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Antibody purification by affinity chromatography based on small molecule affinity ligands identified by SPR-based screening of chemical microarrays

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ABSTRACT

Libraries of small molecules were searched for Fc-fragment selective binders to a recombinant human antibody ("MDJ8", IgG_1-subtype, κ-light chain) via SPR-based screening of chemical microarrays. Identified hit structures were immobilised on NHS-activated Sepharose for the determination of MDJ8 binding and selectivity versus typical proteineous impurities represented by the spend cell culture supernatant. Columns were packed and the most promising ligands further characterized in terms of binding constants, binding kinetics, as well as dynamic and equilibrium binding capacities. The performance of the best ligand, 2A10, was compared to standard Protein A chromatography. Using ligand 2A10 antibody capture from unprocessed cell culture supernatants was possible at similar recovery yield (>90%), purity (>80%), and eluting concentration (approximately 1 g/L) as with Protein A. Affinity constants (K_d) of 2A10 were an order of magnitude higher than for the Protein A material, but still in the nM-range, while maximum binding capacities and binding kinetics were in the same order of magnitude. Ligand 2A10 was also able to capture a murine monoclonal antibody, again with similar efficiency as Protein A, as well as a number of humanised therapeutic antibodies. Antibody elution from the 2A10 column was possible using the Protein A standard protocol, i.e. 100 mM glycine HCl pH 3.0, but also at near physiological pH, when some organic solvent or modifiers were present. Ligand 2A10 thus constitutes a cheaper, more robust alternative to Protein A as possible generic antibody binder. Moreover, the outlined approach to ligand selection could in principle by used to create suitable affinity ligands for other high value biotech products.

1. Introduction

Product isolation, the so-called downstream process, is an acknowledged bottleneck in recombinant protein production [1]. Efficient product capture can be achieved by affinity chromatography and related techniques using specifically interactive ligands that are capable of recognising and retaining the product from a complex raw feed solution [2]. However, suitable affinity ligands are not always known. In consequence, there has been considerable effort in the past to identify and/or synthesize the required affinity ligands. An early approach was the use of dyes, such as Cibacron Blue [3], known to mimic the co-factor of certain enzymes. Of course this approach was strongly limited to a particular substance class. A more general approach is the use of product-specific antibodies or engineered affinity proteins [4,5]. Using proper methods, such antibodies/proteins can be raised against most molecules of interest regardless of whether they occur in nature or not. Possible drawbacks of proteins as affinity ligands are their high cost, their complex production, their limited stability under operating conditions, and in particular the inherent difficulties in finding suitable methods for regeneration and sanitisation of the corresponding affinity columns. More recently, a number of strategies have become available to identify specifically interactive peptides from large libraries; examples include, e.g. phage or ribosomal display techniques [5–8]. However, the stability of the peptidic ligands may again pose a limitation, while the type of interaction motives that can be exploited remains restricted to the chemical groups found in the side chains of the natural protein-forming amino acids.

For certain product classes generic affinity ligands are available. The most prominent example is the use of Protein A for capture of antibodies [9]. A Protein A step is nowadays included in most industrial antibody purification schemes and the availability of this ligand has certainly contributed to the standardisation and streamlining of industrial antibody purification. In order to enlarge

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the group of target biologicals susceptible to generic purification schemes, the concept of an affinity tag has been proposed [10–12]. In such cases the recombinant target protein is expressed as a fusion protein with a peptide sequence, the "tag", to which an affinity ligand exists. The most prominent example for this approach is the HIS_6-tag for use in Ni^{2+}-based immobilised metal affinity chromatography [12]; other examples include the FLAG- or GST-tags. Especially for research and early screening, affinity tags constitute a powerful approach, which is, however, limited to recombinant proteins. Even then in certain cases (e.g. proteins expressed with a signal sequence, protein requiring the free N-/C-terminus for biological activity) positioning the tag can be difficult. Bias of the biological activity and the three-dimensional structure of the target molecule can also not be excluded. Often the tag has to be removed at a later stage by proteases. For production purposes, tag-based affinity purification is therefore not ideal.

Synthetic small molecules would putatively also constitute interesting affinity ligands for the purification of therapeutic proteins due to their generally high chemical stability and low production costs. Proposed general strategies for designing such small synthetic affinity ligands include structure based approaches using NMR or X-ray crystallography data, the modification of known or natural ligands, and last but not least also the screening of small combinatorial libraries [13]. The latter approach would be particularly powerful as it requires comparatively little prior knowledge and is easily adapted to high throughput screening (HTS) formats. However, whereas chemical libraries comprising thousands of compounds are routinely screened in drug discovery to find starting points for further optimisation, this is currently not done in the search for affinity ligands [14]. Moreover, current HTS assay formats are typically designed to address a particular, predefined binding site in a given protein of interest and are therefore not necessarily suited to the unbiased identification of the best affinity ligand for this protein's purification.

In recent years, biophysical methods such as X-ray, NMR and label-free biosensors, e.g. based on surface plasmon resonance (SPR), were adapted as detection principles to the HTS screening formats. Such approaches have been successfully used to identify chemotypes binding specifically to the target of interest. In particular, SPR became increasingly popular and is now recognised as a standard method for screening collections of small molecules. In particular array based systems can provide the necessary throughput for successful screening of large collections. Graffinity Pharmaceuticals GmbH, Heidelberg, Germany, has developed a unique SPR screening technology based on the immobilisation of thousands of compounds on high-density chemical microarrays (9219 compounds per chip). CCD based SPR imaging instrumentation then allows screening of the entire collection of immobilised library compounds in a high throughput manner [15–17]. The platform has so far been mainly applied to drug discovery projects [16,18–20]. However, as a function-blind and label-free assay, SPR screening is not restricted to, e.g. enzymatically active targets and promising ligands for affinity chromatography could in principle be found in a similar manner.

To our best knowledge, however, the identification of an affinity ligand using such an approach has never been described. In this contribution, small molecule libraries available at Graffinity Pharmaceuticals GmbH were screened for suitable affinity ligands for a recombinant human therapeutic antibody. The ligands identified in the primary SPR screening were subsequently immobilised on a Sepharose matrix and the affinity separation developed based on these stationary phases was compared to a conventional approach using Protein A affinity chromatography.

2. Materials and methods

2.1. Materials

Chemicals and standard proteins were from Acros Organics (Geel, Belgium), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma–Aldrich (St. Louis, USA). Plastic materials for cell culture were from Greiner Bioscience (Frickenhausen, Germany). Antibodies Avastin™ (Bevacizumab) and Roactemra™ (Tocilizumab) were from Roche Registration Limited, Welwyn Garden City, UK, antibody Synagis™ (Palivizumab) was from Abbott Laboratories, Queenborough, UK. Purified polyclonal human and rabbit IgGs were from Sigma–Aldrich. Milli Q water was used to prepare all aqueous solutions. Chromatographic buffers were filtered with a nylon membrane filter (0.2 μm pore size, Nalgene, Rochester, USA) and degassed prior to use. Cell culture media and supplements were from PAA Laboratories (Linz, Austria) unless otherwise indicated.

2.2. Analytics

Ligands were quantified as follows: dissolved ligands, appropriately diluted in solvent (20 mM boric acid, 50% DMSO), were incubated with staining solution (1 mM o-phthaldialdehyde (OPA), 20 mM 2-mercaptoethanol in solvent) for 25 min at room temperature. Afterwards, absorption at 340 nm was measured. Measurements were calibrated with OPA-derivatised 1-(3-aminopropyl)imidazole. Protein concentrations were established by Bradford assay (Protein Assay kit, BioRad Laboratories, Hercules, USA) according to the manufacturer's instruction using bovine gamma globulin (BGG) for calibration (range 1.0–20.0 μg/mL, $R^2 > 0.99$, SD < 3.5%). Data points were obtained at least in duplicate and at three different dilutions. Only data points that fell within the range of the calibration curve were used. The purity of the obtained protein fractions was established via SDS–PAGE using Criterion XT Precast gels (BioRad Laboratories) according to the manufacturer's instructions. Gels were silver (SilverSNAPE Stain Kit II, Thermo Fisher Scientific Inc., Rockford, USA) or Coomassie Blue (Bio-Safe Coomassie, BioRad Laboratories) stained according to the supplier's instructions. The molecular weight standard was the Precision Plus Protein Standard All Blue (BioRad Laboratories).

