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Downstream processing of high chain length polysialic acid using membrane adsorbers and clay minerals for application in tissue engineering

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Abbreviations

BDM, dry biomass; DMB, 1,2-diamino-4,5-methylenedioxybenzene; CA, commercially available colominic acid; DP, degree of polymerization; E, eluent; em, emission; ex, extinction; FD, fluorescence detection; polySia, polysialic acid; MWCO, molecular weight cut-off; polySia, polysialic acid; S.E., Standard Error; Q-membrane, quarternary ammonium anion exchange;

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Abstract

Polysialic acid (polySia) is a carbohydrate polymer of varying chain length. It is a promising scaffold material for tissue engineering. In this work, high chain length polySia was produced by an *E. coli* K1 strain in a 10 L bioreactor in batch- and fed-batch mode, respectively. A new downstream process for polySia is presented, based on membrane adsorber technology and use of inorganic anion exchanger. These methods enable the replacement of precipitation steps, such as acetone-, cetavlon- and ethanol-precipitation of the already established purification process. The purification process was simplified, while process efficiency and product qualities were improved. The overall yield of polySia from a 10 L batch cultivation process was 61 % and for 10 L fed-batch cultivation process 40 % with an overall purity of 98 %. The endotoxin content was determined to be negligible (14 EU mg^{-1}). The main advantage of this new downstream process is that polySia with high chain length > 130 DP (degree of polymerization) can be obtained. In fed-batch cultivation chain lengths up to 160 DP were obtained.

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1. Introduction

For the reconstruction of biological tissue, the molecular structure as well as the stability of the carrier material is of utmost importance for the cell-growth. The application of materials in regenerative medicine involves degradation of synthetically produced material without any toxic by-products. A promising scaffold material for this purpose is polysialic acid (polySia), a homopolymer of α2,8-linked sialic acid residues, which is a posttranslational modification to the neural cells existing in all vertebrate species. Some neuroinvasive bacteria like *Escherichia coli* K1 (*E. coli* K1) are encased in a capsule containing polySia, which is similar in structure to that found in human organism.

PolySia is a polymer consisting of monomers of sialic acids, linked by glycosidic bonds. The polymer shows up to 200 units [1]. Polysialic acids have a three-dimensional molecular structure, whose formation is primarily initiated by the negative charge of the carboxyl groups. The polymer forms a helix in which one turn consists of nine monomer units [2]. PolySia is present in lower prokaryotic as well as in higher vertebrates like humans [3]. Polysialic acid usually forms the basic structure in the cell membranes embedded glycoproteins or –lipids [4–6]. In prokaryotes such as *Escherichia coli* K1 and *Neisseria* meningitides, polySia is forming an extra cell envelope [2]. Only 20-50% of the polySia is bound covalently to the cell wall. The remaining part is bound on basis of ionic interactions. In eukaryotes polySia is bound to glycoproteins via O-and N-glycan.

Previously reported purification methods are generally based on precipitation reactions [1, 7, 8] or chromatographic techniques [9].
In the recent literature, the purifications yield is reported to be between 20 % to 56 % [7, 8] for precipitation-based purification of polySia. Other methods include multiple chromatographic steps with high acquisition costs [9]. Here, the yields are in the range of 55-60 %. All these common purification strategies offer several disadvantages as they are expensive, time consuming and include numerous complicated single purifications steps. Only few of them are Compatible for large scale downstream processing.
The main focus of this work is the purification of high molecular weight polySia from *E. coli* high cell density cultivations (fed-batch cultivations). In this paper we present a newly

developed simple purification method based on unit operations such as membrane adsorber technique and cheaper single use technology such as inorganic cation exchange material [10, 11]. This method was established for the pilot scale (10 L) and is very suitable for the production of long chain polySia.

2. Materials and methods

2.1. Material

2.1.1. Bacterial strains

The wild type strain *E. coli* B2032/82 serotype K1 [7] is an original clinical isolate.

2.2 Chemicals and growth media

All bulk chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany). Deionized water was prepared with ARIUM (Sartorius Stedim Biotech, Göttingen, Germany). For the biorector cultivation we have used a basic defined medium [1]. The composition of the batch and feeding media are given in Table 1.

2.3. Cultivation of E.coli K1

2.3.1. Shake flask cultivation and preculture

Precultures were performed in 500 mL shake flasks with 100 mL medium (consisting of yeast extract (10 g L^{-1}), tryptone (10 g L^{-1}) and NaCl (5 g L^{-1})). The bacteria were incubated on a rotary-shaker at 37 ◦C, 130 min^{-1} for 8 –10 h. This cell-suspension was used as inoculum for all bioreactor cultivations. Inoculation volume was 1% of total cultivation volume.

2.3.2. Bioreactor cultivation

The cultivations were carried out in a 10 L stainless steel bioreactor (Biostat C, Sartorius Stedim Biotech, Göttingen, Germany). The temperature was maintained at 37 °C. The pH was kept at 7.5 with 25 % (w v^{-1}) NH_4OH. Control of temperature and pH was carried out by the digital control unit (DCU, Sartorius Stedim Biotech, Göttingen, Germany) of the bioreactor.

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In the batch mode, the air flow rate was 10 L min^{-1} and the stirrer speed was 1,000 min $^{-1}$. The initial glucose concentration in the batch cultivation was 20 g L^{-1}.

