



Single-use membrane adsorbers for endotoxin removal and purification of endogenous polysialic acid from *Escherichia coli* K1

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ABSTRACT

Polysialic acid (polySia) is a promising molecule for various medical applications (e.g., treatment of inflammatory neurodegenerative diseases).

In this study a complete production process for human-identical α -(2,8)-linked polySia was developed using a disposable bioreactor for cultivation of *Escherichia coli* K1 and single-use membrane adsorbers for downstream processing (DSP). The cultivation process was optimized to minimize complex media components and a maturation process after cultivation was established. The maturation led to further product release from the cell surface into the supernatant. Afterwards DSP was established using sodium hydroxide treatment combined with anion exchange membrane adsorbers for endotoxin and DNA depletion.

After downstream processing the final product had neither detectable protein nor DNA contamination. Endotoxin content was below 3 EU mg⁻¹. Investigation of the maximal chain length showed no effect of the harsh sodium hydroxide treatment during DSP on the stability of the polySia. Maximal chain length was \sim 98 degree of polymerization.

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1. Introduction

Polysialic acid (polySia) is a polysaccharide consisting of α -(2,8)- and/or α -(2,9)-linked N-acetylneuraminic acid monomers. PolySia is a promising molecule for application in tissue engineering [1], vaccine development and cancer immunotherapy [2–6]. Furthermore, α -(2,8)-polySia is a potential drug for the treatment of inflammatory neurodegenerative diseases (e.g., age-related macular degeneration) [7,8]. Production of polySia is commonly realized as endogenous product of cultivated bacteria (e.g., *Escherichia coli* K1 or *Neisseria meningitidis* B) [2,9–12]. The polySia of the *Escherichia coli* K1 capsule consists of α -(2,8)-linked N-acetylneuraminic acid and is identical to the polySia found in the human body [13,14]. Recently a process was developed using a disposable bag reactor for the cultivation instead of a conventional stirred tank reactor often used for bacteria cultivation [11]. Single-use systems have become widely accepted for the production of biological agents for pharmaceuticals and medical biotechnological applications [15].

The main advantages of single-use systems are reduced risks for cross-contamination, high flexibility, less complexity of the production plant as sterilization and cleaning steps are reduced as well as easier production in terms of regulatory requirements due to omitted validation and documentation of those sterilization and cleaning steps [16]. Nevertheless, the presence of leachables originating from the plastic material is undesired within the production process [16,17]. Furthermore, costs for the disposable parts constantly occur [16,17], but investment costs are low.

For high quality products not only the production process has to be in accordance to the regulatory requirements of GMP-production (good manufacturing practice), also product purity has to be strictly controlled, especially for products intended for medical application. Therefore downstream processing (DSP) is another important part of a biotechnological production process. All steps which are necessary to purify the target product require a large part of the overall production costs [18,19].

Reported purification processes for polySia are based on precipitation and/or adsorption methods [10,12]. Process-related

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impurities (e.g., host cell proteins, DNA, endotoxins, media components of USP and DSP) as well as product-related impurities (e.g., precursors and degradation products) can affect the product properties. Therefore, different chromatographic methods can be exploited to obtain the desired product purity. Molecules can be separated based on different properties, such as affinity (affinity chromatography), size (SEC = size exclusion chromatography), hydrophobicity (HIC = hydrophobic interaction chromatography) and charge (IEX = ion exchange chromatography). Especially, the depletion of impurities, such as endotoxins, is challenging.

Endotoxins are negatively charged, complex lipopolysaccharides (LPS) and part of the outer membrane of gram-negative bacteria. Even small quantities have a high impact on health of human beings. Endotoxins cause inflammatory response at concentrations greater than $1 \text{ ng kg}^{-1} \text{ h}^{-1}$, based on the body weight [20–22]. The Food and Drug Administration (FDA) regulations define the maximum allowable endotoxin exposure at $5 \text{ EU kg}^{-1} \text{ h}^{-1}$ (EU = endotoxin units). For the removal of endotoxins different techniques are reported: chromatography (such as ion exchange chromatography [23,24], or resins based on immobilized polyethyleneimine [25]), ultrafiltration [26], affinity adsorbents [27–31], Triton X-114 phase separation [32,33], modified membranes [34] and membrane adsorbents [35]. Membrane adsorbents have several advantages compared to classical resin technology, e.g., convective material transport, higher flow rates, easy scale-up and single use. For depyrogenation sodium hydroxide treatment is widely used [36,37].

This study is focused on the development and optimization of a complete production process for long-chain and highly pure polySia. Single-use elements are implemented in the overall process. The downstream processing is improved by a sodium hydroxide treatment and anion exchange membrane adsorbents to obtain highly pure polySia fractions. The produced polySia is analyzed after the production process regarding protein, DNA, endotoxin content and maximal chain length. The production process is compared to the already reported established processes for polySia production.