Biacore binding studies (BIAcore X, GE Healthcare, Uppsala, Sweden) were performed in duplicate at 20 °C using ligand-coated chips (sensor chip type CM5 Research grade, surface covered with carboxymethylated dextran, allowing immobilisation via carbodiimide chemistry). Ligands were coupled to the chip using the amino coupling kit provided by GE Healthcare according to the supplier's instruction. Coupling solution: approximately 10 mM ligand in 200 mM borate buffer pH 8.0, 10 mM N,N-diisopropylethylamine (DIPEA), 8% DMSO. The amount of immobilised ligand was determined via the sensor readout (in relative units, RU, with 1000 RU corresponding to approximately 1 ng of retained material per mm^2 of chip surface). Residual linkage sites were deactivated by 35 μL (flow rate 5 μL/min) of 1 M ethanolamine (pH 8.5). For Biacore measurements the running buffer was 10 mM sodium phosphate buffer pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.005% Tween 20. 35 μL-samples were introduced into the instrument at a flow rate of 20 μL/min. After each measurement, the chip was regenerated with 35 μL of 50 mM NaOH (flow rate 20 μL/min). For data analysis the BIAevaluation software version 4.1 was used.

2.3. Antibody production

Recombinant human antibody "MDJ8″ (h-IgG_1-subtype, κ-light chain) was produced by a Chinese Hamster Ovary (CHO)

cell line (MDJ8) containing the appropriate expression cassettes of the full-length immunoglobulin [21]. A molecular weight of 149.5 kDa had previously been determined for the fully glycosilated antibody via ESI-TOF MS and an isoelectric point (p*I*) of approximately 9.3 via isoelectric focusing gel electrophoresis [22]. The antibody was produced as described previously [23] by suspension culture in a 1 L bioreactor (RALF, Bioengineering, Wald, Switzerland) using a protein-free culture medium (ProCHO5, Lonza, Verviers, Belgium) supplemented with L-glutamine, glucose and penicillin/streptomycin. Bioreactors were harvested after 5 days. Monoclonal antibody "5D10″ was produced as described previously [24] by a mouse hybridoma cell line (5D10). CD hybridoma medium (Invitrogen, Carlsbad, USA) supplemented with L-glutamine, penicillin/streptomycin, and sodium pyruvate was used. Production occurred in 100 and 500 mL spinner flasks. Cultures were harvested after 100 h.

For antibody isolation, supernatants were clarified by centrifugation (4000 g, 1 h, 4 °C), passed through a Minisart single use filter unit (0.45 μm pore size, Sartorius Stedim Biotech, Göttingen, Germany), gently degassed (under vacuum) and subjected to Protein A affinity chromatography (column: HiTrap Mab Select Xtra, 5 mL, GE Healthcare). The Protein A column was equilibrated with 25 mL binding buffer (20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl) at a flow rate of 5 mL/min at 4 °C. Up to 1.2 L of culture supernatant were loaded onto the column at a flow rate of 5 mL/min. For elution the column was transferred to a chromatographic system (ÄKTApurifier workstation including fraction collector Frac-950, GE Healthcare) using Unicorn V4.0 software for system control and data analysis. The column was washed with 5 CV (column volumes) of binding buffer. Elution was by a step (8 CV) of 100% eluent (100 mM glycine HCl, pH 3.0) at 2 mL/min. Fractions of 1 mL were collected in 0.2 mL 1 M sodium phosphate buffer (pH 7.5) to neutralize the acidic elution conditions. Fractions were desalted (5 mL HiTrap Desalting Column, GE Healthcare) to 10 mM sodium phosphate buffer pH 7.0 as quickly as possible. For short-time storage, fractions were sterile-filtered and put at 4 °C. For long-term storage at 4 °C, 20 mM azide and 0.005% Tween 20 were added to the sterile-filtered solutions.

2.4. Antibody fragment preparation

Antibody fragments (Fab-/Fc-fragments) were produced by papain digestion using the Pierce Fab Preparation kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions (0.5 mL antibody solution, concentration ca. 5 mg/mL). Fragments were separated by adsorption of the Fc-fragment on Protein A UNO sphere SUPrA beads (BioRad Laboratories). For this purpose, beads were incubated for 2 min in the supernatant from the papain digest to allow for attachment of the Fc-fragment and recovered by centrifugation. The Fc-fragments were eluted by resuspension in the provided IgG elution buffer. Beads were removed by centrifugation and the supernatant neutralized by the addition of 0.2 mL of 1 M sodium phosphate buffer pH 7.5. In case of antibody MDJ8, fragments were further purified by hydroxyapatite chromatography (CHT type I, BioRad Laboratories). Buffer A was a 10 mM sodium phosphate buffer pH 7.0, buffer B a 10 mM sodium phosphate buffer pH 7.0 containing 1 M NaCl. Elution was in a linear gradient 100% A to 100% B, gradient volume 10 mL, flow rate of 1 mL/min. In case of Bevacizumab, fragments were further purified by gel filtration on Superdex 200 (GE Healthcare) at a flow rate of 0.5 mL/min. Buffer was 20 mM sodium phosphate pH 7.0 containing 0.15 M NaCl. Fractions representing a peak were pooled, desalted, and analyzed by Bradford assay and SDS–PAGE.

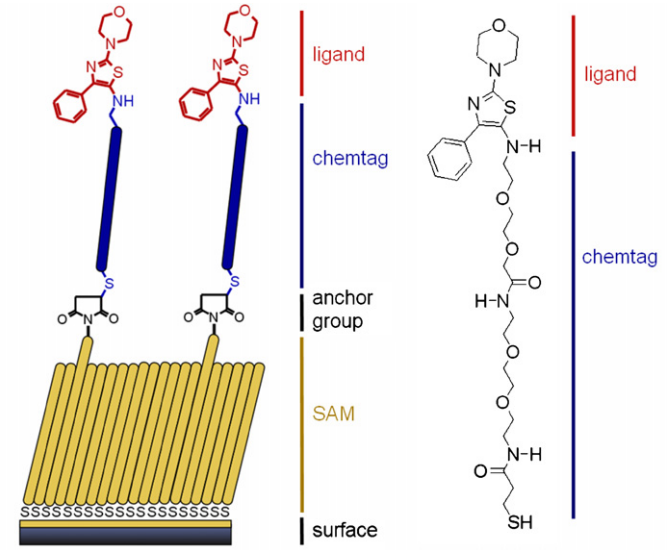

Fig. 1. Scheme of the surface architecture of the chemical microarray used in the screening experiments. The highly defined surface architecture consists of: the glass surface, a self-assembled monolayer (SAM) of two thiols, attached anchor groups that react covalently with the thiol group of the chemtag, the flexible and hydrophilic chemtag linker and the attached fragment molecule. The chemtag is shown in more detail on the right hand side. In total the chemical microarrays comprise 9216 immobilised compounds per arrays and allow to screen a total of 110,000 library compounds.

2.5. Chemical microarrays

For SPR screening of small molecule libraries, the previously described [16] high density chemical microarrays developed by Graffinity Pharmaceuticals GmbH were used (Fig. 1). The arrays were constructed using maleimide-thiol coupling chemistry in combination with high density pintool spotting. In particular, glass plates were microstructured by photolithographical methods in order to define the sensor fields. A subsequently applied gold coating provided the basis for the SPR effect and enabled the formation of a binary, mixed self-assembled monolayer (SAM) of two different thiols. One of the thiols making up the SAM (the "anchor") carried a reactive maleimide moiety for eventual ligand linkage, the second type of thiol in the SAM bore no reactive group for coupling and served as "diluent" to adjust the anchor thiol concentration in the SAM. All library compounds contained a flexible, hydrophilic thiol-containing linker, the "chemtag", see Fig. 1 for details, which reacted covalently with the anchor groups. The resulting microarrays comprised 9216 sensor fields, each containing multiple copies of a defined and quality-controlled array compound. The entire Graffinity library of small, non-peptidic molecules contained a total of approximately 110,000 entities with an average molecular weight of about 320 Da, which were synthesized by two to three step synthesis protocols as described previously [16].

2.6. Microarray screening

For screening, the chemical microarrays were incubated with the target protein under the indicated screening conditions. Protein binding alters the local refractive index adjacent to the sensor surface at the corresponding sensor field. This in turn leads to a shift in the wavelength dependent SPR minimum in comparison to the buffer control. The SPR imaging approach allows to record SPR resonance curves of all 9216 sensor fields per chip in parallel. For the screening experiments the antibody of interest was applied at concentrations between 10 and 60 μg/mL in suitable buffer solu-

Table 1
Conditions used during the screening of the ligand SPR microarrays against the target molecules MDJ8, Bevacizumab (and fragments), Tocilizumab, and Palivizumab.

Target	Type	Screening buffer (optimised)	Ligand density
MDJ8[a]	Full Ab	20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.005% Tween 20	High
Bevacizumab[b]	Full Ab	20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween 20	Medium
Bevacizumab Fab	Fragment	20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween 20	Medium
Bevacizumab Fc	Fragment	20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween 20	Medium
Tocilizumab[b]	Full Ab	20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween 20	Medium
Palivizumab[b]	Full Ab	20 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween 20	High

[a] Human antibody.
[b] Humanised antibody.

tion as indicated in Table 1. Unless indicated otherwise, BSA (bovine serum albumin) served as control for non-specific binding.

