85%, with the principal contaminant being lactoferrin (~5%), whilst Protein G eluted less protein but achieved ~90% purity (Fig. 5; lane 8). The step-wise elution from the Protein G column at pH 3.5 (lane 8) and 2.7 (lane 9) separated the heavy-chain only IgG_3 (42 kDa) from the conventional hetero-tetrameric IgG_1 (heavy chain: 50 kDa, light chain: 25 kDa). Therefore, Protein A would be the better choice for the purification of camelid IgGs from camel milk, whilst Protein G is preferable to resolve the IgG subclasses. Although the purity of the eluted protein achieved by immobilised A2C11I1 is not as high as the Protein A and G adsorbents, further buffer selection revealed potential to improve its performance to purify camelid IgGs specifically.

3.8. Optimisation of bovine IgG elution

The role of the elution buffer is to disrupt the interaction between the immobilised ligand and the target molecule to reduce the binding affinity between them [73]; therefore, the composition of the elution buffer was varied to ascertain if the elution of bovine IgG from the immobilised A2C11I1 adsorbent could be improved. Water-miscible organic solvents, such as ethylene glycol (30–50%, v/v) are commonly used for elution to disrupt hydrophobic interactions [74–77]. Previous experience with immobilised Ugi ligands showed that the interaction between the ligand and the target molecule is likely to be a combination of hydrophobic and electrostatic interactions [78]; therefore, different concentrations

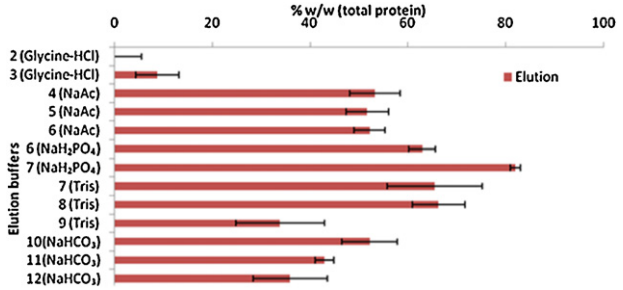

Fig. 6. Effect of pH on the % elution (w/w) of bovine IgG from an immobilised A2C11I1 adsorbent. Ligand-immobilised Sepharose ™ (~24 μmol ligand g^{-1} moist weight gel, 50%, w/v, slurry in 20%, v/v, ethanol) was gravity packed into a column (c.v.: 0.5 ml (0.7 × 1.3 cm)) prepared for chromatographic analysis. A bovine IgG sample (0.16 mg, 0.4 mg ml^{-1}, 0.4 ml) constituted in equilibration buffer (20 mM sodium phosphate, pH 7.0) was loaded onto an equilibrated A2C11I1 adsorbent and each column was eluted with an elution buffer with a different pH value ranging from 2 to 12, all with a molarity of 0.1 M and containing 30% (v/v) ethylene glycol. For each column, 5 ml of the corresponding elution buffer was applied followed by 5 ml of regeneration buffer (0.1 M NaOH, 30%, v/v, isopropanol). The absorbance of each fraction at 600 nm was measured and the percentages (w/w) of elution of total protein applied were calculated. The experiments were conducted in triplicate and the average of the calculated percentages against each elution buffer is summarised with standard deviation as error bars.

of ethylene glycol in the elution buffer (0.1 M $NaHCO_3$, pH 10.0 containing 0–50%, v/v, ethylene glycol in 10%, v/v, increments) were examined. Comparison of the % elution (w/w) of total bovine IgG applied demonstrated that as the proportion of ethylene glycol increased from 0% to 30% (v/v), the % elution increased from 0% to 57% (w/w) correspondingly. However, at higher concentrations of ethylene glycol, i.e. >30% (v/v), little additional effect was observed, except with a slight decrease to 45% (w/w) elution at 50% (v/v) ethylene glycol. This is probably because the higher concentrations of ethylene glycol may alter the conformation of the protein and/or the immobilised ligand, leading to a decreased % elution [79]. Furthermore, inclusion of a high % of ethylene glycol increases buffer viscosity and slows down the elution process; thus, inclusion of 30% (v/v) ethylene glycol was selected to be the optimal concentration.

The other aspect investigated was salt (NaCl) concentration (0–1 M), and the results showed that elution of bovine IgG was not particularly sensitive to the concentrations of NaCl applied, achieving ~65% (w/w) elution in its absence, with an average of ~55% (w/w). Therefore, the addition of NaCl in the elution buffer was not necessary for this purpose.

The elution efficacy of IgG at different pH values (pH 2–12) were examined (Fig. 6) as extreme pH conditions disrupt ionic bonds [73]. All the elution buffers were the same molarity (0.1 M) and contained 30% (v/v) ethylene glycol to disrupt hydrophobic interactions. Some trends were discernible: Low pH values (pH 2 and 3) resulted in <10% IgG elution, but at pH 4 and 5, >50% IgG was eluted, similar to that at pH 10 (Fig. 6). High pH was originally considered to be an effective elution condition for immobilised Ugi ligands [28]; thus, elution buffers with pH 9–12 were expected to achieve the highest elution yield. However, this was not observed for immobilised A2C11I1, since as the pH of the elution buffer increased from 10 to 12, the % elution fell from ~54% to ~37%, and such buffers generally did not perform as well as the elution buffers between pH 6 and 8.

