Faster Analysis of Monoclonal Antibodies Using Silica Monoliths Designed for Bioanalysis

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The chromatographic analysis of biomolecules, such as monoclonal antibodies (mAbs), requires new types of columns providing a good permeability, improved mass transfer and selectivity properties. Silica monoliths with larger mesopores are a powerful tool for the fast separation of biomolecules. In this article, the immobilisation of protein A on those widepore silica monoliths is described for the titre determination of monoclonal antibodies. The protein A modified monolith provided highly reproducible and fast, one minute, separation of mAbs. Furthermore, the high reproducibility of immobilisation method with three batches and the column stability over 5,000 runs are demonstrated.

Introduction

In the last two decades, the pharmaceutical market has changed dramatically from small molecules to protein-based drugs and antibodies, which have a higher potential for targeting extraneous substances [1]. This is supported by the fact that six of the top ten best-selling pharmaceuticals are based on monoclonal antibodies. In comparison to chemical entities, the analysis of mAbs is complex and requires several separation techniques, e.g. affinity chromatography, reversed phase chromatography, size exclusion chromatography and ion-exchange chromatography. Especially, the strong growth in development of biosimilars due to expiring patents of those blockbusters, led to increased requirements concerning column performance in HPLC. Recently, a survey of several HPLC column manufacturers offered the introduction of almost 20 new columns in 2016, addressing biomolecules in different separation modes [2].

The analysis of biomolecules using conventional HPLC columns is usually associated with limited accessibility of surface for larger molecules, slow diffusion leading to extremely broad peaks with severe tailing, and possibly conformational changes during elution. One of the reasons for those observations is the mesopore size ranging from 8-15 nm. The separation quality could be improved by using column materials with wider mesopores of ca. 30 nm or larger [3-5].

During development, process monitoring, and quality control testing of mAbs, fast and precise analytical methods are necessary. The most important technique for observation of mAb titer is affinity chromatography using protein A. Protein A, a cell wall protein from *Staphylococcus aureus*, was the first isolated protein with a high affinity to the Fc region of immunoglobulin G (IgG) [6]. Its high selectivity and high resistance against temperature, pH and tryptic cleavage make protein A a powerful tool in antibody purification [7]. Today, the use of protein A as a ligand on chromatographic media is one the most employed methods for antibody capture during the purification process. In comparison to process protein A media, only few vendors provide analytical protein A columns for process monitoring and quality control. The majority of those columns are packed with particles limited in column backpressure, chromatographic performance and application of 'dirty' samples, such as harvest cell culture fluids. Hence, sample preparation is more complex and the limited column backpressure result in long analysis time due to lower flow rates.

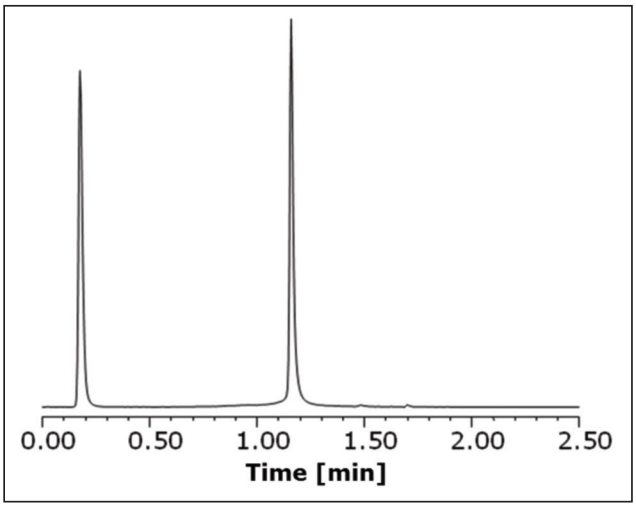

Figure 1: Separation of cetuximab (1 mg/mL) and BSA (1 mg/mL) by immobilised rSPA silica monolith. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; 0.25 min 100/0, 0.25-0.26 min 0/100, 1.00 min 0/100, 1.00-1.01 100/0, 1.01-2.50 min 100/0; flow rate: 2.0 mL/min; detection: 280 nm; injection: 10 µL; temperature: 25°C