2.7. Resynthesis of selected array compounds

The resynthesis of hit compounds was performed by solid phase synthesis starting from 1,3-diaminopropane linked to either trityl or 2-chlorotrityl polystyrene resin. After synthesis, the compounds were cleaved from the solid support using standard protocols and purified by reversed-phase HPLC.

2.8. Ligand stability

For the determination of the chemical stability of the ligands, 1 mM of uncoupled ligand was incubated with either 0.1 M NaOH, 0.5 M NaOH, or 1 M of H_3PO_4 over a period of 8 days. Decay of ligand was measured by LC–MS analysis on a Shimadzu HPLC system including a diode array detector (SPD-M10A VP) and single-quad mass detector (LCMS 2010A) (all Shimadzu Corporation, Kyoto, Japan). Relative concentrations were determined by integration of the UV peak of the ligands (214 nm) at days 0, 1, 2, 4 and 8. Half-lives were calculated by linear regression after logarithmic transformation of the original data.

2.9. Ligand immobilisation

Ligands were coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare). One volume of pre-swollen gel (20 μmol NHS per mL of gel) was mixed with one volume of ligand solution (20–25 mM in 90% DMSO and 10% N-methyl-2-pyrrolidone (NMP) containing 1 M N,N-diisopropylethylamine) followed by incubation for at least 2 h at 25 °C under agitation. After two washes with 10% NMP in DMSO, remaining free binding sites were inactivated with 1 M aqueous ethanolamine. Coupling efficiency was determined indirectly from the amount of ligand remaining in the supernatants (OPA-test) assuming a perfect mass balance.

2.10. Binding studies

Initial binding studies were carried out in 384 well plates fitted with 0.45 μm bottom frits (Pall Life Sciences, Ann Arbor, USA). Equal amounts of resin (30 μL "bed" volume) were loaded into the wells. "Columns" were sealed with a polyethylene top filter (Porex Technologies GmbH, Bautzen, Germany). All tests were done in duplicates. For a typical experiment either 2× 50 μL of pure antibody (0.75 mg/mL in PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.3) or 2× 50 μL undiluted antibody-depleted CHO cell culture supernatant were loaded onto a column. Afterwards, columns were washed with 3× 50 μL PBS. Bound protein was eluted with 3× 50 μL 50 mM glycine pH 2.5. Columns were regenerated with 3× 50 μL 6 M urea and stored in 30% ethanol. Liquids were forced through the microcolumns via centrifugation at 50 × *g* for 1 min. During loading plates were centrifuged at 10 × *g* for 5 min. Bound protein amounts and protein yields were determined by balancing the amount of protein injected and recovered in the flow through (quantification via Bradford assay). Selectivity was expressed as the ratio between bound antibody and bound amount of protein from the culture supernatant.

For initial capture experiments three runs were conducted with (a) antibody-depleted cell culture supernatant alone, (b) antibody alone, and (c) antibody spiked back into the spend cell culture supernatant to a concentration of approximately 20% of the total protein concentration. Purity after chromatography was calculated from the mass of total protein recovered upon elution after injection of the mixture and the mass recovered after injection of the cell culture supernatant.

For optimisation of the elution conditions, the microtitre plate assay was used in a central composite design-of-experiments approach. Bound antibody was eluted with 10 different elution buffers varying in pH-values from 3.0 to 4.5 and in ionic strengths from 20 mM to 500 mM NaCl. Data was statistically evaluated using the Jmp program (SAS, Heidelberg, Germany). Contour plots were created with QtiPlot (http://soft.proindependent.com/qtiplot.html).

For the determination of static isotherms 5 μL of resin were incubated with 100 μL of antibody solution (concentration between 0.03 and 0.5 mg/mL in PBS) in 96 well microtitre plates. Plates were rigorously shaken at 25 °C for 3 h. Afterwards, unbound protein was determined by Bradford assay and used to calculate the bound amounts. Dissociation constants, K_d, and maximal capacities, q_{max}, were determined by direct fitting the obtained data points to the Langmuir isotherm equation. Binding kinetics were measured similarly. In this case 5 μL of resin was incubated with 100 μL of a 0.75 mg/mL antibody solution in PBS pH 7.3 at 25 °C. Concentrations of unbound protein were measured after 0, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 min and fitted with a double exponential function. The time point after which 80% of the initial protein had bound to the beads was referred to as "time scale of binding".

2.11. Chromatography

For small-scale chromatographic studies, 180 μL glass columns (3 mm × 25 mm, Omnifit, Cambridge, UK) were packed with the indicated material using a Knauer K-501 HPLC pump (10 mL head, Knauer, Berlin, Germany), no reservoir was used. The packing solvent was 20% ethanol in water, the stationary phase was prepared as 20% slurry. Packing was done according to an established protocol in steps of 0.1 mL/min (5 min each) until 1 mL/min, followed by 1 mL/min for 0.5 h. Afterwards columns were washed with 5 mL each of 50 mM NaOH and 0.1 M glycine HCl pH 3.0. Breakthrough curves were recorded at flow rates of 0.5 mL/min (420 cm/h) and 0.05 mL/min (42 cm/h) for antibody concentrations of 0.03, 0.15 and 0.75 mg/mL in 10 mM sodium phosphate buffer pH 7.0, 150 mM NaCl (binding buffer). Prior to the measurement, columns were equilibrated with binding buffer for at least 20 min at the indicated flow rate, then the flow was switched to the target molecule solution of the lowest investigated concentration and the breakthrough curve recorded (280 nm). Once a stable plateau was reached the

experiment was terminated, the column was transferred to the Äkta system and the bound antibody eluted using 0.1 M glycine HCl pH 3 and the amount quantified via the peak area. Column regeneration was by 50 mM NaOH, followed by re-equilibration with binding buffer. Results were used to construct dynamic binding isotherms via fitting the data points to the Langmuir equation. Long-term performance tests were performed with the same system.

For experiments involving the direct comparison of the ligand-activated material with Protein A for antibody capture from cell culture supernatant, 1 mL columns were packed (Tricorn high performance column, 55 mm × 5 mm, GE Healthcare) from the ligand-activated material, following a similar protocol as for the small columns. 50 mL of supernatant (filtered, 0.22 μm, protein concentration (Bradford, calibration BGG), ca. 0.3 mg/mL, antibody concentration ca. 0.015 mg/mL) was loaded onto the column at 1.4 mL/min (420 cm/h). Elution, column regeneration and re-equilibration were as described for the small columns.

3. Results and discussions

3.1. Screening of the libraries for specific antibody binders

Library screening was carried out by SPR imaging as described in Section 2. The high throughput design allowed performing the initial experiments in a narrow time frame of a couple of weeks. Before starting the screening campaign against the entire library, a range of screening conditions were tested for the target molecules, including different ionic strengths, detergent concentrations, protein concentrations and surface ligand densities. Eventually, for each target molecule specific screening conditions were identified for which the signal strength and S/N ratio on the arrays were optimal (Table 1). Due to the fact that all targets were of the antibody type, very similar buffer systems and surface dilution of ligands were found to be optimal for screening. The experimental results of the microarray screening campaigns under optimised conditions were analyzed by manual hit selection using in-house developed software routines. Important selection criteria for hit triaging of primary screening hits were: the measured SPR signal strength, the chemical novelty of the identified hits, the selectivity for the target of interest, the structural diversity of the hits and observed structure affinity relationship (SAR) trends. In addition, the SPR signals of all targets investigated were analyzed side-by-side in order to identify common (e.g. Fc-part) binders but also target specific ligands.

In Fig. 2, screening results for antibody MDJ8 are given. The 2D colour coded interaction map (Fig. 2a) represents the 9,216 SPR shifts obtained from one individual array experiment. Blue signals correspond to the background (low SPR shift), while yellow to red signals represent a significant SPR shift and hence indicate protein binding to the corresponding sensor fields. The scatter plot (Fig. 2b) compares the SPR signals of two independent repetition experiments and demonstrates good reproducibility for the results. As this particular type of library was derived from combinatorial synthesis approaches, each row and column contains a common structural motif in combination with 96 other diversities. Hit series identified for MDJ8 clearly appear as coloured rows and columns. The data analysis for the MDJ8 screening thus resulted in a number of hit series, which showed clear binding to the antibody. The hit rate of approximately 0.1–0.5% was comparable to other targets previously screened on the same array platform. From the initial hit list a number of 18 representative binders were selected for hit confirmation by secondary assays.