In the fed-batch mode, the initial culture conditions were as follows: initial culture volume = 8 L, air flow rate = 8 L min^{-1}, stirrer speed = 1,000 min^{-1}. The fed-batch cultivation was started with an initial glucose concentration of 10 g L^{-1}. After consumption of the initial glucose, indicated by an increase of the dissolved oxygen concentration, the fed-batch phase was started. The dissolved oxygen was maintained at 30% of air saturation by increasing the stirrer speed from 150 to 1,250 min $^{-1}$ and aeration from 1.5 L min $^{-1}$ to 10 L min $^{-1}$. To control the fed-batch cultivation, a process model based on the Monod model with limited substrate and for an ideal stirred tank bioreactor was used (Eq. 1) [12]. This differential equation system describes the cell growth in a fed-batch stirred tank reactor.

$$\frac{dS}{dt} = -\frac{\mu_{\max} S}{K_M + S}\frac{X(t)}{Y_{\frac{X}{S}}} - \frac{\dot{V}_F(t)}{V_R(t)}(S_0 - S)$$ Eq.1

In glucose limited fed-batch cultivations, the substrate concentration inside the bioreactor is assumed to be constant i.e. $\frac{dS}{dt} = \mathbf{0}$. Therefore from equation 1 the feed rate at time t can be derived as:

$$\dot{V}_F(t) = \frac{\mu_{\max} S}{K_M + S}\frac{X(t)}{Y_{\frac{X}{S}}}\frac{1}{S_0 - S}V_R(t)$$ Eq. 2

$X(t)$ is the concentration of biomass at time t (g L^{-1}); S is the concentration of substrate (glucose) (g L^{-1}); μ_{max} is the maximum specific growth rate (h^{-1}); K_M is the Monod substrate saturation constant (g L^{-1}); V_F (t) is the feed rate at time t (L h^{-1}); $V_R(t)$ is the actual volume at time t (L); S_0 is the substrate concentration of feed solution (g L^{-1}) and $Y_{X/S}$ is the yield coefficient (g g^{-1}).

A constant specific growth rate was adjusted during the fed-batch phase. The programme used for controlling the feeding is known as Neu-ork (Institute for Technical Chemistry, University of Hannover), executed under MS-DOS [12]. The initial values for the Neu-ork program were obtained from previous cultivations (data not shown). Thel values for $X(t_0)$, V_R (t_0), $S(t_0)$ and S_0 are 7.2 g L^{-1}, 8 L, 0.1 g L^{-1} and 200 g L^{-1} respectively. The values obtained from previous

cultivation for $Y_{X/S}$, and K_M are 0.5 g g^{-1} and 0.01 g L^{-1}, whereas the growth rate was set to 0.3 h^{-1}.

2,4 Downstream processing

2.4.1 Purfication via precipitation steps

After harvesting, separation of biomass was achieved by cross-flow microfiltration (0,45 µm, Sartorius Stedim Biotech, Göttingen, Germany) Afterwards, the supernatant was concentrated 20-fold by cross-flow ultrafiltration using membrane with a MWCO of 10 kDa (HydroSart, Sartorius Stedim Biotech, Göttingen, Germany).
Acetone (45 % v v^{-1}) was added to the retentate of the ultrafiltration at 8 °C for 24 h. The acetone was then removed from the supernatant by vacuum distillation (150 mbar) at 40 °C and the polymer was precipitated in a second step by addition of cetavlon to a final concentration of 10 g L^{-1}. After 24 h at 8 °C the brown precipitates was centrifuged (4,000 min^{-1}, 15 min, 4 °C) and dissolved in 1 M NaCl. Afterwards the product was precipitated with ethanol (80 % v v^{-1}). The white precipitate was dissolved in water and dialysed against 5 L NaCl-solution (1 g L^{-1}) for one day and 5 L deionized water (both pH 9) for two days using Visking dialysis membranes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with a MWCO of 10 kDa. Finally, the product was recovered by freeze-drying (Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) at 1 mbar.

2.4.2 Determination of polySia and protein binding carpacity of Q-membran

Experiments were performed with quarternary ammonium Q anion exchange adsorber units, in thr following stted as Q-membrane (Sartobind Q 100 cm², Sartorius Stedim Biotech, Göttingen, Germany). Therefore, a Q-membrane was connected to a FIA-Station (Flow Injection Analysis). For the determination of the binding capacities polySia samples were diluted with the following buffers (1:1): 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7; sodium phosphate (NaPB), pH 7; 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), pH 8 and pH 9; N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) pH 10. Each buffer was applied in three different concentrations (50 mM, 100 mM and 200 mM). After loading (20 mL polySia-sample) and washing (10 mL buffer), the elution was performed with

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1 M NaCl (10 mL). The flow rate during the loading, washing and eluting process was kept at 3 mL min^{-1}.

2.4.3 pH-dependence of the adsorption of proteins and polySia

The clay mineral EX M 1753 (experimental product name 1753, Südchemie, Moosburg, Germany) 40 mg was suspended in 4 mL buffers solution (see below) and equilibrated to the adequate pH by shaking in buffer (different concentration) for 1 h at room temperature. After centrifugation for 10 min at 4,000g the supernatant was discarded. The clay mineral was then suspended in 1 mL concentrated *E. coli* culture supernatant (Retentate of the 10 kDa ultrafiltration) of a polySia-sample. For adsorption, the clay mineral was shaken with polySia retentate for 0.5-3 h at room temperature. After centrifugation (10 min at 4,000g), supernatant was tested for protein and polySia concentration. The pH was adjusted with the following buffers: pH 4 and pH 5 with acetate, pH 6 with MES, pH 7 with HEPES and NaPB , pH 8 and 9 with Tris and pH 10 with CAPS. Three different buffer concentrations (50 mM, 100 mM, 200 mM) were used.

2.4.3 Pilot scale purification

After cross-flow microfiltration (pore size: 0,45 µm) the optimized parameters gained from 2.4.2 and 2.4.3 for the purification process were applied for the pilot scale.10 L culture supernatant from the bioprocess was diluted with NaPB (100 mM, pH 7) 1:1 (total volume of 20 L) and then was transferred on the Q-membrane (Sartobind® SingleSep 5, membrane area 2,500 cm^2 Sartorius Stedim Biotech, Göttingen, Germany). The elution was performed using 1M NaCl solution. The membrane purification was performed in a pilot plant (see supporting information, [13]).

The pilot plant consists of a loop (black arrows), whereby the membrane can be loaded with the sample in a cycle. In addition, the system has a linear pumping system (gray arrow), whereas the membrane can be flushed with wash- or elution- solution. The programmable pump control valves can switch automatically between the cycle and the linear pumping system. To determine the protein and polySia concentration samples were measured offline colorimetrically.

The complete downstream cycle can be divided into the loading step (10 min, flow rate 6 L min^{-1}), washing step (100 mM NaPB, 2 min, flow rate 2 L min^{-1}), eluting step (1 M NaCl,

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2 min, flow rate 2 L min^{-1}) and washing step (100 mM NaPB, 2 min, flow rate 2 L min^{-1}). For 20 L supernatant four (batch) or seven (fed-batch) process cycles were required.

