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Full Paper

Synthesis of strong cation exchange macroporous polymer cluster for convective protein chromatography

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Alkyne-terminated polyelectrolytic brushes based on 3-sulfopropyl methacrylate are synthesized by reversible-addition fragmentation chain transfer (RAFT) and subsequently linked to macroporous polymeric microclusters by Cu(I)-catalyzed azide alkyne cycloaddition. It is shown that a column packed with the obtained materials enables convective cation-exchange chromatography of proteins, exhibiting flow rate independent resolution and dynamic binding capacity (8 mg mL^{-1}). These flow characteristics are similar to those of monoliths and membranes and therefore excels the mass transport of existing chromatography resins used in the downstream process of therapeutic proteins, while the positive aspects of chromatographic beads are maintained facilitating implementation in well-developed platform technologies.

1. Introduction

The number of approved therapeutic proteins, e.g. monoclonal antibodies, is continually increasing, and thus these biopharmaceuticals have become the fastest growing sector in the pharmaceutical market.[1-2] In recent years, also the first biosimilar products have penetrated the market, and given many first-generation biopharmaceutical patents due to expire, this trend is expected to continue, demanding for more productive manufacturing.[3] So far, tremendous developments in cell culture and cell line engineering result to titers above 20 g L^{-1} and masses of 100 kg per batch.[3-6] On the other hand, this enhancement in fermentation technology causes limitations in the following downstream process (DSP), which have become the bottleneck in protein production. Despite the low productivities due to mass transfer limitations, chromatography is still the favored technique in protein purification owing to better resolutions compared with other technologies.[7] In protein chromatography, high-efficiency media with small bead size are used to remove contaminants and structural variants of the target molecule.[3] However, these small beads result in higher back-pressures impeding the separation at high flow rates. Conversely, the usage of bigger particles results in longer diffusion pathways and consequently to severe mass transfer limitations owing to the small pore sizes (~ 150 nm) of currently used chromatographic media. In this respect the pore radius should be at least eight times larger than the hydrodynamic radius of the molecule of relevance to avoid excessive diffusional hindrance.[8] While this condition is fulfilled for monoclonal antibodies, this is not the case anymore for larger therapeutic modalities such as pDNA or virus-like-particles.

Convective chromatography resins, as previously reported by our group for a protein A affinity resin, decouple the trade-off between pressure drop and mass transfer resistance.[9] These resins are macroporous and combine similar flow characteristics as known from monoliths and adsorptive membranes with advantages of porous chromatographic beads such as packability and scalability in existing platform technologies. Notably, these convective resins must not be mistaken with perfusive materials that still offer smaller diffusive pores, which are virtually

inexistent in convective resins. Although the applicability of convective resins has been shown for affinity chromatography, the concept has not been transferred to other types of chromatography yet. Therefore, the synthesis of a convective strong cation-exchanger based on poly(3-sulfopropyl methacrylate) brushes that enables protein separation with flow rate independent resolution and binding capacity is the motivation of this work.

2. Experimental Section

2.1 Materials

4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid (CPA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 4-(dimethylamino)pyridine (DMAP), propargyl alcohol, 3-sulfopropyl methacrylate (SPMAK), 4,4′-azobis(cyanovaleric acid) (ACVA), copper bromide, copper sulfate, *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA), 2,2′-Bipyridine (BiPy), Tris[2-(dimethylamino)ethyl]amine (Me_6TREN), sodium ascorbate, potassium bromide, sodium dihydrogen phosphate, sodium hydrogen phosphate, chymotrypsinogen A, cytochrome C, and lysozyme were purchased from Sigma Aldrich. Sodium chloride, dichloromethane, and dimethylformamide were purchased from Merck KGaA.