2. Materials and methods

2.1. Bacterial strains and stock cultures

As previously reported *E. coli* B2032/82 serotype K1 was used for the experiments [10–12]. The wild type strain *E. coli* B2032/82 serotype K1 is an original clinical isolate [10]. Stock cultures were prepared as previously reported [11].

2.2. Chemicals and growth media

Bulk chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany). Oxygen was purchased from Linde (Pullach, Germany). Deionized water was prepared with Arium[®] (Sartorius Stedim Biotech, Göttingen, Germany). For the preculture used for bioreactor inoculation and the main bioreactor cultivation a defined salt medium was used. The composition was reported previously [12].

2.3. Cultivation of *E. coli* K1

2.3.1. Shake flask cultivation and preculture

First stock cultures were transferred in complex medium and cultivated for 8 h on a rotary shaker at 37°C and 130 rpm. Complex medium composition was reported earlier [11] and complex medium was only used for the first shake flask cultivation to inoculate the preculture for the main bioreactor cultivation. Afterwards $20 \mu\text{L}$ of this culture (shake flask cultivation in

complex medium) was transferred into 100 mL defined salt medium and incubated for 10–12 h at the mentioned conditions. These cells were used as inoculum for the bioreactor cultivation. For inoculation 300 mL preculture was used ($3\% \text{ v v}^{-1}$ of total cultivation volume).

2.3.2. Disposable bag reactor

For the main bioreactor cultivation a disposable bag reactor with wave-induced mixing and a total volume of 20 L (maximal working volume: 10 L) was used (BIOSTAT[®] CultiBag RM 20 optical, Sartorius Stedim Biotech, Göttingen, Germany). Cultivation conditions were used as previously reported [11]. For effective and efficient fermentation control online parameters were analyzed and after offline sampling analysis are made describing the actual culture conditions [38].

2.3.3. Offline sampling

Offline samples were analyzed during the cultivation. Measurements of optical density, cell dry weight and glucose concentration were performed as previously reported [11].

2.4. Downstream process

2.4.1. Maturation process and cell separation

For maturation the produced cells were stored for 17 h at 8°C . After maturation process cells were separated by continuous centrifugation as previously reported [11].

2.4.2. Product concentration via cross-flow ultrafiltration

After cell separation the supernatant was concentrated to a final volume of 250 mL with a cross-flow device (Sartoflow[®] Smart, Sartorius Stedim Biotech, Göttingen, Germany). The filter cassette had a molecular weight cut-off (MWCO) of 10 kDa (Hydrosart[®], Sartorius Stedim Biotech, Göttingen, Germany). The inlet pressure towards the filter cassette was regulated to maximal 2 bar by controlling the flow rate of the pump.

2.4.3. Precipitation with ethanol

Precipitation of the retentate after cross-flow filtration with ethanol was performed in three subsequent steps. For precipitation $80\% \text{ v v}^{-1}$ ethanol was used. The precipitate was spun down at 4816 g and 4°C for 15 min after the first, for 20 min after the second and for 60 min after the third precipitation step (Multifuge X3 FR, Thermo Scientific, Waltham, USA). The pellet was dissolved in water after each precipitation step.

2.4.4. Purification with clay minerals

Calcium bentonite EX M 1753 (experimental product name 1753, CAS registry number: 1302-78-9, Clariant, Moosburg, Germany) was used for protein adsorption as previously reported [11].

2.4.5. Sodium hydroxide treatment

After purification with calcium bentonite the sample was treated with sodium hydroxide. The pH was set to pH 13 with 2 M NaOH and the solution was mixed at room temperature for 18 h. Afterwards the pH was adjusted to pH 7.4 with 1 M HCl, 10 mM triethanolamine was added and the solution was filtered with a $0.2 \mu\text{m}$ bottle top sterile filter (Sartolab[®], Sartorius Stedim Biotech, Göttingen, Germany).

2.4.6. Purification of polySia with anion exchange membrane adsorbents

2.4.6.1. Evaluation of membrane adsorbents in an FPLC system. The purification process using anion exchange membrane adsorbents

was evaluated in an FPLC system (ÄKTA pure 25 L, GE Healthcare, Chicago, USA) with Sartobind[®] Q 75 (membrane area: 75 cm², column volume (CV): 2.1 mL). The system control software Unicorn[™] 6.4 was used. The membrane adsorber was equilibrated with 5 CV running buffer (100 mM triethanolamine, pH 7.4). Afterwards, the pretreated sample was loaded onto the membrane adsorber, followed by a washing step with 5 CV running buffer. A linear gradient elution was performed over 20 CV with elution buffer (1 M NaCl). The column was re-equilibrated with 5 CV running buffer before the next run. The flow rate was set to 15 mL min⁻¹ during the chromatographic run. During the whole process fractions were collected for further analysis.

