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Monolith peptide affinity chromatography for quantification of immunoglobulin M

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ABSTRACT

We have developed a method for quantification of a specific monoclonal IgM directed toward embryonic stem cells based on a peptide affinity monolith. A peptide affinity ligand with the sequence C–C–H–Q–R–L–S–Q–R–K was obtained by epitope mapping using peptide SPOT synthesis. The peptide ligand was covalently immobilized by coupling the N-terminal cysteine to a monolithic disk that was previously modified with iodated spacer molecules. The monolithic disc was used for quantification of purified IgM and for IgM present in mammalian cell culture supernatant. We observed 17% unspecific binding of IgM to the monolithic disk and additionally a product loss in the flow through of 20%. Nevertheless, calibration curves had high correlation coefficients and inter/intra-assay variability experiments proved sufficient precision of the method. A limit of quantification of 51.69 μg/mL for purified IgM and 48.40 μg/mL for IgM in cell culture supernatant could be calculated. The binding capacity was consistent within the period of the study which included more than 200 cycles. The analysis time of less than 2 min is an advantage over existing chromatographic methods that rely on pore diffusion.

1. Introduction

Convective interaction media (CIM) monolithic disks have been increasingly recognized as quantification tools for large molecules due to their mass transfer independent separation properties [1–3]. Monoliths show a very low band dispersion in the column. The major contribution to bandspreading is the extra column contribution [4,5] and thus sharp zones can be obtained independent of the flow rate enabling short separation time [6–8]. The chemical stability and the large inner channel diameter of 1200–1500 nm make this support especially attractive for separation of large biomolecules. Affinity ligands for affinity chromatography on monoliths are synthetic, inorganic, or, in most cases, biological molecules [9]. Principles of bioaffinity chromatography on monolithic supports have been recently reviewed by Tetala et al. [10]. Protein A and G are the most common affinity ligands for antibody capture and analysis [11–13]. Small synthetic peptides have some advantages over these natural immunoglobulin binding ligands. Peptidic affinity ligands are easily synthesized [14], robust, and can be manufactured in large quantities at low cost. Combinatorial peptide synthesis offers fast and simple methods for peptide ligand screening [15]. Although peptide affinity sorbents often exhibit low binding capacity and low selectivity, numerous efficient applications have been developed in the past [16–21]. Moreover, for analytical purposes, a high capacity is not required. The application of peptidic affinity ligands for preparative purposes is possible as well. Fassina et al. developed a tetrameric tripeptide that mimicks protein A [22]. This peptide has been identified by a multimeric peptide library and could be successfully applied for the purification of IgG, IgY, IgA, IgE and IgM [23–26]. Due to the binding affinity which is greater for IgM than for the other subclasses, Fassina et al. considered that the tetrameric tripeptide has potential for separation of IgM from samples that contain IgG. This ligand has never been commercialized.

At the Bioprocessing Technology Institute (BTI, Singapore), antibodies targeting surface markers on undifferentiated human embryonic stem cells (hESC) have been generated [27]. One of these antibodies (mAb 84) induced cell death of undifferentiated hESCs within 30 min of incubation. Cell death of differentiated cells was not observed. The antigen for this immunoglobulin M (IgM) is Podocalyxin-like-protein-1 (PODXL-1), a surface protein on hESCs. Preliminary experimental data indicate that this cytotoxic antibody is useful in eliminating residual undifferentiated hECSs from differentiated cells for clinical applications, thus minimizing the risk of teratomas. A two-step purification strategy for this antibody has already been developed by Tscheliessnig et al. [28].

Goal of this work was to identify an affinity ligand for IgM quantification. Other analytical methods such as enzyme-linked immunosorbent assay (ELISA) or analytical size exclusion chromatography are currently used for mAb 84 quantification, but these

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methods are time consuming and therefore a method for faster quantification was required. We screened for a peptide ligand for quantification of purified mAb 84 as well as for quantification of mAb 84 in cell culture supernatant. With this ligand, a high performance monolith peptide affinity chromatography method was developed. An epoxy-activated monolithic CIM disk was used for covalent immobilization of a spacer molecule and the peptide ligand. The chromatographic method was evaluated by testing different buffers, different flow velocities, and unspecific adsorption of control proteins. Calibration curves were generated and the precision of the method was determined.

2. Materials and methods

Chemicals for buffer preparation were of analytical grade. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany) if not mentioned otherwise.