Silica monoliths, which consist of one continuous piece of silica, are an alternative to fully porous or superficially porous particles, and could be used [8]. Silica monoliths are preferred for the application and fast separation of 'dirty' samples due to their bimodal pore structure consisting of large through pores (macropores) and smaller micro- or mesopores [9]. They are prepared according to a sol-gel process leading to a bimodal pore structure where both macropores and mesopores could be controlled individually [9, 10]. The design of larger macropores offers good flow

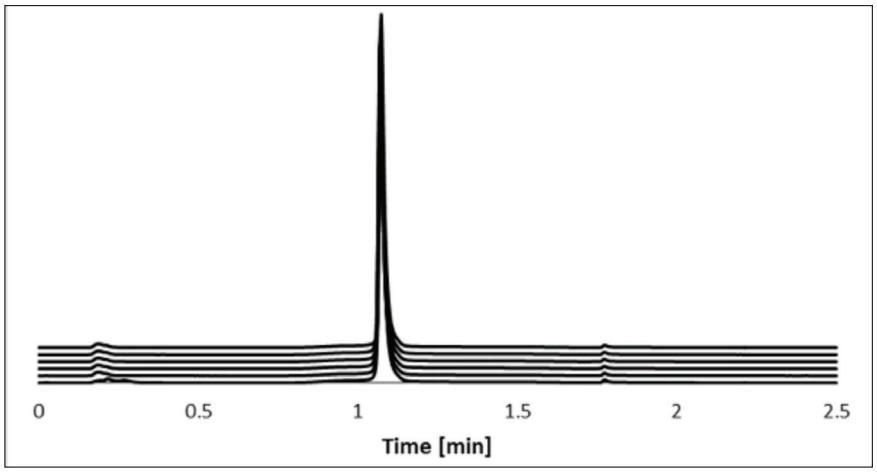

Figure 2: Analysis of 50 injections of cetuximab (1 mg/mL) on immobilised rSPA silica monolith. Only first and every 10th chromatogram is shown. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; 0.25 min 100/0, 0.25-0.26 min 0/100, 1.00 min 0/100, 1.00-1.01 100/0, 1.01-2.50 min 100/0; flow rate: 2.0 mL/min; detection: 280 nm; injection: 10 µL; temperature: 25°C

characteristics resulting in much lower column backpressure compared to columns packed with particles, and maintaining column performance even at higher flow rates [11-13]. Due to the hydrodynamic volume of mAbs (mostly 5-6 nm), the alteration of mesopore development up to 30 nm is necessary allowing them to enter the pores for improved chromatographic separation and to exclude separation effects by size. Recently, the development of silica monoliths containing larger mesopores and its application for the analysis of biomolecules has been reported [14].

Another continuously growing sector in chromatography is the coupling of desired ligands for specific interactions with certain analytes e.g. antibodies, aptamers or enzymes [15-20]. The majority of described protein immobilisations were performed on polymeric particles or monoliths and silica particles. In the last decade, silica-based monoliths were also used for the immobilisation of several ligands to enable their use in the applications of affinity chromatography, for chiral separations or as on-column bioreactors [21-26]. The development of wide pore silica monoliths enhanced the column properties for the immobilisation and analysis of larger molecules. In this study, recombinant staphylococcal protein A (rSPA) is immobilised onto a silica monolith containing larger mesopores suitable for the separation of mAbs. The analysis performed on the monolithic column is characterised by different parameters including detector response linearity, reproducibility and long-term stability.

Experimental

Chemicals

Sodium phosphate dehydrate (≥99.5%), ammonium sulphate (≥99.5%), sulphuric acid (w=98%), ortho-phosphoric acid (w=85%) and sodium hydroxide (w=50%) for analysis were purchased from Merck (Darmstadt, Germany). Pure water was obtained from Milli-Q system from Merck (Darmstadt, Germany). Native recombinant staphylococcal protein A (rSPA) ligand was purchased from Repligen (Waltham, MA, USA) and bovine serum albumin (BSA) was obtained from VWR (Darmstadt, Germany). Pure cetuximab stock solution was a research sample from Merck (Darmstadt, Germany) and gammanorm IgG was obtained from Octapharma (Heidelberg, Germany).