In addition to MDJ8, three more full-size antibodies, namely Bevacizumab, Tocilizumab, and Palivizumab (all humanised therapeutic antibodies) were screened against the library in order to identify generic, most likely Fc-specific, binders together with the Fc- and Fab-fragments of Bevacizumab obtained by papain cleavage. BSA (bovine serum albumin) served as negative control in these experiments. For each of the antibody targets the individually determined optimal conditions (Table 1) were used during screening. For BSA screening conditions were chosen to be identical to those of the corresponding antibody experiments. In Fig. 3 the array screening results for all targets are exemplified by interaction maps of the antibodies and controls against the same fraction of a selected library. The comparison of the interaction maps revealed distinct differences between the four investigated antibodies. For example, Bevacizumab (Fig. 3b) showed binding to some ligands, which were not identified as hits for MDJ8, Tocilizumab, or Palivizumab. Since the present study focused on the development of general binders for the affinity purification of antibodies, binders with affinity only to a specific antibody were not perused further. However, since the target protein as a whole rather than specific predefined binding sites was used during screening, such specific ligands also became available. A typical example for a "generic binder candidate", on the other hand, is ligand 2A10, highlighted in Fig. 3. Ligand 2A10 shows binding to all investigated antibodies (Fig. 3a, b, e, and f) as well as to the Fc-fragment of Bevacizumab (Fig. 3d), but only negligible binding to the Fab-fragment of Bevacizumab (Fig. 3c) and to the negative control BSA (Fig. 3g). Ligand 2A10 was therefore classified as Fc-specific ligand and investigated further together with a number of additional promising Fc-binders identified in an analogous manner.

3.2. Ligand re-synthesis and coupling

To facilitate the immobilisation of the ligands to the chromatographic matrix, structures were re-synthesized with the chemtag replaced by a 1,3-diaminopropyl linker. As a result, the orientation of the ligand on the future chromatographic affinity resin should be the same as in the initial SPR screening and changes in binding behaviour in subsequent chromatography experiments due to altered orientation of the ligands should thus be minimized. Note that the linker did not serve as a spacer, but simply assured the proper orientation of the ligand after immobilisation. In preliminary experiments a longer linker molecule was also used for this purpose (C6 instead of C3), which resulted in a lower stationary phase capacity. It is possible that the higher flexibility provided by the longer linker reduced the percentage of correctly presented ligand molecules. The chosen matrix in this study was NHS-activated Sepharose 4 Fast Flow, i.e. a commercially available, process compatible material, which allows for fast and reproducible ligand immobilisation. All ligands could be coupled to the resin using the established protocol with final ligand densities between 10 and 15 μmol/mL of gel.

Typically, antibodies are eluted from affinity resins by low pH [9]. On the other hand, alkaline conditions are used in column sanitisation and cleaning-in-place procedures. To investigate the stability of the identified ligands towards both of these conditions, we determined half-lives of a subset of ligands in the presence of 0.1 and 0.5 M NaOH or 1 M H_3PO_4. All tested ligands showed excellent chemical stabilities under acidic conditions, Table 2. In fact, no significant decay was measured for any of the ligands in the pres-

Table 2
Ligand half-life in hours under the indicated alkaline and acidic conditions.

Ligand	0.1 M NaOH (h)	0.5 M NaOH (h)	1 M H_3PO_4
1A4	553	337	∞
2A8	557	77	∞
2A9	314	50	∞
2A10	∞	288	∞

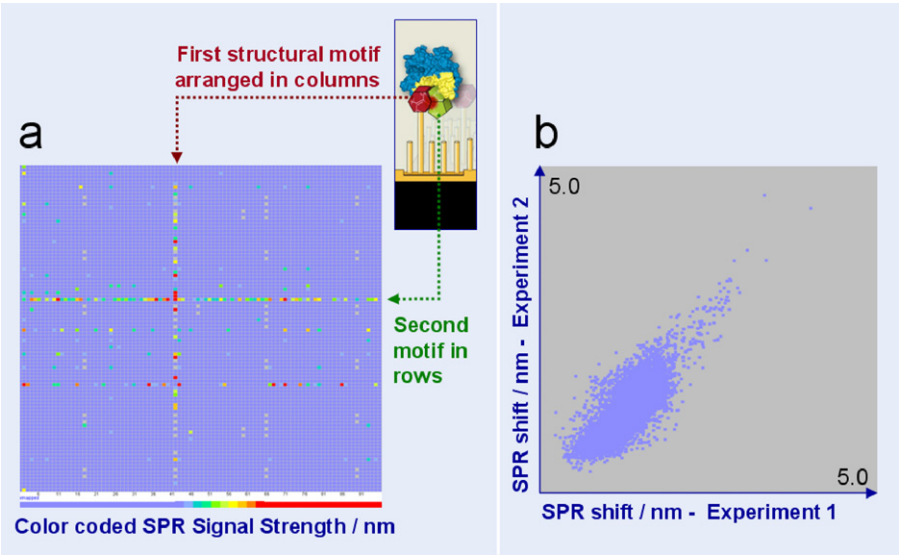

Fig. 2. SPR screening result for antibody MDJ8. (a) Shown are the colour-coded SPR shifts obtained during screening of MDJ8 against one library (9216 sensor fields). Hit series binding to MDJ8 appear as rows and columns. (b) Comparison of two independent repetition experiments.

ence of 1 M H_3PO_4 for the investigated duration of 8 days. Towards 0.5 M NaOH, ligands 1A4 and 2A10 were also extremely stable. With half-lives of 337 h and 288 h, respectively, common elution and regeneration procedures should be applicable without difficulty. In the case of some other ligands, e.g. 2A8 and 2A9, stability was rather poor in 0.5 M NaOH. Here, regeneration with either acidic treatment or with lower NaOH concentrations (e.g. 0.1 M) would be recommended.

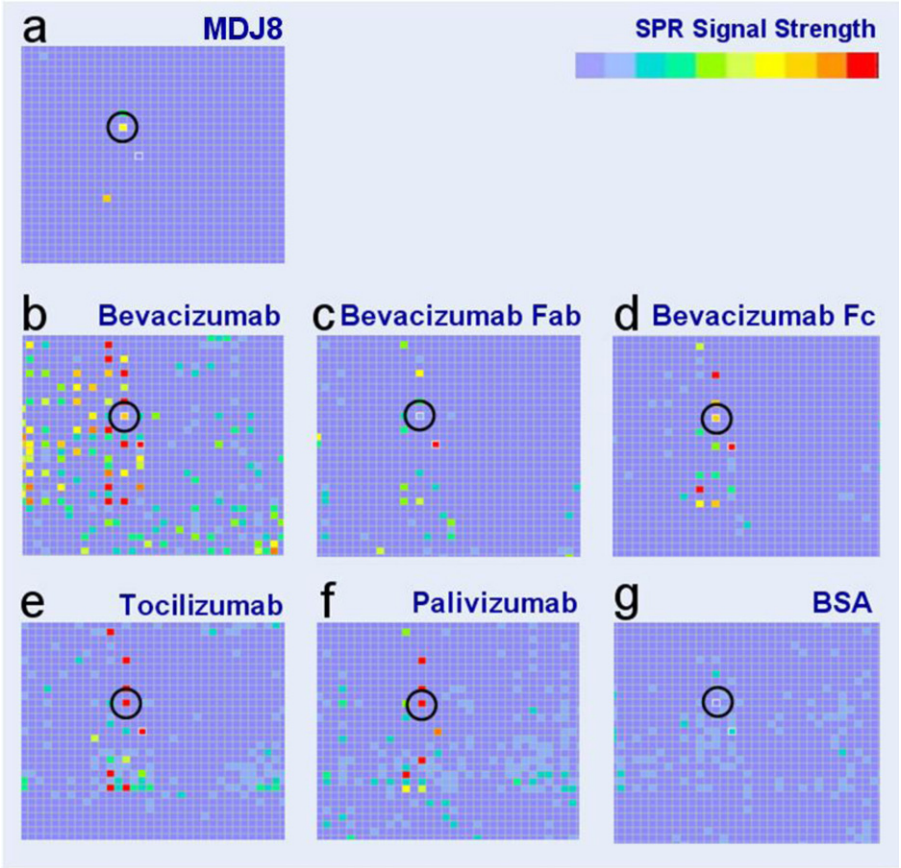

Fig. 3. SPR screening results for (a) MDJ8, (b) Bevacizumab, (c) the Fab-fragment of Bevacizumab, (d) the Fc-fragment of Bevacizumab, (e) Tocilizumab, (f) Palivizumab, and (g) BSA. The circle indicates a putative Fc-specific ligand.