2.1. Peptide SPOT synthesis

For epitope mapping, peptide arrays covering the entire sequence of PODXL-1 (Swiss – Prot accession number O00592-1) were synthesized with the MultiPep Multiple Peptide Synthesizer from Intavis AG (Koeln, Germany) according to the method first published by Frank [29] and modified by Pflegerl et al. [30]. Briefly, di-β-alanine was used as a linker coupled to the cellulose sheet and peptide assembly was carried out by using a conventional 9-fluoromethoxycarbonyl technique. After coupling of the last amino acids, the N-termini were acetylated and all side chain protecting groups were cleaved. For the first screening round, two types of peptide membranes were synthesized. One membrane type consisted of 94 deca-peptide spots with overlaps of four amino acids and the other membrane type consisted of 56 pentadeca-peptide spots with overlaps of five amino acids. In the second screening round, 195 deca- and pentadeca-peptides were selected from the first screening round and synthesized with overlaps of eight or thirteen amino acids. Each membrane contained additional negative control peptide spots (deca-alanine) and positive control peptide spots (WSHPQFEK) that bind streptavidin [31].

2.1.1. Binding assays with peptide membranes

Cultivation of the hybridoma cells and purification of mAb 84 was performed as described by Tscheliessnig et al. [28]. Purified mAb 84 was provided by BTI (Singapore) at a concentration of 1.6 mg/mL in storage buffer (30 mM phosphate, 100 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, 0.05% Tween 80 and 2.5% trehalose, pH 7.4). The protein was labeled using a 20-fold molar excess of 20 mM biotinamidohexanoic acid *N*-hydroxysuccinimide ester in dimethylformamide (Fisher Scientific, Loughborough Leicestershire, UK). After 1 h incubation at room temperature, a buffer exchange into PBS was performed using PD 10 columns from GE Healthcare (Little Chalfont Buckinghamshire, UK). Binding assays were performed with a slight modification as described by Duerauer et al. [32]. Briefly, peptide membranes were blocked with bovine serum albumin, incubated with 2 μg/mL biotinylated mAb 84, followed by incubation with streptavidin-horseradish peroxidase conjugate. The bound mAb 84 was detected with a chemiluminescence detection system. Between the incubation steps, the membranes were stringently washed. Unspecific binding of streptavidin-horseradish peroxidase conjugate to the peptide spots could be eliminated by reducing the incubation time from 1 h to 15 min. The imaged spots were normalized and negative and positive control peptide spots were defined as 0% and 100%, respectively. Signal intensities (%) of binding to PODXL-1 peptide spots were then calculated.

2.2. Parallel peptide synthesis on resin

For synthesis of free peptides selected from the second screening round (see Section 2.1), the MultiPep Multiple Peptide Synthesizer from Intavis AG (Koeln, Germany) was used. Tentagel amide resin (Intavis AG, Koeln, Germany) (50 mg) was placed in each well of the reaction plate. For the automated predefined 10 μmol scale method, 0.6 M amino acids (Bachem, Bubendorf, Switzerland) were used. The amino acids Fmoc–Ala–OH, Fmoc–Cys(Trt)–OH, Fmoc–Asp(OtBu)–OH, Fmoc–Phe–OH, Fmoc–Gly–OH, Fmoc–Ile–OH, Fmoc–Leu–OH, Fmoc–Met–OH, Fmoc–Asn(Trt)–OH, Fmoc–Gln(Trt)–OH, Fmoc–Arg(Pmc)–OH, Fmoc–Ser(*t*Bu)–OH, Fmoc–Thr(*t*Bu)–OH, Fmoc–Val–OH, Fmoc–Trp(Boc)–OH and Fmoc–Tyr(O*t*Bu)–OH were prepared in dimethylformamide and the amino acids Fmoc–His(Trt)–OH, Fmoc–Lys(Boc)–OH and Fmoc–Pro–OH were prepared in *N*-methylpyrrolidone (Applied Biosystems, Foster City, CA, USA). For activation, PyBop (0.6 M) in dimethylformamide and 4-methylmorpholin (4 M) were used. Fmoc deprotection reagent was 20% (v/v) piperidine, cap reagent was 5% (v/v) acetic anhydride in dimethylformamide and all washing steps were performed with dimethylformamide. Cleavage of side chain protecting groups and cleavage of the peptide from the resin was performed with an aliquot (500 μL) of 6% (w/v) phenol, 2% (v/v) triisobutylsilane, 4% (v/v) thioanisole and 4% (v/v) distilled water in trifluoroacetic acid in each reaction well for 3 h. Afterwards, the trifluoroacetic acid was evaporated and the peptides precipitated and washed with diethylether. Mass analysis was performed by using offline ESI Q-TOF MS on a Waters Micromass Q-TOF Ultima Global (Waters, Milford, MA, USA). Data analysis was performed with MassLynx 4.0 SP4 Software.