Epoxy-modified widepore silica monolith (25 mm x 4.6 mm) columns (Chromolith® WP 300 Epoxy) were prepared as research samples at Merck (Darmstadt, Germany).

Apparatus

All modification and cleaning steps necessary for the immobilisation of rSPA ligand on epoxy-modified columns were performed at a Merck-Hitachi L-6200 HPLC Pump (Darmstadt, Germany). The following chromatographic studies were performed on an Ultimate 3000 HPLC system consisting of LPG-3400RS HPLC pump, WPS-3000TRS autosampler, TCC-3000RS column oven compartment and VWD-3000 UV detector from Dionex (Germering, Germany).

Preparation of rSPA Silica Monoliths

Wide pore silica monoliths were prepared according to the sol-gel process [27, 28] possessing a macropore size of 2 µm. Thermal treatment under alkaline conditions allowed mesopore formation to a size of 30 nm. All silica monoliths were cladded with solvent-resistant polymer (polyether ether ketone [PEEK]) housings. The silica monoliths were chemically derivatised to epoxy-modified monoliths following a procedure described elsewhere [22].

The immobilisation of rSPA on wide pore epoxy-modified monoliths was done according to a dynamic process circulating ligand solution through the column. The rSPA ligand solution was dissolved in 6 mL immobilisation buffer (50 mM sodium phosphate + 1.9 M ammonium sulphate pH8.0) resulting in a rSPA concentration of 2 mg/mL. Before immobilisation, columns were equilibrated with 50 mL immobilisation buffer. The protein solution was circulated at a flow rate of 0.2 mL/min for 4 hours. Finally, rSPA silica monolith was washed with 50 mL of 100 mM sodium phosphate buffer pH7.4 and remaining epoxy functions were hydrolysed with 150 mM phosphoric acid pH 1.5.

Results and Discussion

General Column Evaluation

Before the immobilisation process, the silica monoliths were characterised by mercury intrusion porosimetry and nitrogen

Table 1: Chromatographic data of separated IgG by immobilised rSPA silica monolith from different batches. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; 0.25 min 100/0, 0.25-0.26 min 0/100, 1.00 min 0/100, 1.00-1.01 100/0, 1.01-2.50 min 100/0; flow rate: 2.0 mL/min; detection: 280 nm; injection: 10 µL; temperature: 25°C

Batch No.	Retention time [min]	Peak Symmetry (USP)	Peak Width (10%) [min]	Column back pressure [bar]
1 (n=6)	1.15 ± 0.01	1.57 ± 0.15	0.037 ± 0.003	10 ± 0
2 (n=6)	1.16 ± 0.00	1.53 ± 0.12	0.035 ± 0.003	10 ± 0
3 (n=6)	1.15 ± 0.00	1.52 ± 0.06	0.035 ± 0.003	10 ± 0
Total (n=18)	**1.15 ± 0.00**	**1.54 ± 0.11**	**0.036 ± 0.003**	**10 ± 0**