Then, the purified culture eluat was concentrated and desalted by cross-flow ultrafiltration (cut off 10 kDa) to 200 mL. This was followed by the adsorption of proteins from the retentate (200 mL) with EX M 1753 (8 g). For a complete removal of the buffer components, the sample was dialysed for one day against 5 L NaCl-solution (1 g l^{-1}) followed by two days dialysis against 5 L deionized water (both pH 9) using Visking dialysis membranes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with a MWCO of 10 kDa. At the end, polySia was recovered from the aqueous phase by freeze-drying (Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) at 1 mbar.

2.5. Assays and methods

2.5.1. Analysis of polySia content

The amount of polySia in the samples was determined colorimetrically using a modified thiobarbituric acid assay as reported previously [7]. The sample volume was 50 µL with a total amount of 1.5–50 µg polySia. In a first step the polySia was hydrolyzed by adding 200 µL 50 mM H_3PO_4 and incubating the solution for 18 h at 70 °C followed by addition of 100 µL 0.1 M NaOH. The oxidation of sialic acid residues was achieved by 100 µL periodic acid (0.2 M) in 0.5 % (v v^{-1}) H_3PO_4.
The oxidation was stopped after 30 min of incubation at 37 °C by addition of 500 µL $NaAsO_2$-solution (0.38 M) in 2% (v v^{-1}) H_2SO_4. Finally the sample was heated to 95 °C for 15 min after adding 500 µL thiobarbituric acid-solution (0.2 M) in 1.2% (v v^{-1}) NaOH. The pink dye was extracted with cyclohexanone and the extinction was measured at 549 nm (Multiskan Spectro, Thermo Scientific).

2.5.2. Analysis of protein

Proteins were quantified using the Bradford method [14]. 10 µL of the sample were transferred into a 96-well plate and mixed with 300 µL of the Bradford-reagent (0.1 g L^{-1} Coomassie-brilliant-blue, 50 ml L^{-1} ethanol (96%), 100 ml L^{-1} phosphoric acid (85%)). The

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samples were carefully shaken and incubated at room temperature for 10 min. The protein concentration was measured at 595 nm (Multiskan Spectro, Thermo Scientific).

2.5.3. Glucose and biomass concentration

The glucose concentration was measured with a YSI 2300 STAT Glucose & Lactate Analyzer (Yellow Springs Institutes, Ohio, USA). The cell dry weight was determined gravimetrically after drying the cell pellets for 24 h at 100 °C.

2.5.4 Endotoxine determination

Endotoxin was quantified using the EndoSafe PTS system (Endosafe-PTS™, Charles River Laboratories, Boston, MA, USA).

2.5.5. DMB-HPLC-Analysis

Polysialic acid (50 mM) in 80 µL trifluoroacetic acid (TFA, 40 mM) was dissolved in 80 µL DMB (1,2-diamino-4,5-methylenedioxybenzene) reaction buffer (7 mM DMB; 12.5 mM Na_2SO_3; 0.8 M β-mercaptoethanol and 20 mM trifluoroacetic acid) and incubated for 24 h at 8 °C under constant shaking [15]. The reaction was stopped by adding 20 µL of 1 M NaOH. Samples (25 µL) of the obtained DMB polysialic acid were separated by a DNAPac PA-100 column (Dionex, Idstein, Germany) using a FD HPLC system. MilliQ water (Millipore, Eschborn, Germany) and 4M ammonium acetate (E2) were used as eluents at a flow rate of 1 mL min^{-1} [16]. The fluorescence detector (Jasco FP-1520, Gross-Umstadt, Germany) was set to 372 nm for extinction and 456 nm for emission. Elution was performed by the following gradient: $T_{0\ min}$ = 0% (v v−1) E2; $T_{5\ min}$ = 0 % (v v^{-1}) E2; $T_{15\ min}$ = 10 % (v v^{-1}) E2; $T_{20\ min}$ = 13 % (v v^{-1}) E2; $T_{35\ min}$ = 17 % (v v^{-1}) E2; $T_{55\ min}$ = 20 % (v v^{-1}) E2; $T_{100\ min}$ = 26 % (v v^{-1}) E2; $T_{180\ min}$ = 30 % (v v^{-1}) E2.

3. Results and discussion

3.1. Bioreactor cultivation of *E. coli* K1

At first *E. coli* K1 was cultivated in batch- and fed-batch mode to produce polySia. Figure 1 displays the off-line data of the batch (A) and fed-batch (B) culture. The batch cultiviation (Figure 1A) was performed with an initial glucose concentration of 20 g L^{-1}. In this mode bacterial growth was finished after 9 h with a final biomass concentration of 8.4 g L^{-1}.The polySia concentration in the medium was 0.37g L^{-1} and the protein concentration about 200 mg L^{-1}.

In the fed-batch (Figure 1B) was the initial glucose concentration 10 g L^{-1}. After consumption of the initial glucose (8 h), indicated each by an increasing of the dissolved oxygen concentration (data not shown), the fed-batch was started. Feeding was carried out at a specific growth rate set point of 0.3 h^{-1} with the Monod-kinetic (see Section 2.3.2). During the fed-batch phase the dissolved oxygen was maintained at 30 % air saturation. The glucose concentration during the fed-batch was kept at 0.03 g L^{-1}, which led to a maximal yield of dry biomass (BDM) 18.4 g L^{-1}. The final polySia concentration was 1.3 g L^{-1} and the final protein concentration was 460 mg L^{-1}. The fed-batch cultivation was stopped after 16 h at a bioreactor volume of 10 L.

The cultivations were stopped directly after the consumption of glucose or feed solution in order to avoid a strong cell lysis. The cell lysis leads to a strong contamination of the culture broth. It was possible to achieve higher yield by a factor of 3.5 via fed-batch approach, in comparison to batch cultivation of same volume. The next approach was to purify polySia without losing much product.

3.2 Purification of polySia

The novel strategy for the purification of polySia with unit operations based on adsorber materials was first applied on a batch cultivation in a 10 L bioreactor. Subsequently, it was also applied to a fed-batch cultivation. Generally, after the cultivation 60-80% of the *E. coli* K1-polySia is released into the fermentation medium.