2.2 Polymer Synthesis

2.1.1. Synthesis of azide-containing macroporous microclusters

The macroporous microclusters were produced by reactive gelation from azide-containing polymeric core-shell nanoparticles as previously reported.[10]

2.1.2. RAFT-polymerization of 3-sulfopropyl methacrylate

First, the alkyne-containing RAFT agent was synthesized. In a 100 mL round flask a solution of CPA (499.2 mg, 1.79 mmol, 1 eq) and propargyl alcohol (0.4 mL, 6.9 mmol, 4 eq) in 20 mL of DCM was stirred and cooled to 0 °C. After 1h, EDC (1.021g, 5.3 mmol, 3 eq) and DMAP (217 mg, 1.77 mmol, 1 eq) were added and the mixture was stirred for 24 h at room temperature. The solution was washed and extracted with water (2× 50 mL) and DCM (2× 50 mL), dried over magnesium sulphate, vacuum filtered, and the solvent removed to give a red oil. The oil was purified by liquid chromatography, with 42 g silica gel and a mobile phase of 6:1 hexane/ethyl acetate in a column with pore size of 2 and a diameter of 32 mm (the product can easily be identified

in the column as the darkest red fraction). The yield was 250 mg (44 %). Afterwards, the RAFT-polymerization of 3-sulfopropyl methacrylate was done. In a 100 mL two-necked round flask a solution of (234.5 mg, 0.74 mmol, 1 eq), SPMAK (1.19 g, 4.83 mmol, 6.5 eq), DMF (4.6 g), and phosphate buffer (20 mM, pH 7) (8.4 g) was heated to 65°C under nitrogen flow and constant stirring. After 1 h ACVA (60 mg, 0.21 mmol) was added. The reaction mixture was stirred for 24 h and a brown solid obtained. The product was washed with acetone and DMF, dried, and mortared resulting in a brown powder. This was, again, washed with DMF and acetone and dried. Yield: 1.42 g.

2.1.3. Linkage of brushes by click chemistry

A mixture of the polymeric brush (B1, 201 mg), polymeric microcluster (131 mg), DMF (20 mL), and water (100 mL) was stirred for 1h in a 100 mL two-neck round flask under nitrogen atmosphere at 70 °C. Then, a solution of the copper salt (101 mg) and sodium ascorbate (403 mg) in water (2 mL) was added and stirred for 72 h.

The inhomogeneous reaction mixture was filtrated and exhaustively washed with water and acetone to remove the catalyst and residual brushes. After drying, a greyish solid was obtained.

2.3 Methods

The polymeric materials were slurry packed into a GE Tricorn column of 50 mm length and 5 mm inner diameter with a flow rate up to 9 mL min^{-1}. The yield of the click reaction was determined by FT-IR spectroscopy by analyzing the characteristic asymmetric azide stretch at 2095 cm^{-1}, which disappears after the 1,3-dipolar cycloaddition yielding a triazole ring. Since the aromatic C-H stretch remains unaffected by the cycloaddition it can be used as an internal reference for semi-quantitative yield determination. To do this, the sample (5 wt%) was mixed with KBr and analyzed using a Tensor 27 spectrometer (Bruker Optics). The degree of polymerization and the conversion were determined by ^{1}H-NMR using a 300 MHz Spectrometer (Bruker). HPLC experiments were

carried out on an Agilent 1100 series HPLC, equipped with an auto-sampler, a column thermostat, a diode array detector, an online degasser and a quaternary pump. The model protein mixture consisted of chymotrypsinogen A, cytochrome C and lysozyme (each 1 g L^{-1}). The clarified monoclonal antibody was expressed and purified inhouse (1 g L^{-1}). Linear gradient elution experiments were carried out at flow rates of 0.5 mL min^{-1} and 1.0 mL min^{-1} with a gradient of 0 % B to 100 % B (1 M NaCl, 25 mM phosphate) lasting for either 10 CV or 30 CV. Different pH values were investigated. Upon sample injection (50 µL), the column was washed with buffer A (25 mM phosphate at the respective pH) and the gradient started. Binding capacities were determined by feeding a lysozyme solution (1 g L^{-1} in 20 mM phosphate, pH 7.0) at 0.5 mL min^{-1} and 1.0 mL min^{-1} until achieving a constant UV signal at the column outlet. Dynamic binding capacities were determined at 10 % breakthrough based on the UV signal at 280 nm. Static binding capacities were evaluated as the area between the final constant UV signal and the breakthrough curve. The dead volume of system and column were taken into account.