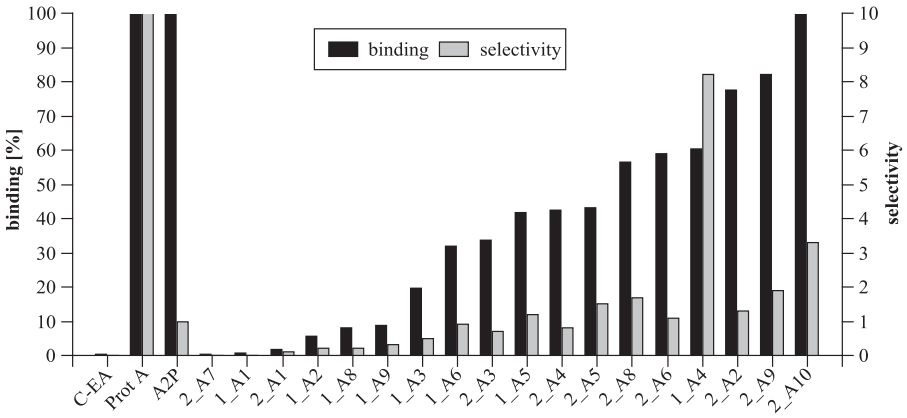

Fig. 4. Binding, recovery yields, and selectivities of Sepharose materials activated with the indicated ligand in comparison to Protein A, Mabsorbent A2P, and inert Sepharose (C-EA). Experiments were done in the 384 well microtitre plate. 100 μL of pure MDJ8 (0.75 mg/mL in PBS) was loaded on the "columns". Bound protein was eluted with 50 mM glycine pH 2.5. Bound protein and yield were determined by mass balances. The experiment was repeated with spend culture supernatant lacking the antibody. Selectivity was defined as the ratio between bound MDJ8 and bound cell supernatant protein. Due to experimental precision the value for selectivity was truncated for values above 10.

3.3. Initial binding/selectivity studies using ligand-activated chromatographic material

Initial binding and selectivity studies with the ligand-activated chromatographic matrix were carried out in 384 microtitre plates with fritted bottoms to allow for enforced gravity flow of liquid phase through the "columns" (ca. 30 μL bed volume each). This approach allowed per plate the evaluation of up to 30 ligands at up to 10 conditions per ligand, while protein consumption was kept very low. In total, 18 ligands from the screens were selected according to the above-indicated criteria for these studies. Protein A Sepharose 4 FF and Mabsorbent A2P (Prometic BioSciences), i.e. a dedicated antibody capture material activated by a synthetic, aromatic triazine derivative ligand, were used as reference media. NHS-Sepharose completely saturated with ethanolamine ("C-EA") served as control for non-specific interaction of the stationary phase backbone. A spent, antibody-depleted (by Protein A chromatography) CHO-MDJ8 cell culture supernatant served as source of impurities putatively present in the raw feed solution (positive control for "unspecific binding"). The eluent was 50 mM glycine HCl pH 2.5 in all cases. The results in terms of affinity and selectivity are shown in Fig. 4.

Nearly all ligand-activated Sepharose materials showed higher affinity to the antibody than C-EA, but also a varying degree of unspecific binding of proteins from the cell culture supernatant. Except for the 1A5-activated material, complete recovery of the bound protein was possible in all cases. Based on these results, ligands were classed as unspecific binders binding cell supernatant components as good as the antibody (selectivity index ≤1), binders, which had higher preferences to the antibody than to the impurities (selectivity index >1), and very selective antibody binders such as 1A4, which bound only minor amounts of the protein impurities and were nearly as selective for the antibody as the Protein A material. Interestingly, at least in our study, Mabsorbent A2P also showed little selectivity for the antibody. From these experiments ligands 1A4, 2A8, 2A9 and 2A10 emerged as most interesting structures as they combined good capture of the antibody with high selectivity.

Binding studies in the microtitre plate format were subsequently repeated for these four ligands using Bevacizumab as target molecule as well as for the Fab- and Fc-fragments of this antibody prepared by papain digestion. All four ligands were shown to bind the full antibody with an affinity and selectivity similar to Protein A (Table 3). In case of the antibody fragments, ligands 1A4, 2A8 and 2A9 showed no preference and bound both fragments to a similar extent, albeit at a markedly reduced amounts compared to the whole antibody. It is thus possible that these ligands bind somewhere to conserved parts in the hinge region. In case of ligand 2A10, however, the Fc-part could be verified as the major binding site, as this ligand bound the Fc-fragment to a similar extent as the full antibody, while showing no affinity for the Fab-fragment. The potential of 2A10 as a generic antibody-binder was further demonstrated with two additional human antibodies (Tocilizumab and Palivizumab) and a human poly-IgG mixture. All three were quantitatively bound by 2A10. The same was the case for a poly-IgG mixture derived from rabbit serum, which also was bound to nearly 100%, all data found in Table 3.

For a first test for binding under chromatographic conditions, 180 μL columns were packed with 2A10-activated Sepharose FF. From these columns, the Fc-fragment of antibody MDJ8 eluted in the glycine HCl step at the same position as the intact antibody, while the Fab-fragment eluted unretained in the flow through (Fig. 5a). Finally, a monoclonal antibody of murine origin ("5D10″), which recognises the c-myc receptor on the surface of cancer cells [25] and is also known to interact with Protein A via its Fc-part was chromatographed. Fig. 5b shows elution of antibody 5D10 in a

Table 3
Binding and recovery yields for humanised therapeutic antibody, as well as poly-IgG mixtures from human and rabbit on selected affinity ligands in comparison to Protein A.

Ligand	Bevacizumab (%)				MDJ8 (%)		Tocilizumab (%)		Palivizumab (%)		(h)poly-IgG (%)		(r)poly-IgG (%)	
	IgG		Fab	Fc										
	Bound	Yield	Bound	Bound	Bound	Yield	Bound	Yield	Bound	Yield	Bound	Yield	Bound	Yield
ProtA	100	100	7	100	100	90	100	95	100	100	95	79	100	100
1A4	100	92	28	29	–	–	–	–	–	–	–	–	–	–
2A8	100	92	48	39	–	–	–	–	–	–	–	–	–	–
2A9	100	96	59	58	–	–	–	–	–	–	–	–	–	–
2A10	100	92	24	100	100	90	100	97	100	92	100	90	100	100

h, human; r, rabbit.

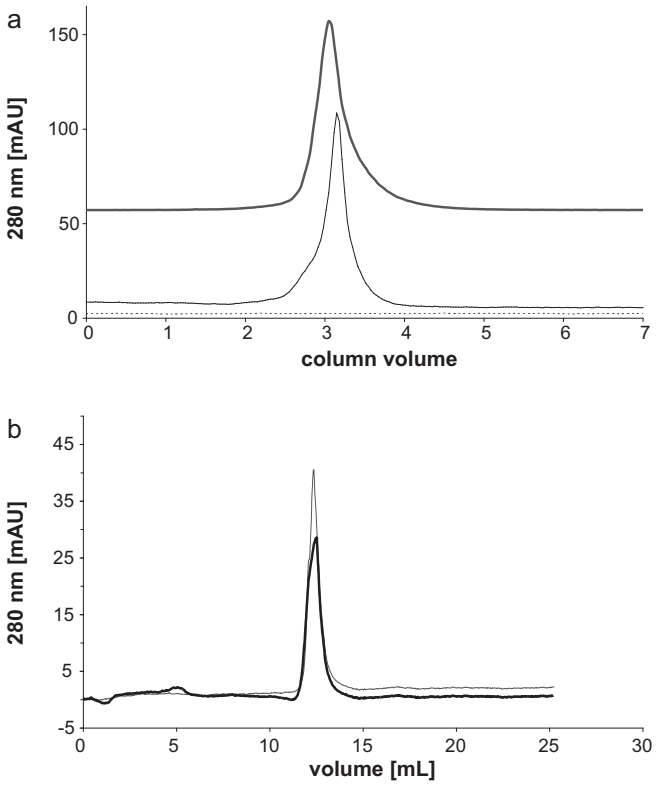

Fig. 5. (a) Elution of the purified Fab- and Fc-fragments of antibody MDJ8 by 0.1 M glycine HCl pH 3.0 in comparison to the elution profile of the whole antibody. Ligand 2A10, column volume 1 mL, flow rate 1 mL/min, thick line: MDJ8 (full antibody), thin line: MDJ8 Fc-fragment, dotted line: MDJ8 Fab-fragment. (b) Elution profiles for monoclonal antibody 5D10 from a column activated with ligand 2A10 compared to elution from a standard Protein A column. Eluting agent 0.1 M glycine HCl pH 3.0, column volumes 1 mL, flow rate 1 mL/min, thick line: 2A10-activated column, thin line: Protein A activated column.