2.3. Enzyme-linked immunosorbent assay

The synthesized peptides (see Section 2.2) were dissolved in dimethylsulfoxide and diluted in 0.1 M carbonate buffer, pH 9.6, to a final concentration of 100 μg/mL. To coat the sample wells, aliquots (100 μL) of each peptide were pipetted in duplicates in wells of a Nunc Immobilizer™ Amino 96 well plate (Nunc, Roskilde, Denmark). The plate was incubated overnight at 4 °C. For all further steps, the washing buffer was phosphate buffered saline (PBS), pH 7.2, and the incubation buffer was washing buffer with 1% (w/v) bovine serum albumin, fraction V (BSA). The plate was blocked for 2 h in washing buffer with 3% (w/v) BSA and afterwards was incubated for 1 h with biotinylated mAb 84 (50 μg/mL) (see Section 2.1.1) in incubation buffer. The plate was then incubated for 1 h with 1:3200 diluted streptavidin-horseradish peroxidase conjugate (RPN4401 V, GE Healthcare, Little Chalfont, Buckinghamshire, UK) in incubation buffer. Between all incubation steps, the plates were stringently washed with washing buffer. The color reaction was induced in each well with 100 μL of 100 μg/mL *o*-phenylenediamine dihydrochloride in 35 mM citric acid monohydrate, 67 mM disodium hydrogen phosphate dihydrate, pH 5, and 3.6% (v/v) hydrogen peroxide. After 5 min the reaction was stopped with 1.25 M sulfuric acid and absorption was measured at 492 nm with a reference wavelength of 620 nm on the Genios Pro multimode micro plate reader (Tecan, Groedig, Austria). For evaluation, values of blank wells (prepared as described before but without the coated peptide) were subtracted from all other values.

2.4. Peptide purification

The peptide CCHQRLSQRK, selected from ELISA, was ordered in large scale with N-terminal acetylation and C-terminal amidation from JPT (Berlin, Germany). The crude peptide was dissolved in distilled water, filtered with a 0.22 μm nitrocellulose filter (Milli-

pore, Bedford, USA), and purified with the Agilent 1200 preparative HPLC system (Vienna, Austria). The reversed phase column was a Luna 15 μ C18 from Phenomenex (Aschaffenburg, Germany) and the multiple wavelength detector was set at 210 nm. Solvent A was 5% acetonitrile and 0.1% trifluoroacetic acid in ultra-pure water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The flow rate was 30 mL/min. Firstly, the column was equilibrated 5 min in solvent A, and followed by a linear gradient over 55 min from 0% to 55% solvent B. Fraction collection was performed within the linear gradient. Finally the column was regenerated for 5 min with 90% solvent B and stored in solvent A. Afterwards, acetonitrile in the peptide fraction was evaporated and the peptide was lyophilized.

2.5. Coupling the peptide on a monolith

An epoxy-activated CIM® Monolithic Column from BIA Separations (Ljubljana, Slovenia) with a disk volume of 0.34 mL was used as the chromatographic support. To fill the pores of the monolith with coupling reagents, syringes were connected at the inlet and outlet of the housing to enable flushing the monolith in both flow directions. This dynamic procedure was done in order to overcome limitations of diffusion and to achieve higher immobilization efficiency as shown by Bencina et al. [33] by comparison of dynamic and static immobilization of an enzyme. Firstly, the disk was equilibrated with distilled water and afterwards incubated with 1 M bis-3(aminopropyl) amine in distilled water overnight at 4 °C. After washing with distilled water, the spacer was iodated with 1 M iodoacetic anhydride in dimethylformamide for 30 min and the disk was washed with 20 mM MES, pH 6. The purified peptide was dissolved in 1 mL 20 mM MES to a concentration of 3 mg/mL and injected into the disk. The coupling efficiency was monitored over 60 min by taking samples at different time points for assays with a BCA protein assay kit (Thermo Scientific, Rockford, USA). After washing the disk with distilled water, the reactive groups were blocked with 2-mercaptoethanol. Then the disk was washed again with distilled water and stored in 20% (v/v) ethanol. In this manuscript, this disk will be referred to hereafter as the peptide disk. A second disk, hereafter called the blank disk, was prepared in exactly the same way, but instead of peptide in MES buffer, only MES buffer was used.