Samples were analyzed by GPC using a Merck-Hitachi LaChrom system, consisting of a Merck-Hitachi LaChrom L-7100 HPLC pump, a LaChrom L-7360 column oven, a LaChrom L-7400 UV detector, and a LaChrom L-7490 RI detector.

3. Results and Discussion

3.1. Synthesis of polyelectrolytic brushes by RAFT-Polymerization

3.1.1. Synthesis of clickable RAFT-agent

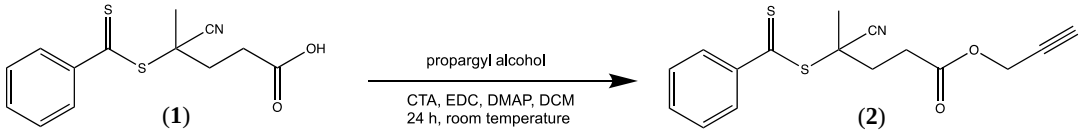

Figure 1: synthesis of the clickable RAFT-agent.

An alkyne-containing RAFT agent was synthesized from 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid **(1)** via Steglich-esterification with propargyl alcohol using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent and 4-dimethylaminopyridine (DMAP) base, giving (**2**) as a red oil with a yield of 44 % upon purification.

3.1.2. RAFT-polymerization of 3-Sulfopropyl methacrylate

Using (**2**) as chain transfer agent, several polyelectrolytic brushes have been synthesized by RAFT-polymerization of 3-sulfopropyl methacrylate. Table 1 shows the obtained degree of polymerization (DP) of the synthesized brushes, which equals the net charge of the single brush. It is also apparent that the brushes are considerably longer than the theoretical values, which is attributed to the low initiator efficiency of 4,4′-Azobis(4-cyanovaleric acid) (ACVA), which is only about 33 % as calculated using Equation 1, where $[M]_0$ and $[RAFT]_o$ are the initial concentration of monomer and chain transfer agent, p represents the monomer conversion, and M_M and M_{RAFT} are the molecular weights of the monomer and the chain transfer agent, respectively.

$$M_{\mathrm{th}} = \frac{[M]_0 p M_M}{[RAFT]_o} + M_{RAFT}$$

Table 1: Overview of brush-length and molecular weight of synthesized brushes

	$\frac{[M]}{[RAFT]}$	$\frac{[RAFT]}{[I]}$	$\frac{[M]}{[I]}$	DP*	χ	M_{th}	M_{NMR}	M_{GPC}	PDI
B-1	3	3	9	9	99	744	2231	3380	1.31
B-2	6	3	18	17	99	1487	4214	4290	1.27
B-3	12	3	36	37	99	2975	9172	8010	1.12

* The degree of polymerization was determined by ^{1}H-NMR

Figure 2 shows the dependency of the degree of polymerization on the monomer conversion, which is, as expected, linearly increasing with the monomer consumption, proving the livingness of the polymerization. Although in all cases the polymerization was run until full conversion, the polydispersity index (PDI) remained considerably small, ranging from 1.12 (longest brush) to 1.31 for the shortest brush, indicating that a very small fraction of dead chains was produced.

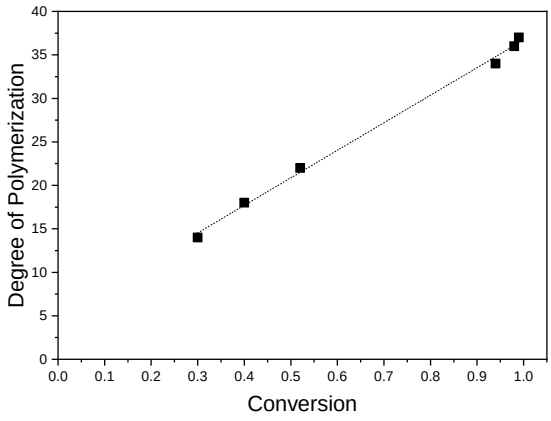

Figure 2: Dependency of the degree of polymerization on monomer conversion.