2.4.6.2. Upscale of membrane adsorber technique for polySia purification. The upscale of the downstream processing was carried out with Sartobind[®] Q 75 mL (membrane area: 2700 cm², CV: 75 mL) (Sartorius Stedim Biotech, Göttingen, Germany). The chromatographic protocol was described in the previous section. Only the sample elution was changed to an isocratic elution of 75% elution buffer (5 CV). The flow rate was set to 100 mL min⁻¹ during the chromatographic run.

Detailed information about the membrane adsorber, sample volume and composition are described in the “Results and discussion” chapter.

2.4.7. Dialysis

The eluate was splitted into 250 mL fractions and each fraction was dialyzed against 5 L deionized water with 0.02 M NaCl for 24 h at 8 °C and afterwards twice against 5 L deionized water for 24 h at 8 °C for each (every time pH 9, set with NaOH) using a Visking dialysis membrane (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with a MWCO of 14 kDa.

2.4.8. Lyophilization

Freeze drying was performed as previously reported [11].

2.5. Analysis

2.5.1. Standard analysis

During cultivation, downstream processing and for the final characterization of the product several analysis were performed. The polySia concentration was measured with a modified thiobarbituric acid assay, protein concentration was determined

by the Bradford method, DNA concentration by UV-vis absorption (NanoDrop 2000, Thermo Scientific, Waltham, USA), endotoxin concentration with Endosafe-PTS[™] system (Endosafe-PTS[™], Charles River Laboratories, Boston, USA) and the chain length characterization with DMB-HPLC analysis (DMB = 1,2-diamino-4,5-methylenedioxybenzene). Cell disruption was performed with ultrasonic treatment. All methods were performed as previously reported [11].

2.5.2. Polymerase chain reaction and gel electrophoresis for DNA analysis

For further DNA analysis polymerase chain reaction (PCR) and gel electrophoresis were performed. PCR reactions were conducted as follows: 10 µL 5× Green GoTaq[®] Reaction Buffer (Promega, Fitchburg, USA), 4 µL dNTP mix (2 mM each) (Thermo Fisher Scientific, Waltham, USA), 2 µL primer mix (*E. coli* forward: 5'-GAGCGCAACCCTATCCT-3', *E. coli* reverse: 5'-GGTCCCCTACGGT-TAC-3') (MWG-Biotech AG, Ebersberg, Germany), 1 µL template and 0.25 µL GoTaq[®] G2 DNA Polymerase (Promega, Fitchburg, USA) in a total volume of 50 µL (add H₂O). Amplification was performed for 40 cycles after initial denaturation at 95 °C for 5 min as follows: 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s. After 5 min at 72 °C PCR was stopped and the samples were stored at 4 °C.

For gel electrophoresis 1.5% w v⁻¹ agarose gels were prepared and 0.005% v v⁻¹ HDGreen[™] (Intas Science Imaging Instruments GmbH, Göttingen, Germany) was added for staining. Gel electrophoresis was performed for 1 h at 110 V.

3. Results and discussion

In the following the results of the cultivation process, downstream processing and product characterization are shown. The polySia production process is shown in Fig. 1.

3.1. Cultivation process in a disposable bag reactor

In contrast to a previously reported cultivation process [11], the preculture was also prepared in a defined media as used in the cultivation process. This step was implemented to avoid contamination of defined medium with complex compounds (e.g., yeast extract) and other byproducts of the preculture.

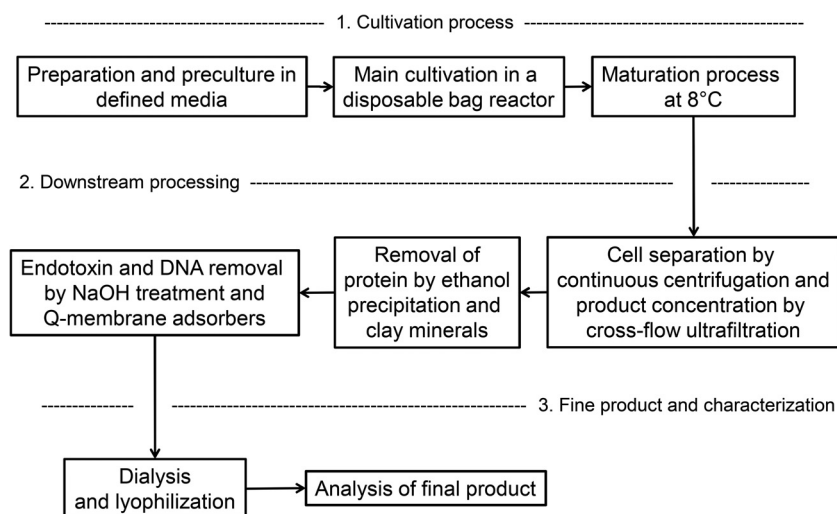


Fig. 1. Process scheme.