2.6. Chromatographic experiments

Chromatographic experiments were performed with the Agilent 1100 analytical HPLC system (Agilent, Vienna, Austria) and later continued with the Agilent 1200 preparative HPLC system (Vienna, Austria). MAb 84, human IgM from serum, and bovine IgG from serum were diluted in equilibration buffer and, for spiking experiments, these same proteins were diluted in a serum free hybridoma cell culture supernatant (monoclonal antibodies in the supernatant were previously purified at BTI, Singapore and the chromatographic flow through was provided). If not mentioned otherwise, in all experiments 100 μl samples with 100 μg/mL protein were injected onto the equilibrated peptide disk. Unbound proteins were washed out with 3 mL equilibration buffer and bound proteins were eluted with 3 mL elution buffer. Afterwards a re-equilibration was performed with 3 mL equilibration buffer. For experiments with the preparative HPLC system, the re-equilibration volume was extended to 8 mL equilibration buffer due to the higher void volume of the system. The absorbance was measured at 280 nm and the amount of protein in the flow through and the eluate was calculated by peak integration. The column was cleaned with 2.5 M guanidine–HCl after approximately every 30th run. For equilibration buffer screening, (1) PBS pH 7.2 and (2) 30 mM sodium phosphate with 1 M sodium chloride, pH 7.4, were used. For elution buffer screening, (1) 10 mM HCl, pH 2, (2) PBS with 2 M NaCl, (3) PBS with 4 M NaCl, (4) PBS with 4 M NaCl, pH 2, and (5) 2.5 M guanidine–HCl were used. For experiments with the analytical HPLC system, a flow velocity of 1 mL/min was used in all experiments. For experiments with the preparative HPLC system, flow velocities between 1 mL/min and 10 mL/min were tested and 7.5 mL/min was used in all other experiments. All experiments were performed at least in triplicate.

3. Results and discussion

3.1. Identification of a peptide ligand

The first two screening rounds for the selection of potential mAb 84 peptide affinity ligands were performed with peptide SPOT membranes. Fig. 1A shows identified epitope regions from the first screening round while Fig. 1B shows all peptides that were bound by mAb 84 in the second screening round. Based on these results, twenty peptide candidates were selected for a third screening round based on ELISA. The results of this third screening round are presented in Fig. 1C. In order to achieve sufficient signal, a relatively high concentration of mAb 84 (50 μg/mL) was required for ELISA. The need for the high levels of mAb 84 is explained by the lack of a spacer between the peptides and the surface of the well plates, as previously shown by Andresen et al. [34] with immobilized peptides on glass surfaces. The peptide with the highest affinity for mAb 84 and therefore the selected affinity ligand for chromatographic experiments was the peptide C–C–H–Q–R–L–S–Q–R–K. Due to the two arginines and the lysine, the peptide is positively charged under chromatographic conditions below pH 10.5 (pKa K = 10.5, pKa R = 12.5). This may lead to certain unspecific adsorption. Especially the terminal R–K doublet can be expected to confer strong anion exchange properties. The interaction is definitely not based on a pure electrostatic interaction because other peptides also containing this amino acid doublet did not bind the IgM. So we conclude that the interaction is also based on the structure of the peptide and thus leading to this affinity. The two cysteines can be further used for the N-terminal immobilization.

3.2. Peptide coupling on the CIM disk

The poor accessibility of the peptide when bound on a surface as observed by ELISA (see Section 3.1) prompted us to introduce a spacer molecule between the CIM disk and the peptide ligand, although spacer molecules are usually not necessary for monolithic sorbents [35]. The coupling reaction of the peptide to the spacer was completed after 5 min. Measurement of peptide concentrations in the liquid phase prior to and after coupling showed a ligand density of 3.6 μmol/mL sorbent. This ligand density is comparable to procedures where peptides are directly coupled to epoxy-activated monoliths [36].

3.3. Peptide affinity chromatography

3.3.1. Specificity of the peptide ligand

In order to determine whether the peptide affinity sorbent was specific for mAb 84 or if it was capable of binding IgM in general, samples of human serum IgM were compared to samples of mAb 84. Additionally, bovine serum IgG was used as a control sample. The experiments were performed with the analytical HPLC system. In the superimposed chromatograms of the three samples (Fig. 2), the peak of unbound mAb 84 sample in the flow through can be explained by impurities or conformational variants of IgM which do not bind to the ligand. Such “impurities” cannot be detected by electrophoresis and size exclusion chromatography. Another possibility is the association of IgM and DNA or DNA fragments. The interaction of the highly charged DNA molecule may impair the