3.2. Linkage of polyelectrolytic brushes to macroporous microclusters via click chemistry

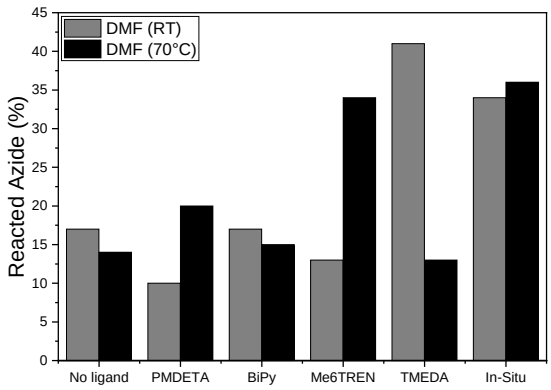

Figure 3: Screening of different Lewis bases in combination with CuBr and the comparison with in situ generation of Cu(I) using copper sulfate and sodium ascorbate (and no additional Lewis base).

Even if many transition metals (e.g. ruthenium[11], silver[12]) have been reported to catalyze the formation of metal-acetylide complexes, the usage of Cu(I) remains the gold standard in azide-alkyne cycloaddition. Since Cu(I) is a Lewis acid exhibiting an intermediate softness, it can partner with a variety of ligands.[13-14] Thereby, it should be noted that the primary roles of the ligands are the prevention of the formation of polynuclear copper complexes and the enhancement of the solubility of such complexes. However, as shown in Figure 3, where CuBr was tested with four different amine ligands, the usage of ligands revealed in three out of four examples similar click yields as the reaction without any ligand usage. Only Me_6TREN for reactions at room temperature and TMEDA for 70 °C resulted in increased click yields, but a generalization that the usage of ligands can facilitate the reaction cannot be made. The *in situ* generation of the Cu(I) source shows the highest yield, both at room temperature and 70 °C. Given that Cu-complexes are very electron rich and therefore susceptible to oxidation, the additional reducing agent (sodium ascorbate), which was used for the *in situ* generation of Cu(I), led to the highest yields. This indicates that the reaction rate for the click step to a solid support is too low to achieve maximum conversion prior Cu(I) oxidation.

3.3. Preparation of chromatographic materials for protein separation

This section deals with the fabrication of a chromatographic column packed with a macroporous polymer that bears negatively charged poly(3-sulfopropyl methacrylate) brushes on its surface. As depicted in Figure 4, two pathways were followed. In the first example (C-1), the polyelectrolytic brushes were clicked on the azide-containing porous polymer scaffold in bulk and subsequently packed in the column. In the second example (C-2) the execution was carried out vice-versa, meaning the azide-containing polymeric microclusters were packed in a chromatography column and afterwards the click reaction was conducted within the column by circulating a mixture of 3-sulfopropyl methacrylate, $CuSO_4$, sodium ascorbate, and DMF through it. Even if a higher yield for the click reaction was attained following the first route, it was found that the packing in the column was extremely slow and also revealed high pressure drops (4-5 bar at 2.5 mL min^{-1}), potentially caused by copper residues that were trapped within the polymer. On the other hand, the pristine azide-containing microclusters could easily be packed at 9 mL min^{-1} showing a marginal pressure increase of only 2-3 bar. Moreover, the

copper removal in a column is rather convenient as it only requires the constant flow of a saturated ethylenediaminetetraacetic acid (EDTA) solution for 24 hours.