Precipitation-based polySia purification is highly suitable for batch cultivations [7]. However, the preciptation method provides some disadvantages. It results in a fairly decreased polySia

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yield. Furthermore, it is not applicable on fed-batch cultivations since many more contaminantes such as proteins, lipids, DNA and endotoxines are present in the fermentation broth after the longer cultivation in fed-batch mode. Table 2 summarizes the efficiency of modified precipitation reactions in batch and fed batch purifications.

Precipitation purification in batch cultivations delivers a yield of 50-55 %. A previous study used centrifugation as unit operation, which was replaced by an microfiltration step to enable the purification of higher volumes [7]. Using microfiltration, a two-fold amount of polySia was obtained compared to the purification described by Rode et al. Furthermore, this purification method was applied to a culture supernatant originating from a fed-batch cultivation, resulting in a yield of 5-11%. The filtration and cetavlon precipitation step remained to be critical, due to the high loss of polySia observed within these steps. The loss of product during the microfiltration step indicates the presence of long- as well as short-chained products in fed-batch cultivation.

In order to maximize the yield, a new purification process based on adsorber unit operations was developed. At first, the polySia purification was optimized using an anion exchanger membran and clay mineral, respectively. Afterwards, both adsorbers were combined in order to further improve the purification efficiency.

3.2.1 Optimization of purification of polySia using anionic exchanger (Q membrane)

The aim of this study was a more effective purification of polySia and thus also requires an efficient trapping of proteins during purification [17, 18]. Since polySia has anionic properties, a Q-mebrane was used to separate the target molecule from proteins. In this set up the inference of pH values on binding or rather breakthrough of polySia (on or through the membrane) was examined and buffer concentrations were varied in the different pH-ranges. In order to compare the results directly, samples from the batch with an initial concentration of 510 µg mL^{-1} polySia and a protein concentration of 655 µg mL^{-1} were used. In principle a separation effect was obtained at all pH values (Table 3), but the breakthrough of polySia and proteins occurred at different times. The most optimal separation effect is obtained at high dynamic binding capacities of polySia and low dynamic binding capacities of proteins to the membrane. The dynamic binding capacity for each buffer system is estimated by taking the amounts bound to the membrane as 100 %.

The experiments show that binding capacity of the membrane decreased with increasing pH value. An effective purification of polySia was achieved in a pH-range of pH 7-8. The best results were obtained with the 100 mM Tris binding buffer (pH 8). The binding capacity of polySia was observed at 282,2 ± 25,3 µg $(cm^2)^{-1}$ and protein binding capacity at 62,7 ± 5,6 µg $(cm^2)^{-1}$

For further studies, NaPB should be used in order to ensure the effective purification of polySia in pilot plants as well as in industrial scale. This buffer gives the advantage to carry out the purification directly after the cultivation without changing the pH value. Due to this fact the breakthrough of polySia was further investigated at pH 7. Hereby NaPB was applied at various concentrations (50 mM, 100 mM and 200 mM) (Table 3).

The concentration of 100 mM NaPB (pH 7) demonstrates the best separation effect (Table 3). It exhibits the most significant difference between the the binding capacity for proteins and polySia. In addition, the deployed NaPB is economically applicable in pilot and industrial scale due to its low costs.
Concerning the binding capacity for polySia, the Q 100 membrane shows very different results at various pH values and buffer concentrations (Table 3). Although HEPES and NaPB were set to the same pH range of pH 7, big differences were obtained. While HEPES resulted in an earlier breakthrough of polySia and thus no separation effect, the purification with NaPB was possible due to the higher binding capacity. This leads to the suggestion that buffer components also influence the separation efficiency due to different interactions with the functional groups of the membrane and the proteins.

3.2.2 Optimization of the purification of polySia with clay mineral (EX M 1753)

In this section, the purification of polySia using the clay EX M 1753 (experimental product name 1753, Südchemie, Moosburg, Germany) is described. The aim is to achieve effective trapping of proteins and endotoxins while the adsorption of polySia is undesirable. The EX M 1753 is a clay mineral (Magnesium-Aluminium silicate, particle size ≥45 µm), with a high protein binding capacity due to its structure and surface charge [19]. Because of the severe swelling in aqueous media this material is not suitable as a filling material for columns. Therefore, the experiments could only be performed under static conditions. For this reason, to investigate adsorption properties, the adsorbent was incubated with a sample solution in a closed reaction vessel.

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To investigate the influence of the pH on the adsorption behavior of EX M 1753 different buffers were used with varying concentration (see section 2.4.3). The initial concentration of the proteins in the samples was 649 mg L^{-1} (polySia sample originating from batch cultivation). Figure 2A shows the unbound protein amount as a function of pH after 30 min of adsorption on EX M 1753. Generally, adsorption is observed at all pH values. The highest amount of proteins was adsorbed at pH 7 (NaPB) and a buffer concentrations of 50 mM.

Polysaccharides are adsorbed or partially intercalated because of electrostatic interactions with the clay mineral surface [20]. In order to proof whether this finding also applies for polySia, the amount of polySia was determined after 30 min of adsorption. The polySia initial concentration was 0.78 mg mL^{-1} (Figure 2B).

At all investigated pH ranges, proteins are depleted with EX M 1753. One explanation for the adsorptive behavior of EX M 1753 can be the positive excess charge of the octahedral layer**,** resulting in further interactions such as hydrogen bonds with negatively charged proteins [10].The adsortion of proteins decreases with increasing buffer concentration (Figure 2A). Probably during the interaction, the ions of the buffer intercalate in the structure or the surface of the EX M 1753. As a result a lower amount of protein can be bound on this material. The highest protein adsorption was measured at pH 7 (NaPB) and pH 10 (Figure 2A).

Figure 2B shows a low adsorption of polySia at all pH values. The concentration of unadsorbed polySia varies from 0.65 to 0.72 mg mL^{-1}. The greatest recovery rate of polySia was achieved with the 50 mM buffer (NaPB, pH 7) with 0.75 mg mL^{-1} (96 %).

3.2.3 Pilot scale

In the pilot scale, the Q-membrane was applied together with EX M 1753 in order to achieve an even higher adsorption efficiency of the purification process. Preliminary tests revealed that this combination is the most effective approach (data not shown). In Figure 3 this newly developed purification strategy is shown schematically.