100 mM glycine HCl pH 3.0 step from the 2A10 column in comparison to the elution profile from the corresponding Protein A column. The peak obtained for the Protein A column is somewhat sharper, however, this is due to the difference in the number of plates between the two columns. Otherwise both affinity columns appear equally suited to isolate the antibody. In particular, recovery yields and obtained purities were similar.

Ligand 2A10 thus can be considered a potential alternative to Protein A as a generic antibody-capturing agent. At this point it would be interesting to pan this ligand against various human and murine antibody subtypes, to see whether the pattern of binding strength differs from that of Protein A. However, since antibody purification has been for such a long time dominated by Protein A chromatography it is difficult to find a commercially available antibody of a subtype that is not interacting with Protein A.

3.4. Binding equilibria and kinetics

For a quantitative investigation of the binding behaviour, promising ligands from the initial screening campaigns were immobilised on Biacore chips and the dissociation equilibrium constants (K_d-values) determined for antibody MDJ8 as target and BSA as negative control. Three ligands, 1A5, 1A6, and 2A10 gave K_d-values in the nM-range, while showing little affinity to BSA, several others had K_d-values in the low μM-range, some also with good selectivities. Two more ligands, namely 1A8, and 2A8, also showed good binding to the target, but also a somewhat elevated affinity for BSA.

Table 4
Affinity constants (K_d), maximum static binding capacities (q_{max}) and "time scale of binding" ($t_{80\%}$), i.e. the time required to bind 80% of the maximum amount, determined for different affinity resins in static batch adsorption experiments using Bevacizumab as probe. Affinity constants and maximum binding capacities were calculated by fitting experimental data to the Langmuir equation.

Ligand	K_d (nM)	q_{max} (mg/mL)	$t_{80\%}$ (min)
Protein A[a]	33.3	41	8.9
1A4	1353.3	30	6.4
2A8	966.7	30	8.9
A9	546.7	41	6.7
2A10	506.7	37	9.8

[a] Protein A reference material: rProtein A Sepharose FF, GE Healthcare.

Then ligands 1A4, 2A8, 2A9, and 2A10 were immobilised on Sepharose FF and affinity constants (K_d), maximal binding capacities (q_m) and binding kinetics ("time scale of binding") were determined in comparison to the Protein A-material using the batch adsorption experimental approach (96 well plate format). Fixed amounts of resin and varied concentrations of Bevacizumab (0.05–0.5 mg/mL for the K_d and q_{max}-measurements, 0.75 mg/mL for the kinetics) were used. Data points were directly fitted to the Langmuir isotherm equation (Table 4). Not surprisingly, the highest affinity was found for Protein A. However, for ligands 2A9 and 2A10 K_d-values were also in the sub-micromolar range, while for 1A4 and 2A8 these values were in the low micromolar range. With 41 mg/mL the highest static equilibrium capacity of the in-house resins was calculated for the material activated with ligand 2A9, which was thus in the same range as Protein A. Static capacities of the other investigated resins were slightly lower and ranged from 30 mg/mL to 37 mg/mL. All tested resins bound 80% of the available antibody within 10 min of incubation time. In certain cases (1A4: 6.4 min, 2A9: 6.7 min) binding was faster than for Protein A (8.9 min).

The chromatographic binding behaviour of the Fc-binding ligand 2A10 was in addition characterized under dynamic conditions, again in comparison to Protein A. Breakthrough curves were recorded at a flow rate of 50 cm/h for the 180 μL columns packed with the respective materials using Bevacizumab (1 mg/mL) as probe. Dynamic binding capacities were determined at 10% breakthrough. For the determination of the corresponding dynamic equilibrium capacities, breakthrough curves were extrapolated assuming sigmoidal functions, which resulted in values of 28.4 mg/mL and 20.2 mg/mL for Protein A and 2A10, respectively. For antibody MDJ8 breakthrough curves on the 2A10 columns were determined at two different flow rates, 42 cm/h (0.05 mL/min) and 420 cm/h (0.5 mL/min), for protein concentrations between 0.05 and 0.75 mg/mL, BSA was used as negative control. In all cases the breakthrough curve of the antibody was fairly broad, an example is shown in Fig. 6. While this may be attributed to low column efficiency – always a potential problem in the case of small home-packed columns – the much sharper breakthrough curve of the BSA also shown in Fig. 6, indicates that it is less the column efficiency but rather some heterogeneity of the interaction, which is at fault. Moreover, when the breakthrough curve data were used to construct isotherms, the one recorded at 420 cm/h was suppressed compared to the one recorded at 42 cm/h save for the highest investigated concentration. This indicates that mass transfer effects also influence the chromatographic behaviour.

The dynamic isotherm data were subsequently fitted to the Langmuir equation and used to calculate K_d and q_{max} (Table 5). For comparison, values measured for a 180 μL column packed with the Protein A material (UNOsphereSUPrA, BioRad Laboratories) were also determined. Again, several of the selected ligands showed affinity constants in the nM range. In particular ligand 2A9 came very close to Protein A. The dynamic capacity of the Protein A

Table 5
Affinity constants (K_d) and binding capacities (q_{max}) determined under flow conditions for different affinity resins. Parameters were calculated from fitting the breakthrough curve data obtained at flow rates of 420 and 42 cm/h (0.5 and 0.05 mL/min) for the 180 μL columns to the Langmuir equation. Antibody MDJ8 was used as probe.

Ligand	K_d (nM)420 cm/h	K_d (nM)42 cm/h	q_{max} (mg/mL)420 cm/h	q_m (mg/mL)42 cm/h
1A4	–	267	–	0.06
2A8	–	132	–	0.38
2A9	–	49	–	1.05
2A10	622	306	8.25	7.2
Protein A[a]	–	47	–	26.7

[a] Protein A reference material: UNOsphereSUPrA, BioRad Laboratories.

material, on the other hand, was higher than that of any of the ligand-activated materials. If, based on the ligand immobilisation results, we assume a ligand density of 12–15 μmol/mL of resin and a 1:1 type of interaction between the antibody and the ligand, the maximum capacities given in Table 5 correspond to a ligand efficiency (ratio between active ligand and immobilised ligand) below 0.5% even in the best cases. For commercially available affinity materials ligand efficiencies are typically an order of magnitude higher. It is possible that the ligand density was too high for the efficient interaction of all available ligand molecules with a target molecule. Given the size of the ligands, however, it is also possible that some ligands were immobilised in areas of the stationary phase where the antibody could not reach.

Finally, the multi-well column format was used in case of ligands 1A4, 2A8, 2A9, and 2A10 to screen for favourable elution conditions. Different experiments were performed according to the Design of Experiment (DoE) method. Via an initial full factorial design, pH and ionic strength were identified as the main factors to influence elution of the antibody from the in-house resins using Bevacizumab as probe (data not shown). Afterwards, the impact of these factors was characterized in detail by a central composite design over pH and ionic strength. Contour plots of the elution profiles of the ligands are shown in Fig. 7. With all in-house ligands, elution of the antibody was supported by low pH and low ionic strength. In the absence of additional salts, complete elution was possible between pH 3.4 (1A4) and pH 3.9 (2A10). While glycine HCl pH 3.0 can be used to elute the antibody from the ligand-activated columns, elution should in principle also be possible at near-physiological pH when some organic solvent or other modifiers are present. Elution of antibody MDJ8 from 2A10 was, e.g., also possible with 50% ethylene glycol at pH 7.4 (97% recovery). This could be an interesting option for the elution of pH sensitive antibodies, which are notoriously difficult to purify by Protein A affinity chromatography.

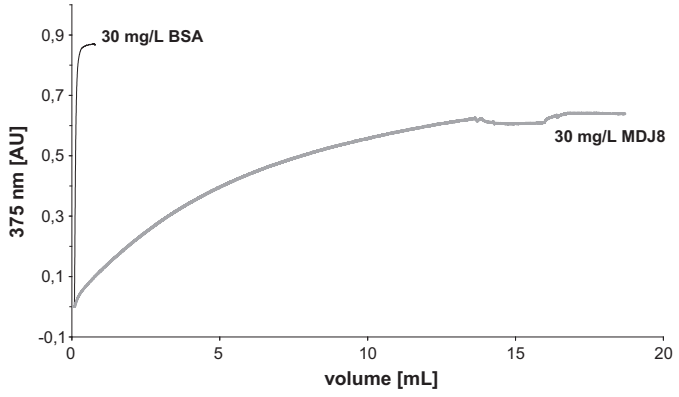

Fig. 6. Breakthrough curve recorded for BSA and antibody MDJ8 (both 30 mg/L in 10 mM sodium phosphate buffer, 150 mM NaCl) on a 180 μL (3 mm × 25 mm) column packed with 2A10-activated Sepharose FF material; flow rate: 0.5 mL/min (420 cm/h).