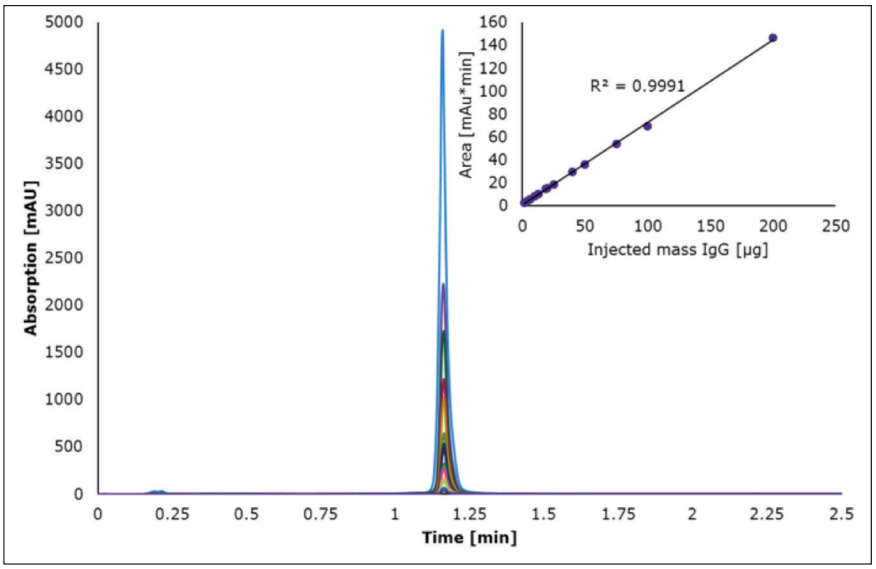

Figure 3: Calibration curve of Cetuximab on rSPA silica monolith ranging from 1.25 µg to 200 µg. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; 0.25 min 100/0, 0.25-0.26 min 0/100, 1.00 min 0/100, 1.00-1.01 100/0, 1.01-2.50 min 100/0; flow rate: 2.0 mL/min; detection: 280 nm; injection: 20 µL; temperature: 25°C

adsorption/desorption (BET). They consisted of macropores of 1.83 µm and mesopores of 28.6 nm resulting a total surface area of 120 m^2/g silica. The column was immobilised with rSPa ligand circulating through the column for four hours. The final column possessed a dynamic binding capacity of 2.75 (± 0.11) mg for monoclonal IgG.

The rSPA modified silica monoliths were used for separation of monoclonal antibodies from their related impurities. The separation of cetuximab from BSA impurity was performed in around one minute and a typical chromatogram is shown in Figure 1. The unbound BSA eluted first followed by cetuximab which was released due to the pH shift to pH2.5. The rSPA silica monoliths provided very sharp peaks with peak widths < 0.040 minutes in a reasonable run time which was supported by the hydrophilic column surface reducing undesirable backbone interactions with the analytes.

Another important criteria for analytical protein A columns is column performance reproducibility. Therefore, cetuximab sample was injected 50 times and all chromatographic parameters were evaluated especially with respect to their relative standard deviation (RSD). An overlay of all 50 chromatograms is shown in Figure 2 indicating a robust and reproducible column performance. The RSD of the retention time of eluted cetuximab was < 0.1% with the peak width being equal for every run. The peak area RSD was 0.4% supporting the previous data and assumptions of a robust column modification and reproducible column performance.

Furthermore, different batches were synthesised under exactly same conditions and six columns of each batch were immobilised with rSPA ligand, respectively. The summary of chromatographic data of each batch are shown in Table 1. The data revealed only slight differences between columns from the same batch as well as minor differences between different batches. Those results demonstrate that the immobilisation process via epoxide functions is very robust and reproducible.

Column Linear Range

For titre determination, linear range of the analytical column is a key property for its application. In antibody production, the used bioreactors contain antibody concentrations between 0.5-7 g/L, which is the minimum requirement for an analytical protein A column. The rSPA modified monolith was evaluated by analysing different concentrations of pure cetuximab ranging from 0.0125 mg/ml to 10 mg/mL. The 15 used calibration standards revealed a broad analysis range from 1.25-200 µg of injected cetuximab for the immobilised rSPA silica monolith. The retention time of eluted cetuximab concentrations varied by only 0.1% and was confirmed by an overlay of all calibration chromatograms as shown in Figure 3. The correlation coefficient was higher than 0.999 supporting the hypothesis of a high column capacity.

Flow rate

Since monolithic silica columns enable a low column backpressure due to their high flow through pores, the separation capability of immobilised rSPA silica monoliths was evaluated at higher flow rates. The comparison of column performance was done with pure cetuximab spiked with BSA. It was expected that retention time and peak width of eluted cetuximab would decrease with increasing flow rate.

In Table 2, the most important data were shown for flow rate comparison. It was visible that cetuximab column binding was not effected by flow rate and the relative peak area of eluted cetuximab was constant. Additionally, column backpressure increased linearly (R^2 = 0.998) enabling high flow rates and even shorter run times with constant column performance and without any loss in binding efficiency. The high-speed separation of monoclonal antibodies at high flow rates was enhanced by the high mass transfer properties of the macropores. The flow rate data demonstrated the large time savings and high separation efficiencies obtained by using silica monoliths.