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The purification process is described in section 2.4.4 of the batch and fed-batch cultivation respectively. In Figure 4A and 4B the results of the pilot scale purification strategy are displayed. PolySia yields and protein contents of each unit operation were compared. The values of total protein contents and polySia present in the fermentation broth were chosen as reference (100%). For the experiment, the culture supernatants of a batch and fed-batch cultivation were used.

The results of the combination of Q-membrane and EX M 1753 in Figure 4A show, that an effective protein removal was achieved for the polySia containing supernatant originating from the batch cultivation. The polySia yield was 61% (2.02 g from 10 L bioreactor). The biggest loss was observed during the purification step using the membrane adsorption and cross flow ultrafiltration, here 23% of polySia were lost. Since the adsorptive interactions between the clay mineral EX M 1753 and proteins are very high compared to polySia, only a small loss of polySia was obtained in this purification step (1-2%). After purification with Q-membrane most proteins were depleted.

The initial concentration of polySia after fed-batch cultivation was 0.72 g L^{-1}. By cross-flow microfiltration (0,45 µm) the amount of protein could be reduced by 23 % but the product loss of 19 % polySia was relatively high. The purification with Q-membrane resulted in a protein-elimination of up to 70%. In this unit operation, the product loss was also 19 %. By ultrafiltration polysialic acid was concentrated in the retentate. Here a product loss of 5 % was observed. This loss is due to the low molecular weight polySia, which accumulates in the permeate. This polySia can be recovered with a 2-kDa membrane. These low molecular polySia consists of max. DP (degree of polymerization) 35 (data not shown). Furthermore the bar chart (Figure 4B) shows that the proteins were concentrated as well. The protein recovery rate rose from 7 % to 12 %. In the adsorption step with EX M 1753 the proteins were completely removed. Up to max. 1% polySia was adsorbed during this step of the purification. A final dialysis was performed in order to desalt the purified polySia. The desalted polySia solution was lyophilized and isolated as a solid. In these two unit operations, the product loss was minimal and less than 7 %. The final yield of purified polySia was 2.9 g, equivalent in terms of the culture broth to a yield of 40%, the endotoxin content was determined to be 14 EU mg^{-1}, which is negligible. The yield of the batch broth (61 %) compared to the yield of the precipitation reaction (50-55 %, Table 2) could be increased by

6-11 %. In the case of the fed-batch broth the yield of the precipitation reaction (5-11 %) and the adsorber technique (40 %) showed a difference of 29-35 %.

3.3 DMB-HPLC-Analysis

To characterize the product quality in case of polySia chain length is an important aspect. The polySia with high chain length is suitable for the synthesis of 3D-scaffolds in tissue engineering [21]. The distribution of the obtained polySia was investigated by DMB-HPLC. This method was developed to determine chain lengths of polySia with a detection threshold of 1.4 femtomol [15].

The commercially available colominic acid (DP 70) was used as a reference (Figure 5). While the batch-polySia exhibits a maximum chain length of 110 units (purification strategy I from batch cultivation). The polySia chain length from fed-batch purification strategy I was significantly higher (120 DP). Normally an exact determination of chain length is difficult due the signal overlap of DPs higher than 100 (Nakata und Troy 2005). The measurement of the fed-batch supernatant of the pilot scale showed almost no overlap, which is because of the fact that the purified polySia shows discrete peeks for each chain length. Therefore, chain lengths of around 160 DP have been reliably determined. In summary the purification in pilot scale was successful. The polySia-sample of the batch precipitation had a maximum chain length of 110 DP which is 20 DP lower than the batch-sample purified with the adsorption technique. With this new method we successfully revealed that the produced and purified samples of the batch and fed-batch polySia consist of high molecular chains, with high purity and high efficiency.

4. Conclusion

We have developed a new purification strategy for polySia based on adsorber unit operations. The newly developed strategy has been shown to be a suitable method for the purification of polySia from pilot scale production (10L) to obtain high molecular polySia with high yield and purity at large scale.

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The purification was started with an ion exchange membrane adsorption (Q-membrane). In order to investigate the binding and eluting characteristics of polySia on this membrane, parameters such as buffer concentration and pH were determined at laboratory scale. The optimal buffer concentration of NaPB for binding was found to be 100 mM and pH 7. The advantage in the utilization of NaPB buffer is that a buffer exchange of the culture medium is not necessary and a cost-effective production is ensured.

Furthermore, it was shown that the low-cost adsorbent EX M 1753 was effectively depleting the protein content, due to its high adsorption capacity of protein, i.e. 300 mg under static conditions. To optimize the adsoption, experiments were carried out at different pH-values. The most effective protein adsorption was determined at pH 7 (NaPB) and pH 10 (CAPS). Up to 97% of the total protein amount could be adsorbed under these conditions. The combination of Q-membrane unit operation and EX M 1753 clay adsorber resulted in a new purification method which could be successfully applied to both batch and fed-batch cultivation. The up scaling to pilot plant scale provided a purity of 98% and a yield of 61 % in the batch cultivation and 40 % in the fed-batch cultivation, which shows higher yields compared to the precipitation method. In addition, polySia (35 DP) with low molecular weight was obtained from the permeate of the ultrafiltration.
Due to this optimized combination of membrane adsorber and clay adsorber, significantly longer polySia chains up to 160 DP were produced and purified. In the analytical characterization of the molecular units, an influence of the cultivation process on chain length was observed. It could be shown that polySia obtained from fed-batch cultivations shows higher chain length than obtained from batch cultivation. PolySia obtained from pilot scale fed-batch supernatant provides chains containing up to 160 DP. Therefore, a fed-batch cultivation is highly recommended for future biotechnological production of polySia. Long-chained polySia produced in large scale cultivations is worth to be further investigated as polySia-based scaffolds representing a promising alternative for existing materials in reconstructive medicine e.g. of nerves.

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The authors have declared no conflict of interest.

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5. References

[1] L. B. Rodríguez-Aparicio, A. R. A. I. O. u. J. M. L.: Effect of physical and chemical conditions on the production of colominic acid by Escherichia coli in a defined medium. In: Applied Microbiology and Biotechnology 27 (1988) 5-6, S. 474–83.