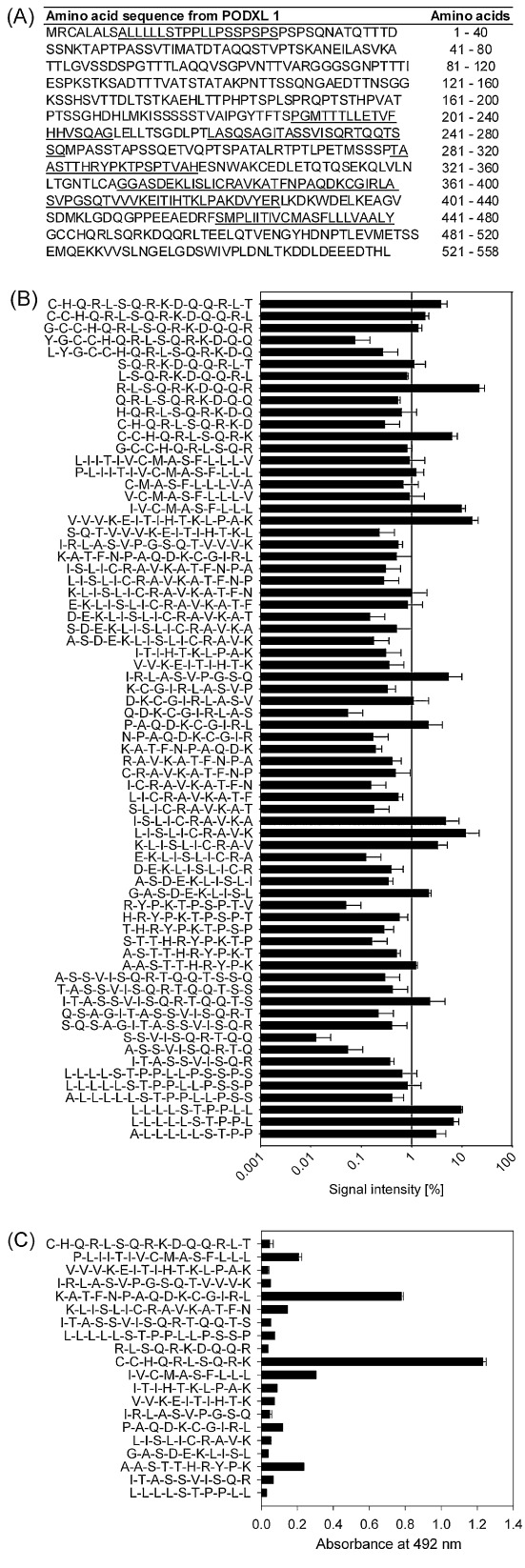

Fig. 1. Results from three peptide screening rounds. (A) The whole amino acid sequence from PODXL-1. Underlined amino acids indicate potential binding epitopes, found in the first screening round by using peptide SPOT membranes. (B) Signal intensities from the second screening round on peptide SPOT membranes. Only peptides that were bound by mAb 84 are illustrated. The scaling of the signal intensity is logarithmic. (C) All peptides that were used for ELISA and the respective absorbance in the binding assay.

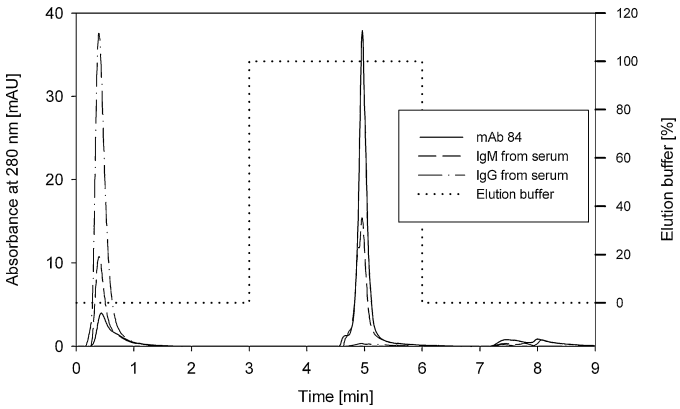

Fig. 2. Elution profile of 100 μg/mL mAb 84, IgM from serum, and IgG from serum on the peptide disk.

ability of the ligand to recognize and bind the IgM component, at least a subpopulation thereof, which would consequently appear in the flow-through. This has been already shown for IgM in ion-exchange chromatography [37]. Further studies are required to identify the structure of IgM in the flow through in order to judge if the ligand is able to discriminate between different conformational variants.

About 39% of human serum IgM and 100% of bovine serum IgG flowed through upon application. In order to prove that mAb 84 was bound specifically by the peptide ligand and not by the CIM disk or the linker molecule, experiments with a blank disk containing only the linker molecule but not the peptide ligand were performed. The chromatogram of mAb 84 injected on a blank disk showed that the whole amount of mAb 84 appeared in the flow through (Fig. 3).