Stability

The examination of column long-term stability was performed with IgG as a control. The stability of the monolithic structure, column modification and protein

Table 2: Chromatographic data of separated IgG (1 mg/mL) and BSA (1 mg/mL) by immobilised rSPA silica monolith at different flow rates. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; same gradient was used as shown in Figure 2 and was adjusted based on used flow rate; flow rate: 1.0-5.0 mL/min; detection: 280 nm; injection: 10 µL; temperature: 25°C

Flow rate [mL/min]	Retention time [min]	Peak Width (10%) [min]	Unbound area	Bound area	Column back pressure [bar]
1.0	2.31	0.058	39%	61%	3
2.0	1.16	0.032	39%	61%	6
3.0	0.77	0.024	39%	61%	10
4.0	0.58	0.021	39%	61%	13
5.0	0.47	0.020	39%	61%	17

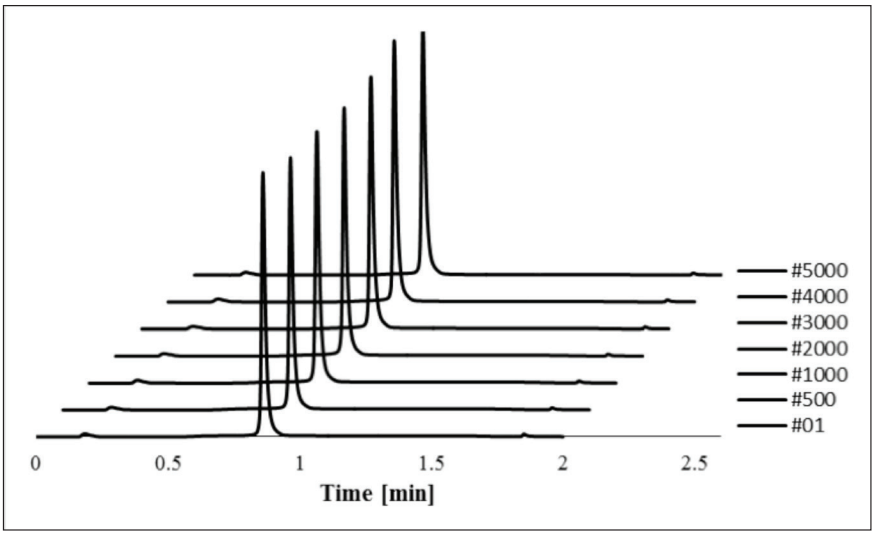

Figure 4: Stability test of immobilised rSPA silica monolith against 10.000 pH shifts using gammanorm IgG (1 mg/mL) as control for column performance. Chromatographic conditions: Stepwise gradient: 100 mM sodium phosphate pH 7.4/100 mM sodium phosphate pH 2.5; 0.05 min 100/0, 0.05-0.06 min 0/100, 1.10 min 0/100, 1.10-1.15 100/0, 1.15-2.00 min 100/0; flow rate: 2.0 mL/min; detection: 280 nm; injection: 10 µL; temperature: 25°C

A linkage were extensively investigated by more than 5,000 runs including 10,000 pH shifts which was corresponding to more than 53,000 CV applied to the column.

The results of IgG control are shown in Figure 4. Immobilised rSPA silica monolith provided constant analysis of IgG during the complete stability test remaining retention time, peak area and peak width nearly unchanged. The RSD of IgG retention time was smaller than 0.5% whereas for the peak area of eluted IgG the RSD was below 1.1% indicating no loss of binding capacity.

Conclusions

Recombinant protein A was covalently attached to the surface of silica monoliths with larger mesopores suited for biomolecules. The immobilised rSPA column was successfully utilised in the separation of monoclonal antibodies by affinity chromatography. The applied method for immobilisation yielded to a high dynamic binding capacity leading to broad range of applicable antibody concentrations. Furthermore, stability test revealed a strong linkage between silica monolith and rSPA ligand due to the constant analysis of monoclonal antibodies. In conclusion, silica monoliths with bimodal pore structure immobilised with rSPA ligand are ideally suited for the chromatographic separation of monoclonal antibodies.

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