[2] Baumann, H.; Brisson, J. R.; Michon, F.; Pon, R.; Jennings, H. J.: Comparison of the conformation of the epitope of alpha(2--8) polysialic acid with its reduced and N-acyl derivatives. In: Biochemistry 32 (1993) 15, S. 4007–13.

[3] Angata, T.; Varki, A.: Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. In: Chemical reviews 102 (2002) 2, S. 439–69.

[4] Gotschlich, E. C.; Fraser, B. A.; Nishimura, O.; Robbins, J. B.; Liu, T. Y.: Lipid on capsular polysaccharides of gram-negative bacteria. In: The Journal of biological chemistry 256 (1981) 17, S. 8915–21.

[5] Tzeng, Y.-L.; Datta, A. K.; Strole, C. A.; Lobritz, M. A.; Carlson, R. W.; Stephens, D. S.: Translocation and surface expression of lipidated serogroup B capsular Polysaccharide in Neisseria meningitidis. In: Infection and immunity 73 (2005) 3, S. 1491–505.

[6] Whitfield, C.: Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. In: Annual review of biochemistry 75 (2006), S. 39–68.

[7] Rode, B.; Endres, C.; Ran, C.; Stahl, F.; Beutel, S.; Kasper, C.; Galuska, S.; Geyer, R.; Mühlenhoff, M.; Gerardy-Schahn, R.; Scheper, T.: Large-scale production and homogenous purification of long chain polysialic acids from E. coli K1. In: Journal of biotechnology 135 (2008) 2, S. 202–09.

[8] Liu, J.-L.; Zhan, X.-B.; Wu, J.-R.; Lin, C.-C.; Yu, D.-F.: An efficient and large-scale preparation process for polysialic acid by Escherichia coli CCTCC M208088. In: Biochemical Engineering Journal 53 (2010) 1, S. 97–103.

[9] Kapre, S. V. (. I. S. U.: Highly pure polysialic acid and process for preperation thereof (2010) 20100086974. Abrufdatum 04.11.2011.

[10] Ralla, K.; Sohling, U.; Suck, K.; Sander, F.; Kasper, C.; Ruf, F.; Scheper, T.: Adsorption and separation of proteins by a synthetic hydrotalcite. In: Colloids and surfaces. B, Biointerfaces 87 (2011) 2, S. 217–25.

[11] Kökpinar, O.; Harkensee, D.; Kasper, C.; Scheper, T.; Zeidler, R.; Reif, O.-W.; Ulber, R.: Innovative modular membrane adsorber system for high-throughput downstream screening for protein purification. In: Biotechnology progress 22 4, S. 1215–19.

[12] Chen, R.; John, J.; Rode, B.; Hitzmann, B.; Gerardy-Schahn, R.; Kasper, C.; Scheper, T.: Comparison of polysialic acid production in Escherichia coli K1 during batch cultivation and fed-batch cultivation applying two different control strategies. In: Journal of Biotechnology 154 (2011) 4, S. 222–29.

[13] Graf, A.-M.; Graf, A.-M.; Steinhof, R.; Lotz, M.; Tippkötter, N.; Kasper, C.; Beutel, S.; Ulber, R.: Downstream-Processing mit Membranadsorbern zur Isolierung nativer Proteinfraktionen aus Kartoffelfruchtwasser. In: Chemie Ingenieur Technik 81 (2009) 3, S. 267–74.

[14] Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. In: Analytical biochemistry 72 (1976), S. 248–54.

[15] Inoue, S.; Lin, S. L.; Lee, Y. C.; Inoue, Y.: An ultrasensitive chemical method for polysialic acid analysis. In: Glycobiology 11 (2001) 9, S. 759–67.

[16] Nakata, D.; Troy, F. A.: Degree of polymerization (DP) of polysialic acid (polySia) on neural cell adhesion molecules (N-CAMS): development and application of a new

strategy to accurately determine the DP of polySia chains on N-CAMS. In: The Journal of biological chemistry 280 (2005) 46, S. 38305–16.
[17] Suck, K.; Walter, J.; Menzel, F.; Tappe, A.; Kasper, C.; Naumann, C.; Zeidler, R.; Scheper, T.: Fast and efficient protein purification using membrane adsorber systems. In: Journal of biotechnology 121 (2006) 3, S. 361–67.
[18] Plate, K.; Beutel, S.; Buchholz, H.; Demmer, W.; Fischer-Frühholz, S.; Reif, O.; Ulber, R.; Scheper, T.: Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. In: Journal of chromatography. A 1117 (2006) 1, S. 81–86.
[19] Ralla, K.; Anton, F.; Scheper, T.; Kasper, C.: Application of conjoint liquid chromatography with monolithic disks for the simultaneous determination of immunoglobulin G and other proteins present in a cell culture medium. In: Journal of chromatography. A 1216 (2009) 13, S. 2671–75.
[20] Ruiz-Hitzky, E.; Darder, M.; Aranda, P.: Functional biopolymer nanocomposites based on layered solids. In: Journal of Materials Chemistry 15 (2005) 35-36, S. 3650.
[21] Haastert-Talini, K.; Schaper-Rinkel, J.; Schmitte, R.; Bastian, R.; Muehlenhoff, M.; Schwarzer, D.; Draeger, G.; Su, Y.; Scheper, T.; Gerardy-Schahn, R.; Grothe, C.: In Vivo Evaluation of Polysialic Acid as Part of Tissue-Engineered Nerve Transplants. In: TISSUE ENGINEERING PART A 16 (2010) 10, S. 3085–98.

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Table

Table 1: Medium composition for batch and fed-batch cultivation.

Components	batch medium	feed solution
Glucose	20[a] / 10 g L^{-1}	200 g L^{-1}
K_2HPO_4	13.3 g L^{-1}	
KH_2PO_4	0.5 g L	
NaCl	1.2 g L^{-1}	
K_2SO_4	1.1 g L^{-1}	
$(NH_4)_2SO_4$	10 g L^{-1}	
$MgSO_4 \cdot 7\ H_2O$	0.15 g L^{-1}	0.3 g L^{-1}
$FeSO_4 \cdot 7H_2O$	0.001 g L^{-1}	0.002 g L^{-1}
$CuSO_4 \cdot 5H_2O$	0.001 g L^{-1}	0.002 g L^{-1}
$CaCl_2 \cdot 2H_2O$	0.013 g L^{-1}	0.026 g L^{-1}

[a]For batch cultivation only

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Table 2: Average yield of polySia during different downstream unit operations in batch and fed-batch cultivation.