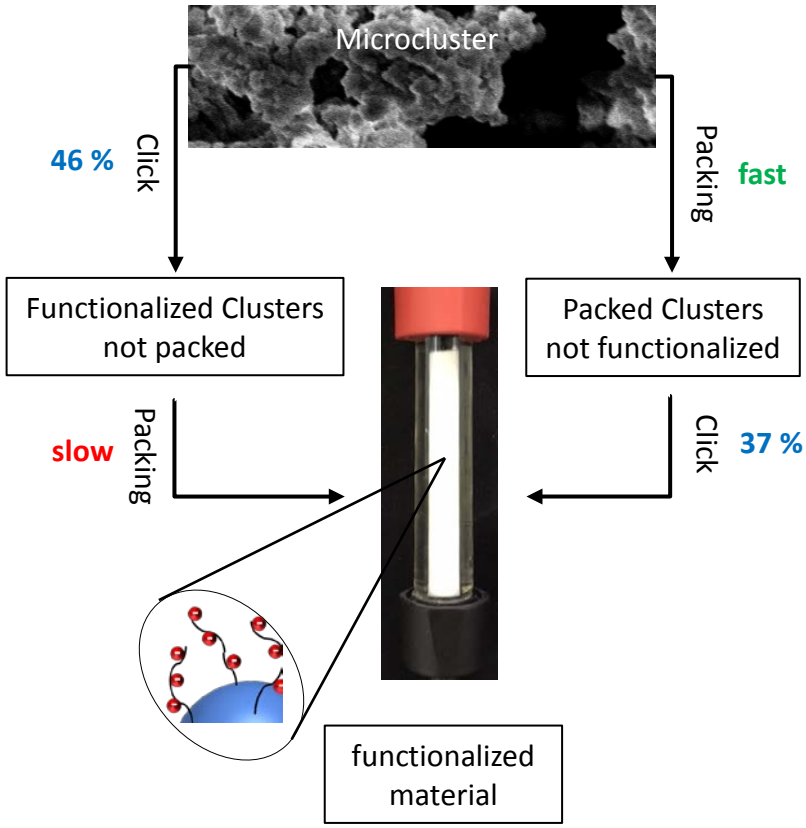

Figure 4: Sketch of the two procedures applied to fabricate a chromatographic column packed with a macroporous material bearing negatively charged brushes on its surface.

3.4. Protein Chromatography

3.4.1. Click in bulk vs. click inside the column

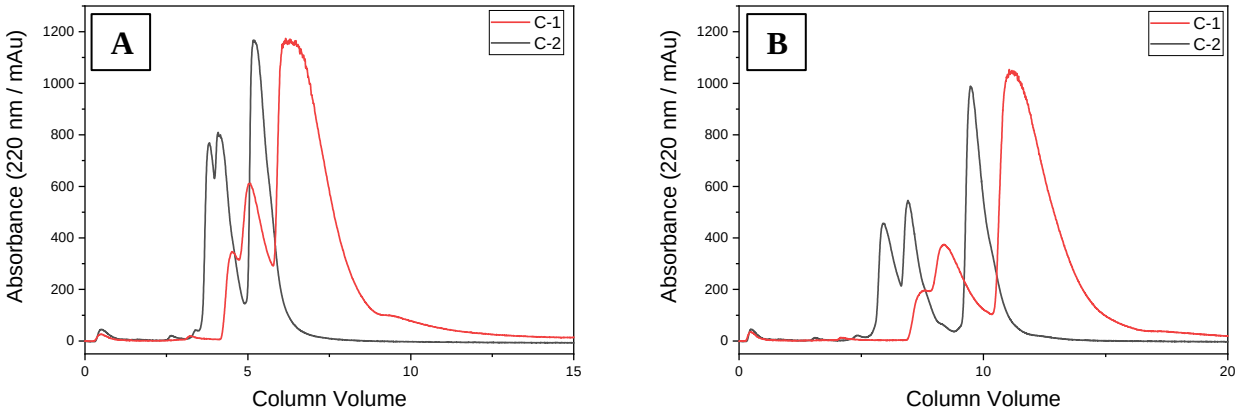

Figure 5: Separation of a protein mixture containing chymotrypsinogen-A, cytochrome C, and lysozyme using a chromatography column packed with materials produced by bulk functionalization (C-1) and by click chemistry inside a column (C-2). The column volumes are corrected to account for the injection time and post-injection wash times (i.e. 0 CV is the gradient start time). A) 10 CV gradient and B) 30 CV gradient, with a flow rate of 0.5 mL/min for both figures.