3.3.2. Determination of recovery

Recovery experiments were performed using mAb 84 in PBS, human serum IgM, and bovine serum IgG as the control sample. The experiments were performed with the analytical HPLC system. Table 1 compares the results of experiments with the empty CIM cartridge, which was used as the 100% reference, experiments with the blank disk, and, finally, experiments with the peptide disk itself.

As expected, the recovery of serum IgG is very high. There is no significant binding to the blank disk or the ligand. With respect to mAb 84, the major loss of recovery resulted from unspecific binding to the blank disk giving a recovery of 86%. Experiments with the peptide disk showed a recovery of 83%, indicating that only 3% loss are caused by unspecific binding to the peptide ligand and 14% loss are caused by the blank disk. The blank disk is a monolithic disc modified with a spacer as described in Section 2.5. Further

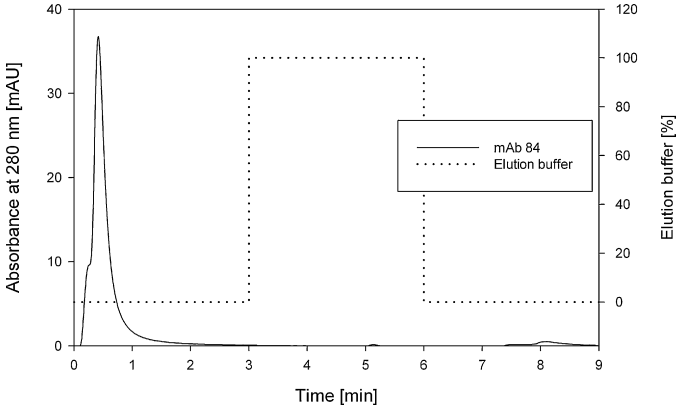

Fig. 3. Elution profile of 100 μg/mL mAb 84 on the blank disk.

Table 1
Determination of the recovery of chromatographic experiments. Experiments were performed in triplicate with each protein sample (100 μg/mL) diluted in equilibration buffer and the responses of the flow through and the eluate were summed in mAU * min. The relative standard deviations were below 1%.

Chromatographic column	Recovery of mAb 84 [%]	Recovery of serum IgM [%]	Recovery of serum IgG [%]
Empty CIM disk cartridge	100	100	100
Blank disk	86	88	95
Peptide disk	83	67	95

investigations concerning monolithic material and different spacer molecules might improve the recovery. Similar to mAb 84, the serum IgM showed recovery of 88% with the blank disk, but also showed a significant loss in recovery with the peptide disk, giving a recovery of only 67%. IgM is a much more complex molecule than IgG with a lager number of charges and also hydrophobic amino acids. Thus a higher unspecific adsorption can be expected. We assume that the material is strongly bound at the surface of the monolith. Tubings and housing can be excluded since 100% was recovered when injecting the antibody into a system with empty CIM-housing.

In order to elute the unrecovered material from the peptide disk, we used 2.5 M guanidine–HCl after approximately every 30th run. The amount of eluted material could not be monitored due to the high absorbance of guanidine–HCl at 280 nm in comparison to the equilibration buffer, but as binding capacity was consistent within the period of the study, we concluded that guanidine–HCl provides sufficient regeneration of the peptide disk.

3.3.3. Buffer screening

The goal of the buffer screening was to concentrate the maximum amount of mAb 84 in the eluate. The experiments were performed with the analytical HPLC system. For the first experiments (see Section 3.3.1), PBS was used as the equilibration buffer and 10 mM HCl, pH 2, was used as the elution buffer. The proportion of the eluate was 79.4%. The remaining 20.6% were unbound antibodies in the flow through. To further optimize the binding, buffer screening experiments with calculated ratios of eluate and flow through areas were performed. The results are shown in Table 2. Higher salt concentrations for the equilibration buffer were considered for later spiking experiments with cell culture supernatant and potential nonspecific binding of host cell proteins to the peptide disk. The higher salt concentration in the equilibration buffer resulted in a dramatic loss in binding affinity of the antibody to the peptide. Thus, no further buffer screening for the equilibration buffer was performed and PBS was used as equilibration buffer for all further experiments. Buffer screening for elution buffer showed that 10 mM HCl (pH 2), PBS with 2 M NaCl (pH 7.4) and PBS with 2 M NaCl (pH 2) produced similar acceptable results, whereas 2.5 M guanidine–HCl is not suited for analytical purposes due to its relative high absorbance at 280 nm in comparison to the equilibration buffer. For further analytical experiments 10 mM HCl, pH 2, was used as elution buffer. For preparative purposes, elution with PBS with 2 M NaCl would be most suitable, particularly with regard to maintenance of activity of the antibody.