Downstreaming step	**Batch**	**fed-batch**
Microfiltration (0,45 µm)	90-99 %	60-90 %
Ultrafiltration (10 kDa)	>70 %	60-70 %
Aceton-precipitation (45 %)	~ 90 %	83-88 %
Cetavlon-precipitation (10 g/L)	> 99%	< 26 %
Ethanol-precipitation (80 %)	> 99 %	> 99 %
Total	50-55 %	5-11 %

Table 3: Binding capacities for the Q 100 membrane at pH 7-10 depending on the buffer concentration 50 mM, 100 mM and 200 mM.

Buffer [mM]	pH	binding capacity [µg cm^{-2}]	
		polySia	Protein
HEPES 50	7	255,1 ± 7,6	22,2±1,1
HEPES 100		179,9 ± 5,4	90,9±4,5
HEPES 200		262,8 ± 7,8	57,58±2,8
Tris 50	8	282,2±25,3	62,7±5,6
Tris 100		220,6±19,8	70,1±6,3
Tris 200		158,7±14,2	116,8±10,5
Tris 50	9	231,2±20,8	151,2±13,6
Tris 100		114,3±10,2	134,0±12,0
Tris 200		211,2±19,0	166,12±14,9
CAPS 50	10	139,6±12,5	124,4±11,1
CAPS 100		122,3±11,0	10,6±0,9
CAPS 200		134,8± 2,1	10,37± 0,90
NaPB 50	7	321,3±22,4	42,2 ± 3,3
NaPB 100		412,2±28,8	35,9 ± 2,8
NaPB 200		312,3±21.8	32,2 ± 2,5

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Figure Legends

Figure 1: Off-line data of biomass, glucose-, polySia and protein concentration during batch (A) and fed-batch (B) cultivation carried in 10 L bioreactor. The cells were grown in batch mode with 20 g L^{-1} glucose as carbon source (A). In the fed-batch, the cultivation was started with initial concentration of 8 g L^{-1} and was maintained at a level of 0.05 g L^{-1} (B).

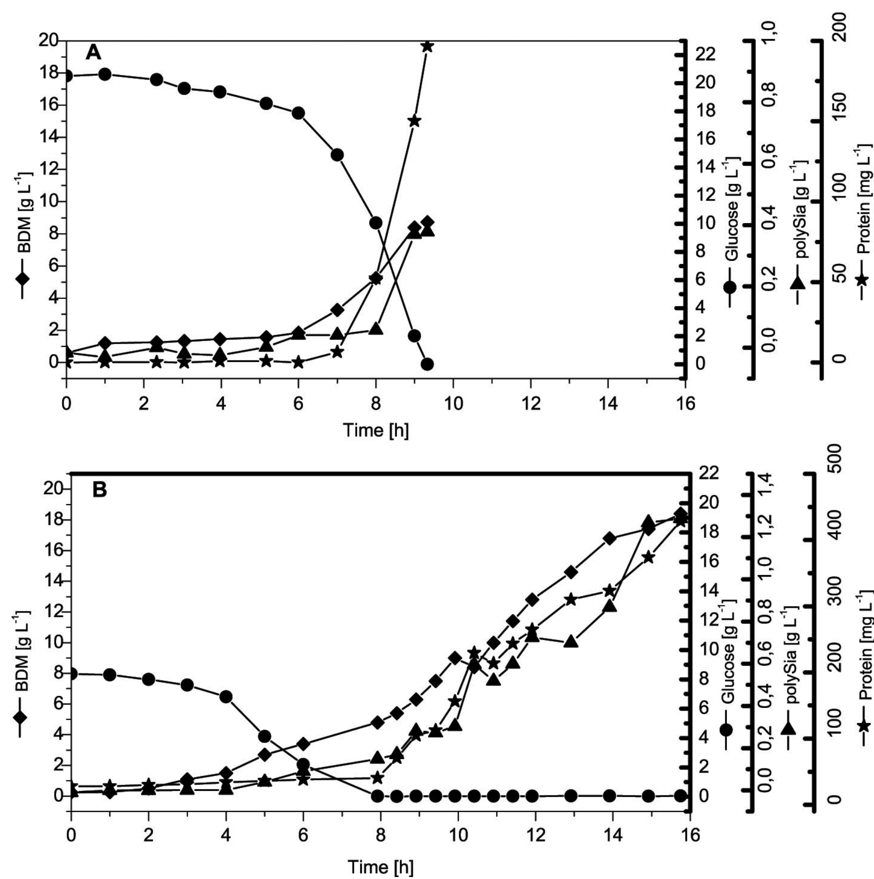

Figure 2: pH-dependent adsorption of a sample originating from batch cultivation on EX M 1753 after 30 min of unbound protein (A) and polySia (B). The adsorption was performed in 50 mM, 100 mM and 200 mM buffer of appropriate pH. NaPB indicated as pH 7*. The initial protein concentration was 649 mg mL^{-1} and initial polySia concentration 0.78 mg mL. Error bars represent the S.E. (n=3).

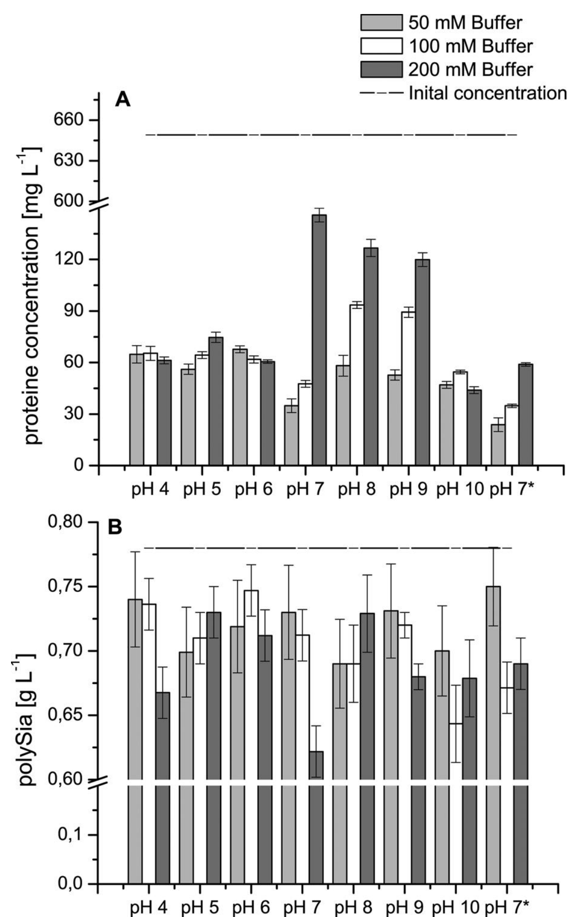

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Figure 3: Flow chart of the developed purification strategy for polySia from *E. coli* K1-cultivation.