Figure 5 shows the chromatographic performance of the two produced columns regarding the separation of three different model proteins (chymotrypsinogen A, cytochrome C, and lysozyme). The ion-exchange chromatography was carried out at pH 8 and a flow rate of 0.5 mL min^{-1} with a gradient over 10 CV or 30 CV. The chromatograms that are depicted in Figure 5-A and Figure 5-B reveal that a better resolution is achieved with the column where the functionalization via click chemistry was conducted inside the packed bed. In Figure 5-B the gradient was increased to 30 CV compared to Figure 5-A (10 CV), which resulted, as expected, in a better resolution. In the case of lysozyme, the strongest retained protein, baseline separation with respect to cytochrome C could be achieved revealing a resolution R_S of 1.7. However, the asymmetry factors (As) which were calculated for lysozyme revealed strong tailing, as summarized in Table 2.

Table 2: Overview of the chromatographic parameters obtained using C-1 and C-2 with different gradients (10 CV and 30 CV). PI represents the isoelectric point of the investigated proteins, t_R the retention time, R_S the resolution, and As the asymmetry factor.

		PI	C-1 t_R [min]	C-1 R_S	C-1 As	C-2 t_R [min]	C-2 R_S	C-2 As
Chymotrypsinogen A	10 CV gradient	8.5	10.1	-	-	8.7	-	-
Cytochrome C		10.0	11.2	0.3	-	9.2	0.3	-
Lysozyme		11.0	13.4	0.5	4.9	11.4	0.9	4.5
Chymotrypsinogen A	30 CV gradient	8.5	17.0	-	-	12.9	-	-
Cytochrome C		10.0	18.0	-	-	14.9	0.6	-
Lysozyme		11.0	23.7	0.8	7.9	20	1.7	2.9

3.4.2. Screening of chromatography conditions

In the next set of experiments, the pH influence was investigated towards the separation quality of C-2 regarding the separation of the model protein mixture. The experiments were carried out at pH 5, pH 6, pH 7, and pH 8 and a flow rate of 0.5 mL min^{-1} with a 10 CV gradient. As shown in Figure 6, it is apparent that the resolution improves with increasing pH value as the ratio of

the differences between the elution pH and the proteins' pI increase. Furthermore, the lower net charge at higher pH results in weaker interactions as proven by the earlier elution.

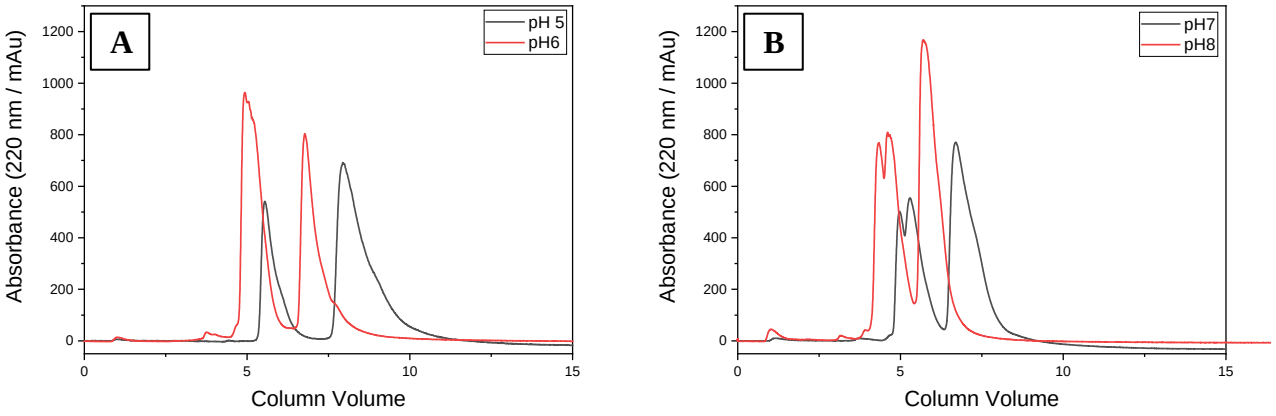

Figure 6: Separation of a protein mixture containing chymotripsinogen-A, cytochrome C, and lysozyme at different pH values (A: pH = 5 and 6, B: pH = 7 and 8) using a chromatography column packed with materials produced by click chemistry inside a column (C-2). The column volumes are corrected to account for the injection time and post-injection wash times (i.e. 0 CV is the gradient start time). The flow rate is 0.5 mL/min in all cases.