Elution buffers with pH values in the range of 6–8 showed the highest elution rates, ranging from 53% to 82%, and averaging ~65% (Fig. 6). This range of pH values was not previously investigated on the immobilised A3C1I1 ligand mimicking Protein L [28], but the effective elution of immobilised A2C11I1 at neutral pH could be highly advantageous, since it provides an alternative to the low pH elution for natural (Protein A and Protein G) and synthetic (triazine 8/7 [5] and 22/8 [22]) affinity ligands.

Depending on the functional groups incorporated in the ligand, both hydrophobic and electrostatic interactions may participate in the interaction with the protein. Since the immobilised A2C11I1 adsorbent carries hydrophobic groups, such as the indole ring of indole-3-acetic acid (C11) and the phenyl ring of 4-aminobenzamide (A2), the adsorption involves a reversible association between the hydrophobic functional groups on the ligand and the hydrophobic moieties on the target protein [80]. Further analysis of the protonation states of ligand A2C11I1 shows that between pH 2 and 11, >98% of the ligand is in the neutral form, which suggests that the elution of IgG from immobilised A2C11I1 is probably not contingent on electrostatic interactions. Thus, it is postulated that hydrophobic interactions may play a dominant role in the binding, which is consistent with the effect of including ethylene glycol in the elution buffer.

The peptide ligand TG19318 (or PAM) was similarly able to elute adsorbed antibodies at two ranges of pH values, 0.1 M acetic acid pH 2.8 or 0.1 M sodium bicarbonate pH 9, and addition of 0.5 M NaCl was reported to generate a sharp elution peak [55], suggesting that the interaction between ligand TG19318 and the antibodies could be predominantly electrostatic; hence, extreme pH and high ionic strength contained in the elution buffers tend to interrupt the binding [73]. This may differ from the triazine ligands and Ugi ligands developed to date, where hydrophobic interactions play a more dominant role. Although neutral pH achieved the highest % elution (82%), pH 10 was reasonably effective and thus the standard elution buffer used to date for immobilised A2C11I1 ligand: 0.1 M $NaHCO_3$, 30% (v/v) ethylene glycol, pH 10.0, was deemed an appropriate eluent. Nevertheless, the use of 0.1 M sodium phosphate, 30% (v/v) ethylene glycol, pH 7.0 might achieve higher elution yields of more stable protein.

Contributions from the spacer arm emerge from both its chemical nature, flexibility and length, which affect the immediate environment of the immobilised ligand [81]. Haigh et al. [28] investigated two spacer arm lengths (3 atoms and 11 atoms) of the lead ligand (A3C1-immobilised Sepharose™) and showed that the longer spacer arm had less than half the binding capacity of the shorter one, which might be explained by generating local steric interference or enhancing interactions between the ligand and matrix backbone [82].

3.9. Partition equilibrium

The maximum binding capacity for bovine IgG (Q_{max}) and its dissociation constant (K_d) to the solid phase A2C11I1 adsorbent were assessed by partition equilibrium. Q_{max} and K_d were calculated from the Scatchard plot ($R^2 = 0.975$) derived from the Langmuir isotherm, which suggests a static binding capacity of $Q_{max} = 24.6$ mg ml^{-1} moist resin and a dissociation constant of $K_d = 4.78 \times 10^{-6}$ M.

4. Conclusions

To date, synthetic Protein G ligands have not been reported. This work exemplifies the application of the Ugi MCR solid phase synthesis to design and evaluate a Protein G mimic (ligand A2C11I1). *In silico* docking supported the rational design, but this at most, is a semi-rational process, because solid phase synthesis introduces numerous unknown factors such as chemical, geometrical and steric constraints of the complex three-dimensional solid matrix environment [83]. Therefore, *in silico* analysis was further supported by chromatographic experiments where immobilised A2C11I1 demonstrated affinity to immunoglobulins from a wide range of species, including the non-conventional camelid IgGs, which was broader than that of Protein A and Protein G. This

allows the A2C11I1 adsorbent to purify IgG species that bind neither to Protein A nor Protein G columns. Studies on optimising the elution buffers suggested that the binding of IgG to immobilised A2C11I1 is predominantly hydrophobic and similar to the triazine ligands. More importantly, the most effective elution was achieved at neutral pH (~82%), which could be a major advantage for the application of solid phase Ugi ligands, because this could provide a credible alternative to the standard acidic pH elution required for a range of natural and synthetic affinity ligands, where such low pH may result in conformational changes of the antibodies [13]. The dissociation constant of the Ugi ligand was in the range of 10^{-6} M, which is suitable for purification purposes [84], since too high an affinity is not ideal for affinity purification due to the need for harsh elution conditions [85]. In conclusion, the solid phase Ugi scaffold provides a credible platform to synthesise affinity ligands with distinct properties, with applicability to the purification of immunoglobulins and other biotherapeutic molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.03.043.

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