3.5. Antibody affinity capture by small molecule affinity ligands versus Protein A

Protein A is currently the gold standard in antibody purification. In order to compare the potential of the selected small molecule affinity ligands, the ability of ligands 1A4, 2A8, 2A9, and 2A10 to selectively bind antibody MDJ8 from a complex matrix was investigated in comparison to Protein A-activated material (Unosphere SUPrA) and C-EA as positive and negative controls. For this purpose the antibody-depleted cell culture supernatant (Protein A processed, 0.55 mg/mL total protein content) was spiked with enough purified MDJ8 to reach a concentration of 0.15 mg/mL of antibody and passed through the microtitre plate microcolumns. For the determination of purity and recovery of the eluted proteins, two additional runs were performed with either antibody-depleted culture supernatant alone or antibody alone. Purities and recoveries were calculated from these data assuming a perfect mass balance (Table 6). With the in-house resins purities between 66% and 74% were achieved. Recovery rates were between 79% and 87%. For comparison, purity and recovery after Protein A chromatography were 88% and >99%.

Ligand 2A10 was finally compared to Protein A for the ability to capture the target antibody MDJ8 directly from an unprocessed CHO cell culture supernatant (antibody concentration ca. 0.015 mg/mL). 1 mL columns were used throughout and 50 mL unprocessed cell culture supernatant was passed through the column at a flow rate of 1.4 mL/min. Elution from the Protein A column was by the standard 100 mM glycine HCl pH 3.0 step. Several 2A10 columns were operated in parallel to the Protein A column and different elution conditions were tested. In all cases we performed a regeneration step with 50 mM NaOH (4 mL at 1 mL/min) and also quantified the amount of protein released during column regeneration. All quantifications were in relative units based on the peak area. Table 7 summarizes the result in terms of relative recovery yields based on the yield for the corresponding Protein A column (using glycine HCl pH 3.0 elution). Purities of the antibody fractions were analyzed by SDS–PAGE. Under optimal circumstances, the 2A10 column yielded similar results in terms of recovery yield (>90%), purity (>80%), and eluting concentration (approximately 1 g/L) as the Protein A column in these experiments.

As predicted by the factorial design experiments, pH and NaCl content of the eluent were most important for the recovery yields. When the pH was, e.g. increased from 3 to 4 and 5 under otherwise identical conditions, antibody recovery yields dropped dramati-

Table 6
Purity and recovery of antibody MDJ8 from cell culture supernatant determined in comparison to the Protein A material (Unosphere SUPrA) in microtitre plate binding studies.

Ligand	Purity (%)	Recovery (%)
Protein A	88	>99
1A4	68	83
2A8	66	79
2A9	70	87
2A10	74	84

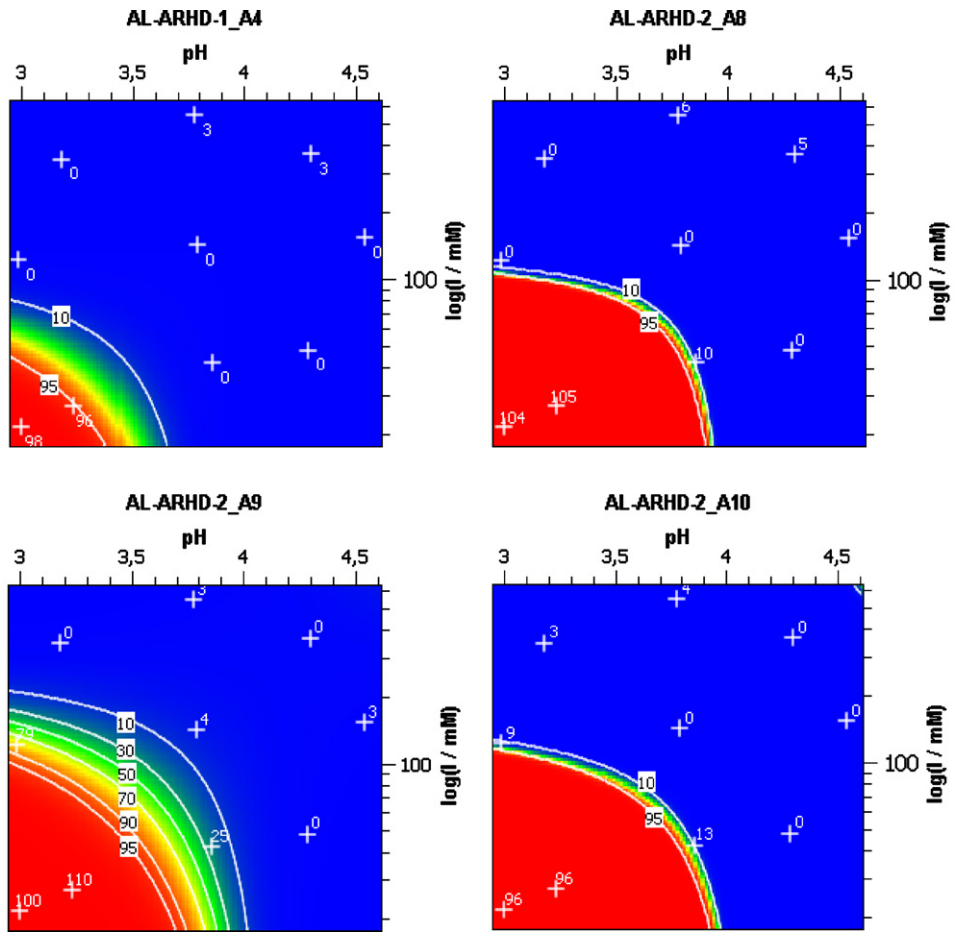

Fig. 7. Contour plots of the elution profiles obtained for the ligands under different pH and ionic strength conditions investigated via a central composite design. Bevacizumab was used as probe.

cally. The same was observed in case of an increase in the NaCl content. As observed before, elution was also improved by the addition of certain organic solvents such as DMSO. A comparison of the relative purities of the obtained antibody fractions also shows a very similar performance in case of ligand 2A10 and Protein A. Fig. 8 shows an SDS–PAGE analysis (reduced and non-

Table 7
Antibody MDJ8 recovery yields obtained for 2A10-activated stationary phases using the indicated elution conditions. Values are given as relative recovery yields taking the amount eluted by a 0.1 M glycine HCl pH 3.0 eluent from a Protein A column operated in parallel to the 2A10 one as 100%. The antibody was captured directly from a cell culture supernatant.

Elution conditions	Relative recovery yield (%)
100 mM glycine HCl pH 3.0	96
100 mM citric acid/sodium phosphate pH 3.0	67
100 mM citric acid/sodium phosphate pH 4.0	63
100 mM citric acid/sodium phosphate pH 5.0	29
100 mM citric acid/sodium phosphate pH 4.0, 0 mM NaCl	63
100 mM citric acid/sodium phosphate pH 4.0, 150 mM NaCl	14
100 mM citric acid/sodium phosphate pH 4.0, 500 mM NaCl	10
100 mM citric acid/sodium phosphate pH 4.0, 1000 mM NaCl	8
10% DMSO in 10 mM sodium phosphate pH 7.0, 150 mM NaCl	40
10% DMSO in 100 mM citric acid/sodium phosphate pH 4.0, 150 mM NaCl	58

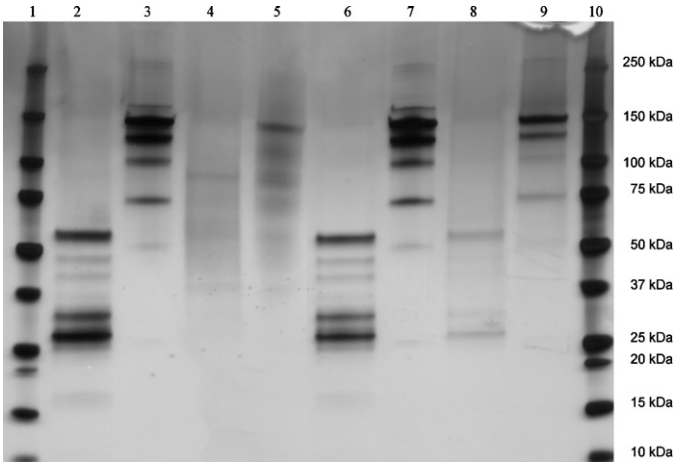

Fig. 8. SDS–PAGE gel comparing antibody fractions eluted from either Protein A or 2A10 activated column materials and the indicated eluent. Lane 1: molecular weight marker Precision Plus Protein Standard All Blue (BioRad Laboratories); lane 2: 2A10 column, eluent 0.1 M glycine HCl, pH 3.0, reducing conditions; lane 3: 2A10 column, eluent 0.1 M glycine HCl, pH 3.0, non-reducing conditions; lane 4: 2A10 column, eluent 50 mM NaOH, reducing conditions; lane 5: 2A10 column, eluent 50 mM NaOH, non-reducing conditions; lane 6: Protein A column, eluent 0.1 M glycine HCl, pH 3.0, reducing conditions; lane 7: Protein A column, eluent 0.1 M glycine HCl, pH 3.0, non-reducing conditions; lane 8: Protein A column, eluent 50 mM NaOH, reducing conditions; lane 9: Protein A column, eluent 50 mM NaOH, non-reducing conditions; lane 10: molecular weight marker.