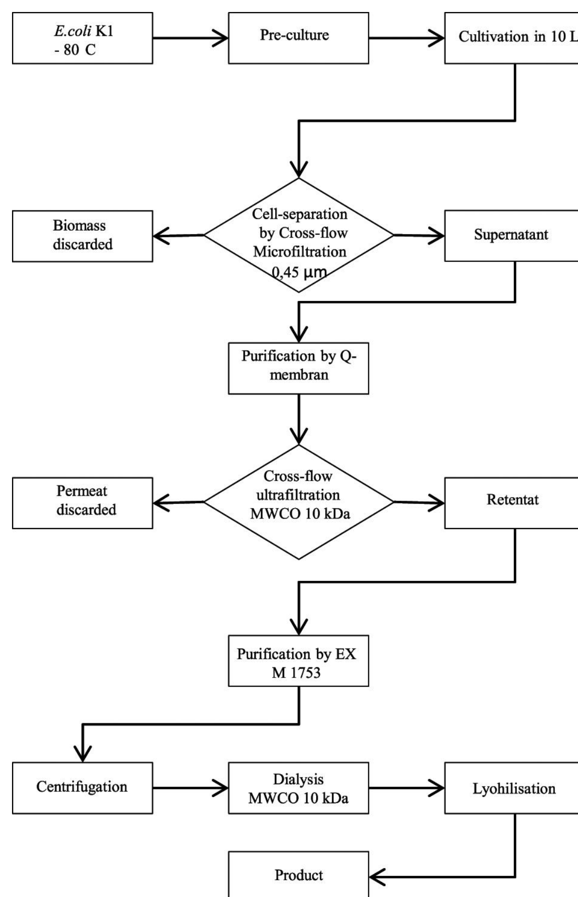

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Figure 4: Results of the pilot scale purification of polySia for the batch mode (A) and fed-batch (B). The polySia-yield and recovery of proteins are shown for each step in the process. Error bars represent the S.E. (n=2).

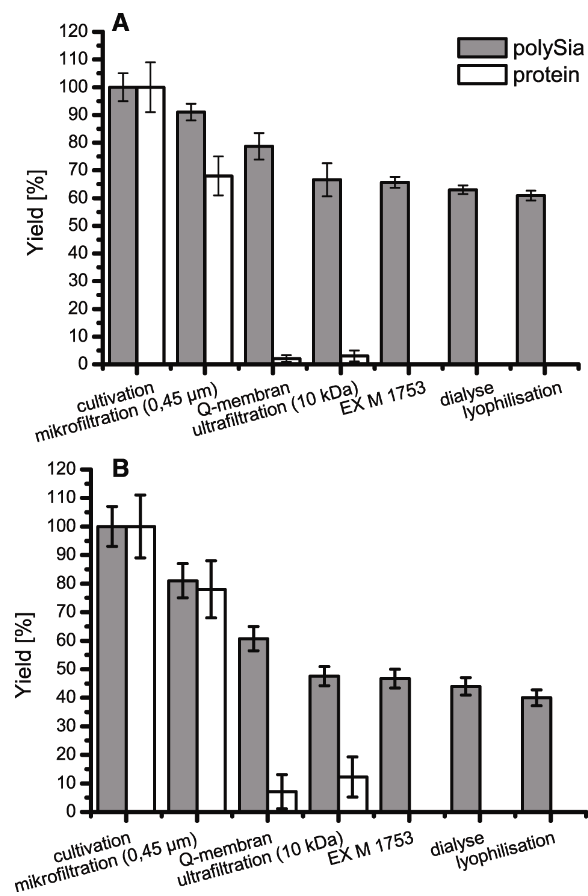

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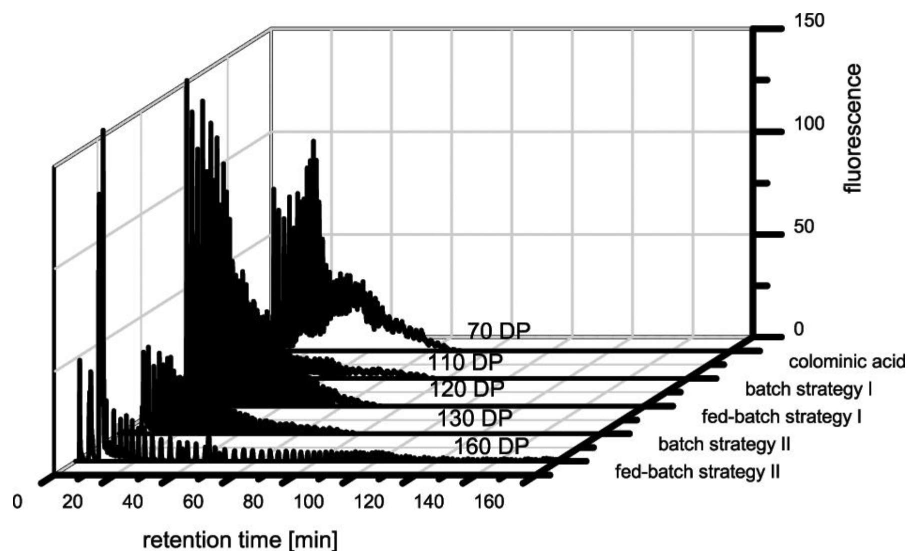
Figure 5: Chromatograms of DMB-tagged commercially available comominic acid and polySia form different purifications. Strategy I: purification with precipitation reaction and strategy II: purification with adsorber in the pilot scale.



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