3.3.4. Influence of flow velocity

Flow velocities above 1.0 mL/min could not be tested with the analytical HPLC system due to the high backpressure of the system. CIM disks are suited for flow velocities of up to 10 mL/min (530 cm/h) which greatly reduce the analysis time. For that reason we continued our experiments with a preparative HPLC system where higher flow velocities could be used that should not affect the binding capacity of the sorbent [38,39]. The focus of the experiments with different flow velocities was to compare the amount of eluted mAb 84 (Table 3). In the case of slow kinetic binding, increasing column residence time by decreasing flow velocity might be expected to improve binding efficiency, with the result of increasing the amount of antibody in the eluate [40]. Experiments showed that the amount of eluted mAb 84 and mAb 84 in the flow through were more or less unchanged with different flow velocities, indicating a rapid binding kinetic. As mentioned above it is likely that the ligand is able to discriminate between different forms of DNA and IgM adducts. For further analyses, a flow velocity of 7.5 mL/min was used which reduced the analysis time to ~2 min. Such analysis time would not be feasible with porous material, since the diffusion into the pores would be to slow. The separation with a porous material would be definitely mass transfer limited and wide peaks can be expected resulting in lower sensitivity.

3.3.5. Evaluation of calibration curves

A calibration curve with purified mAb 84, diluted in equilibration buffer, was generated as well as a calibration curve of spiked mAb 84 in cell culture supernatant. Experiments were performed with the preparative HPLC system. Eleven concentrations ranging from 0 to 500 μg/mL were injected in triplicate. The range of 32–500 μg/mL mAb 84 was confirmed by statistical tests for the calibration curve of mAb 84 in equilibration buffer as well as for the calibration curve of mAb 84 in cell culture supernatant (Fig. 4). For the calibration curve of mAb 84 in equilibration buffer, the slope was 1.94 ± 0.01 and the intercept was -10.7 ± 2.87. We also quantified the peak areas of the flow through from experiments with mAb 84 diluted in equilibration buffer (Fig. 4). As the injected concentrations of mAb 84 increased, the ratio of eluate/flow through increased. This outcome confirmed that adsorption was concentration dependent. For the calibration curve of mAb 84 in cell culture supernatant the slope was nearly identical to that for the studies of mAb 84 in equilibration buffer, but the intercept was higher

Table 2
Buffer screening for chromatographic experiments. Experiments were performed in triplicates with each 100 μg/mL mAb 84 diluted in equilibration buffer and the responses of the flow through and the eluate were calculated in mAU * min. The relative standard deviations were below 1%.

Equilibration buffer	Elution buffer	Ratio of response eluate/response flow through
PBS	10 mM HCl, pH 2	3.84
PBS, 1 M NaCl	10 mM HCl, pH 2	0.41
PBS	PBS, 2 M NaCl	4.22
PBS	PBS, 2 M NaCl, pH 2	4.06
PBS	2.5 M guanidine–HCl	Incalculable

Table 3
Effect of different flow velocities on flow through and eluate responses. Experiments were performed in triplicate with each protein sample (100 μg/mL) diluted in equilibration buffer. The relative standard deviations were below 5%.

Flow velocity [mL/min]	Response flow through [mAU * mL]	Response eluate [mAU * mL]
1	84.9	188.1
2.5	81.0	188.9
5	82.8	187.5
7.5	78.3	216.8
10	83.7	204.0

Table 4
Intra-assay variability of different concentrations of mAb 84 in equilibration buffer. The data represent the mean values of six experiments.

Concentration [μg/mL]	Response [mAU * min]	Relative standard deviation [%]	Calculated concentration [μg/mL]	Relative error [%]
62.5	121.3	1.6	68.2	9.2
125	232.3	0.7	125.8	0.6
250	473.8	0.8	250.8	0.3

Table 5
Inter-assay variability of different concentrations of mAb 84 in equilibration buffer on three consecutive days. The data represent the mean of three experiments.