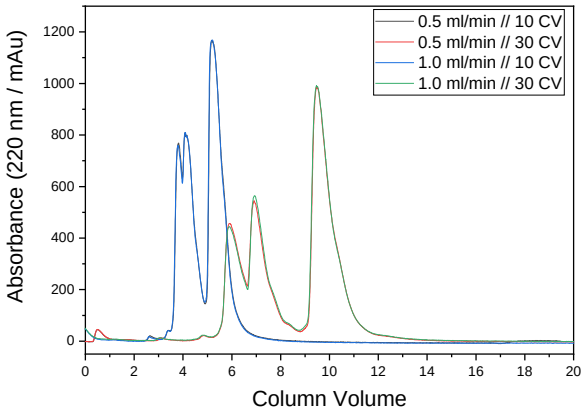

Figure 7: Chromatogram of a protein mixture of chymotripsinogen A, cytochrom C, lysozyme separated using C-2 at 0.5 mL/min and 1.0 mL/min with two different salt gradients (1 M NaCl, 10 CV and 30 CV). The column volumes are corrected to account for the injection time and post-injection wash times (i.e. 0 CV is the gradient start time)

Figure 7 reveals that the resolution is independent of the flow rate, indicating a convective mass transfer behavior of the macroporous base material for two different gradients (1 M NaCl, 10 CV and 30 CV). These findings are similar to those reported in our previous work, in which the same base material was used to make a convective protein A chromatography material, however evaluated only with mAb breakthrough curves[9]. The ease with which the high-throughput base material can be tailored gives rise to applications covering all types of liquid chromatography including, but not limited to strong cation exchangers. However, one should

note that, owing to the macroporosity, the active surface of the used material is low (11.4 $m^2 g^{-1}$)[9] resulting in low binding capacities. Indeed, the binding capacities at 10 % breakthrough of the presented strong cation exchanger were found to be 8.6 mg mL^{-1} at a flow rate of 0.5 mL min^{-1} and 8.4 mg mL^{-1} at 1.0 mL min^{-1} (Table 3).

Table 3: Dynamic binding capacities at 10 % breakthrough and static binding capacities towards Lysozyme at two different flow rates (0.5 mL min^{-1} and 1.0 mL min^{-1}).

	Flow rate [mL min^{-1}]	$DBC_{10\%}$	SBC
Lysozyme	0.5	8.6	10.2
	1.0	8.4	9.9

3.4.3. Antibody loading

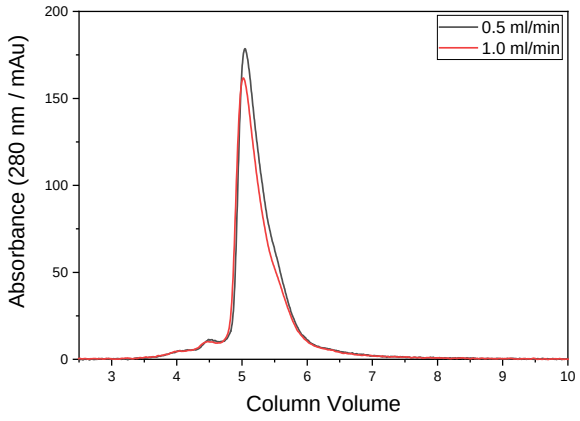

Figure 6: Loading experiment of a monoclonal antibody at two different velocities.

Finally, a monoclonal antibody was loaded and eluted from the column at 0.5 mL min^{-1} and 1.0 mL min^{-1}. Again, it is shown in Figure 8 that the chromatographic performance of the produced material is independent of the flow rate owing to the convective mass transport. Interestingly, a shoulder can be seen at the onset of the peak, which might indicate the presence of charge variants of the injected mAbs. However, further testing would be necessary to prove the feasibility to separate charge isoforms.