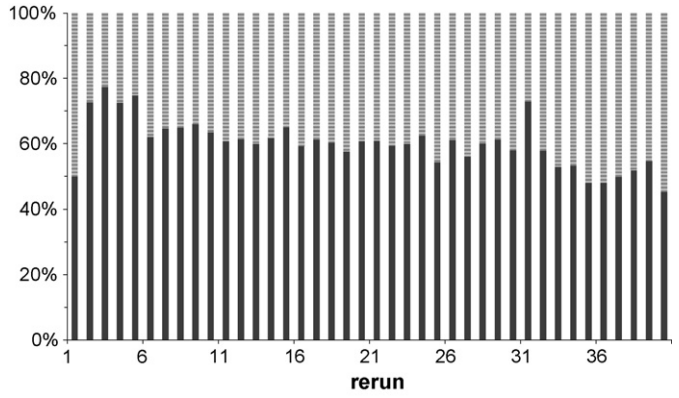

Fig. 9. Stability of the 2A10 column over time. A 180 μL 2A10 column was loaded with 15 mL cell culture supernatant. The flow through was passed through a Protein A column. Both columns were then eluted with 100 mM glycine HCl pH 3.0. Relative protein contents of the peaks were calculated from the integral of the peak area. Values are given in % antibody of the total recovered amount recovered at either of the two steps. 2A10 column: full bars, Protein A column: striped bars.

reduced conditions) of the antibody preparations eluted from the two columns by 100 mM glycine HCl pH 3.0 as well as by the subsequent washing/regeneration step using 50 mM NaOH. In both cases, the antibody eluted from the column is already fairly pure. The multi-band pattern seen in the SDS–PAGE gel is typical for this particular antibody and obtained with various isolation procedures alike. More importantly, while glycine elution seems fairly complete in case of ligand 2A10, a considerably amount of antibody is eluted in case of the Protein A column by the NaOH step.

3.6. Long-term stability

In order to investigate the stability of the ligand-activated column material under conditions of prolonged use, a freshly prepared 180 μL 2A10 column was repeatedly loaded with 15 mL of an antibody containing cell culture supernatant at 0.5 mL/min (420 cm/h). The breakthrough was collected and passed through a 1 mL Protein A column (flow rate 0.5 mL/min). Afterwards, the material retained on the two respective columns was eluted with a glycine HCl pH 3.0 pulse and quantified via the peak area. The results are summarized in Fig. 9. After 5 runs, the initial capacity of the 2A10 column was reduced to about 80% and remained more or less stable over the next 35 runs. After 45 runs the column had to be discarded as the backpressure exceeded the limit of the chromatographic system. The cell culture supernatant loaded onto the column had initially been filtrated through a 0.2 μm filter and aliquoted into volumes of 50 mL. However filtration was not repeated before each individual injection to avoid biasing the antibody content. It is hence possible that feed quality deteriorated with time, which contributed to the increase in column backpressure. Concomitantly, the reduction in antibody recovery yield may to some extent reflect a real deterioration of the antibody in the supernatant.

4. Conclusions

Using the outlined approach it was possible to identify small molecule binders for antibody MDJ8 and other control antibodies with binding constants in the submicromolar range. In addition, these ligands showed a similar ability to distinguish between the target molecule and other components from the cell culture supernatant as Protein A. Compared to Protein A, the proposed chemical ligands are considerably cheaper and presumably less problematic in terms of immunogenicity; dedicated toxicity studies have not been carried out. The molecules are chemically stable and thus compatible with harsh cleaning and sanitisation conditions. At least one of the ligands, 2A10, appears to generally bind the Fc-part of selected human and murine antibodies. This ligand could thus become a generic ligand for antibody affinity chromatography. While Protein A affinity chromatography is restricted to antibody purification, the screening approach as used in this contribution could in principle be applied to any protein product in biotechnology. Moreover, the approach proposed by us would in principle also allow for straightforward screening for dedicated elution protocols, e.g. based on known stability limits of the product.

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References

[1] U. Gottschalk, Biotechnol. Prog. 24 (2008) 496.
[2] K. Mondal, M.N. Gupta, Biomol. Eng. 23 (2006) 59.
[3] Y.D. Clonis, C.R. Lowe, Biochim. Biophys. Acta 659 (1981) 86.
[4] N.E. Labrou, Y.D. Clonis, J. Biotechnol. 36 (1994) 95.
[5] C. Grönwall, S. Stahl, J. Biotechnol. 140 (2009) 254.
[6] C.F. Barbas, D.R. Burton, J.K. Scott, G.J. Silverman, Phage Display: A Laboratory Manual, Cold Spring Habor Laboratory Press, Cold Spring Harbor, NY, 2001.
[7] J. Hanes, A. Plückthun, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 4937.
[8] G. Stocker, D. Dumoulin, C. Vandevyver, F. Hilbrig, R. Freitag, Biotechnol. Prog. 24 (2008) 1314.
[9] C. Vandevyver, R. Freitag, in: G. Subramanian (Ed.), Antibodies: Production and Purification, Kluwer Academic/Plenum Publishers, New York/Boston/Dordrecht/London/Moscow, 2004, p. 133.
[10] K. Terpe, Appl. Microbiol. Biotechnol. 60 (2003) 523.
[11] M. Hedhammar, T. Gräslund, S. Hober, Chem. Eng. Technol. 28 (2005) 1315.
[12] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[13] Y.D. Clonis, J. Chromatogr. A 1101 (2006) 1.
[14] L.M. Mayr, D. Bojani, Curr. Opin. Pharmacol. 9 (2009) 580.
[15] S. Maier, M. Frank, H. Rau, P. Lewandrowski, R. Uhrig, O. Keil, H. Deppe, N. Müller, C. Vanier, H. Mannsperger, S. Zepter, H.D. Junker, QSAR Comb. Sci. 25 (2006) 1047.
[16] T. Neumann, H.D. Junker, K. Schmidt, R. Sekul, Curr. Top. Med. Chem. 7 (2007) 1630.
[17] D. Vetter, J. Cell. Biochem. Suppl. 39 (2002) 79.
[18] S. Dickopf, M. Frank, H.D. Junker, S. Maier, G. Metz, H. Ottleben, H. Rau, N. Schellhaas, K. Schmidt, R. Sekul, C. Vanier, D. Vetter, J. Czech, M. Lorenz, H. Matter, M. Schudok, H. Schreuder, D.W. Will, H.P. Nestler, Anal. Biochem. 335 (2004) 50.
[19] A. Heim-Riether, S.J. Taylor, S. Liang, D.A. Gao, Z. Xiong, E.M. August, B.K. Collins, B.T. Farmer, K. Haverty, M. Hill-Drzewi, H.D. Junker, S.M. Mariana, N. Moss, T. Neumann, J.R. Proudfoot, L.S. Keenan, R. Sekul, Q. Zhang, J. Li, N.A. Farrow, Bioorg. Med. Chem. Lett. 19 (2009) 5321.
[20] T. Neumann, H.D. Junker, O. Keil, K. Burkert, H. Ottleben, J. Gamer, R. Sekul, H. Deppe, A. Feurer, D. Tomandl, G. Metz, Lett. Drug Des. Discov. 2 (2005) 563.
[21] S. Miescher, M. Zahn-Zabal, M. De Jesus, R. Moudry, I. Fisch, M. Vogel, M. Kobr, M.A. Imboden, E. Kragten, J. Bichler, N. Mermod, B.M. Stadler, H. Amstutz, F. Wurm, Br. J. Haematol. 111 (2000) 157.
[22] S. Schubert, R. Freitag, J. Chromatogr. A 1216 (2008) 3831.
[23] S. Schubert, R. Freitag, J. Chromatogr. A 1142 (2007) 106.
[24] C. Vandevyver, S. Canarelli, C. Bossen, I. Fisch, R. Freitag, Biotechnol. Bioeng. 97 (2007) 721.
[25] L. Plessers, E. Bosmans, A. Cox, A.J. Raus, Anticancer Res. 5 (1985) 609.