Day	Concentration [μg/mL]	Response [mAU * min]	Relative standard deviation [%]	Calculated concentration [μg/mL]	Relative error [%]
1	62.5	120.2	2.9	67.7	8.3
	125	237.7	0.9	128.5	2.8
	250	478.5	2.0	253.2	1.2
2	62.5	129.25	1.1	72.4	15.8
	125	235	1.4	127.1	1.7
	250	490	8.7	259.2	3.7
3	62.5	120.75	0.1	68.0	8.7
	125	228	0.1	123.5	−1.2
	250	470.25	0.1	248.9	−0.4

(105.1 ± 4.17). In previous studies with mAb 84 [28] a high DNA contamination was mentioned. The DNA was bound with such a strong affinity to the anion exchanger that it could replace IgM. Therefore, the authors used benzonase to break down the DNA prior to purification. In the present study the L–K doublet of the peptide ligand might have strong affinity for DNA as well. As mentioned above DNA can form complexes with mAb 84 as well as with host cell proteins. The bound DNA, DNA–host cell protein complexes and DNA–mAb 84 complexes could then be co-eluted together with mAb 84 and thereby amplify the signal. This could account for the offset of the intercept of the calibration curve of mAb 84 in cell culture supernatant as compared with that for the purified mAb 84. The concentration dependent amount of mAb 84 in the flow through can be also explained with this hypothesis, because residual DNA fragments after treatment with benzonase could also complex with MAb 84. Bound DNA and complexes could shield mAb 84, impair the ligand to recognize mAb 84 and therefore affect specificity and kinetics. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) are defined as

$$\mathrm{LLOD} = \frac{3 \times \sigma_{\mathrm{RSS}}}{k_{\mathrm{low}}} \quad (1)$$

and

$$\mathrm{LLOQ} = \frac{10 \times \sigma_{\mathrm{RSS}}}{k_{\mathrm{low}}} \quad (2)$$

where σ_{RSS} is the residual standard deviation and k_{low} is the slope of the calibration curve in the lower concentration range. For mAb 84 in equilibration buffer, the LLOD is 15.51 μg/mL and the LLOQ is 51.69 μg/mL. For mAb 84 in cell culture supernatant, the LLOD is 14.52 μg/mL and the LLOQ is 48.40 μg/mL. The lower limits of detection are lower than the lowest values of the calibration curves, but the lower limits of quantification exceed the lowest values of the calibration curves, thus the lower ranges of the calibration curves are not valid for quantification. For our quantitative purposes, to monitor the purification process, a LLOQ of ∼50 μg/mL is sufficient, and therefore we made no further attempts to improve the LLOQ. For the purposes of developing a potential one step preparative process, the adsorption of host cell proteins must be reduced to insignificant values by means such as more intensive buffer screenings or modified peptide ligands.

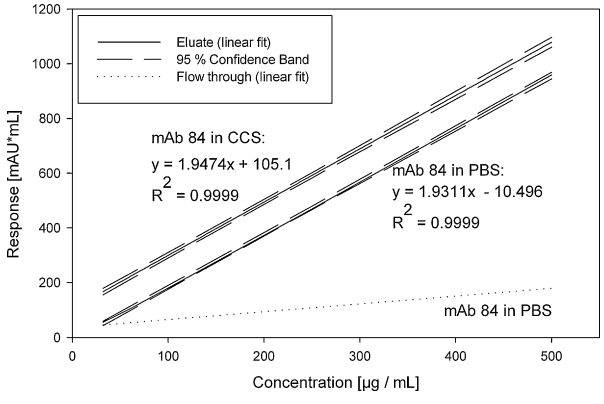

Fig. 4. Calibration curves of mAb 84 in equilibration buffer and mAb 84 in cell culture supernatant (CCS). The linear fit was calculated from experiments performed in triplicate with relative standard deviations below 5%.

3.3.6. Determination of inter- and intra-day variability

Evaluations of the precision of the method were performed with 3 different concentrations utilizing the preparative HPLC system. Table 4 shows results from six consecutive injections and Table 5 shows results from three different days with each of three consecutive injections. Intra- and inter-assay experiments showed relative standard deviations and relative errors below 10%. Only the 15% relative error on day 2 seemed to be critical, but the relative standard deviation in that case was only 1.1% and therefore a sample preparation anomaly was most likely the reason for that outlier.

4. Conclusions

Ligand C–C–H–Q–R–L–S–Q–R–K immobilized on a monolith supports adequate binding and elution efficiency to support useful quantitation of mAb 84. Although less sensitive than ELISA, its 2 min assay time makes it an attractive option for measuring product concentration in cell culture supernatants and in-process fractions from chromatographic purification steps. In the latter case, the chromatographic assay format makes it easy for purification process developers to run the assay on their same equipment and obtain results immediately. Experimental results raise doubts about how applicable this ligand may be for preparative purposes. Additional optimization of process buffers, support material and spacers may overcome this limitation and perhaps make this approach feasible for preparative purification of mAb 84.

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