4. Conclusions

In here, the production of convective strong cation exchange chromatography resins is presented. The negatively charged polyelectrolytic brushes were synthesized by RAFT-polymerization of 3-Sulfopropyl methacrylate at high conversion and with low PDI using 4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid propargyl ester as a transfer agent. Subsequently, the attained brushes (DP = 9) were linked to azide-containing macroporous base materials by Cu(I)-catalyzed azide-alkyne cycloaddition. It was found that the *in situ* generation of Cu(I) gave the highest yields both at room temperature and 70 °C, whereas the usage of CuBr in combination with lewis bases did not lead to such high yields. Two pathways were followed to produce packed bed columns for protein chromatography. In the first endeavor, the click reaction was done in bulk and the material was subsequently packed in a chromatography column. In a different route, the pristine, unfunctionalized material was packed in the column prior to the functionalization revealing a better overall packability but lower yields for the click reaction compared to route one. Owing to the better packing the second route proved to give better chromatograms concerning resolution and peak asymmetry and was investigated towards the separation of a protein mixture containing chymotrypsinogen A, cytochrome C, and lysozyme. It was proven that the chromatographic performance is independent of the flow rate owing to the macroporosity of the used chromatographic base material that allows a convective mass transport throughout the packed bed. It needs to be noted that the achieved binding capacities towards lysozyme are still too low (8 mg mL^{-1}) and further improvement is necessary. Finally, the loading of monoclonal antibodies was also performed, revealing a shoulder at the onset of the peak which could indicate the presence of charged isoforms of the used antibody.

Keywords: convective chromatography, reactive gelation, click chemistry, protein chromatography

5. References

1. Walsh, G., Biopharmaceutical benchmarks 2018. *Nat Biotechnol* **2018,** *36* (12), 1136-1145.
2. R., O.; Santagonisto, A.; Schrader, U., Rapid growth in biopharma: challenges and opportunities. *McKinsey Co.* **2017**.
3. Milne, J. J., Scale-Up of Protein Purification: Downstream Processing Issues. *Walls D., Loughran S. (eds) Protein Chromatography. Methods in Molecular Biology, Humana Press, New York, NY* **2017,** *1485*.
4. Zouwenga, R.; D'Avino, A.; Zijlstra, G., Improving productivity in bioreactors. *Genetic Engineering and Biotechnology News* **2010**.
5. Shukla, A. W., L.; Mostafa, S.; Norman, C., Evolving trends in mAb production processes *Bioengineering and Translational Medicine* **2017,** *2* (1), 58-69.
6. P. Gronemeyer, R. D., J. Strube, Trends in Upstream and Downstream Process Development for Antibody Manufacturing. *Bioengineering* **2014,** *1*, 188-212.
7. Curling, J.; Gottschalk, U., Process chromatography: Five decades of innovation. *Biopharm Int* **2007,** *20* (10), 70-+.
8. Carta, G.; Jungbauer, A., Protein Chromatrography - Process Development and Scale-Up. *Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim* **2010**.
9. Lorenz, M.; Paganini, C.; Vogg, S.; Storti, G.; Morbidelli, M., Convective Protein A resin for high-throughput capture of monoclonal antibodies. *to be submitted* **2018**.
10. Lorenz, M. S., C; Finkelstein, P.; Cingolani, A.; Storti, G; Morbidelli, M, Template-free synthesis of porous polymeric microclusters from core-shell nanoparticles by reactive gelation and their post-functionalization via click chemistry. *submitted*.
11. Johansson, J. R.; Beke-Somfai, T.; Stalsmeden, A. S.; Kann, N., Ruthenium-Catalyzed Azide Alkyne Cycloaddition Reaction: Scope, Mechanism, and Applications. *Chem Rev* **2016,** *116* (23), 14726-14768.
12. Fang, G. C.; Bi, X. H., Silver-catalysed reactions of alkynes: recent advances. *Chem Soc Rev* **2015,** *44* (22), 8124-8173.
13. Hein, J. E.; Fokin, V. V., Copper-catalyzed azide-alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(I) acetylides. *Chem Soc Rev* **2010,** *39* (4), 1302-1315.
14. Hathaway, B. F., Comprehensive Coordination Chemistry. *Wilkinson G., editor, Pergamon, Oxford* **1987,** *5*.