The overall production process of polysialic acid is shown. First cultivation is performed in a disposable bag reactor. After cultivation maturation process is carried out, followed by downstream processing and characterization of the final product.

After the glucose consumption a cell dry weight (CDW) of 7.5 (± 0.8) g L⁻¹, a polySia concentration in the supernatant of 300.9 (± 19.8) mg L⁻¹ and a protein concentration in the supernatant of 108.8 (± 41.5) mg L⁻¹ was reached. Total polySia concentration after cell disruption was 560.5 (± 56.9) mg L⁻¹. Thus, 54 (± 7)% of the polySia was released during cultivation. In total three cultivations were performed. Data are presented as mean \pm standard deviation.

The results of the cultivation process are within the range of the conventional process [11]. Nevertheless, polySia concentration was increased by 23% and protein concentration was decreased by 17% in the supernatant.

3.2. Maturation process after cultivation

The disposable bag reactor provides the opportunity for an easy and sterile storage after cultivation. The culture broth can be stored directly in the reactor containment (bag) and transported to the desired storage/maturation place. In this case the maturation process was performed at 8 °C for 17 h. Whereas in the established process the cells were directly separated after cultivation to avoid cell lysis and host cell protein release into the supernatant [11], the maturation process was implemented to release still bound polySia from the cells into the supernatant and make it available for further downstream processing. Furthermore, the cultivation process can be decoupled from the downstream processing by storage of the culture broth.

The final polySia concentration after maturation process was 332.6 (± 36.3) mg L⁻¹ and protein concentration in the supernatant was 191.6 (± 84.8) mg L⁻¹, which is an increase in product concentration by about 10% but also an increase in protein impurity of about 75%. The total polySia concentration after cell disruption is constant. Thus, polySia was further released from the cells into the supernatant, but not newly produced, during storage.

3.3. Downstream processing of the produced polySia

Afterwards, downstream processing was performed to provide suitable product purity for the desired application. Critical parameters of the product are protein and DNA concentration as well as endotoxin concentration. In previous studies downstream processing based on clay minerals and/or anion exchange membrane adsorbers was performed [11,12]. The processes focused mainly on the removal of protein contamination. Nevertheless, due to use of membrane adsorber treatment also endotoxin was reduced to 14 EU mg⁻¹ [12].

In the following a new downstream process was established, focusing, as well on the removal of protein contamination using the established methods of ethanol precipitation and clay minerals [11,12], as on the removal of endotoxin contamination using sodium hydroxide treatment and anion exchange membrane adsorbers.

3.3.1. Cell separation, product concentration and protein removal

Cell separation after maturation was performed using continuous centrifugation as previously reported [11]. The composition (protein to polySia) of the supernatant after maturation process and cell separation had a ratio of approximately 0.6 g_{protein} g_{polySia}⁻¹.

The 10L supernatant were concentrated 40-fold to 250 mL using cross-flow ultrafiltration. For cross-flow ultrafiltration a Sartoflow[®] Smart device (Sartorius Stedim Biotech) with a filter cassette with MWCO of 10 kDa was used. The system provides flexibility due to exchangeable tubes and feed pump and is accepted in cGMP environments. The concentrate (volume: 250 mL) had a composition of 6417 (± 779) mg L⁻¹ polySia and

2539 (± 783) mg L⁻¹ protein (ratio approximately 0.4 g_{protein} g_{polySia}⁻¹). During concentration with ultrafiltration nearly 50% of the produced polySia got lost. Product was lost in the permeate (polySia with chain length under 35 degree of polymerization, DP [12]), as well as in the dead volume of the system and/or binding to the filter membrane.

Removal of protein was based on a previously reported process [11]. In comparison to the reported process ethanol precipitation was improved. Due to increased centrifugation time instead of altering ethanol concentration from 80% v v⁻¹ up to 90% v v⁻¹ ethanol [11] polySia loss was minimized to less than 10%. After ethanol precipitation, the ratio was approximately 0.3 g_{protein} g_{polySia}⁻¹. Then a subsequent clay mineral adsorption was performed as previously reported [11], yielding polySia with protein impurity left below 0.05 g_{protein} g_{polySia}⁻¹.

The material with low protein contamination was used for further downstream processing to reduce the endotoxin and DNA content of the product. Previous studies showed, that after DSP based on the mentioned treatment with ethanol and clay minerals endotoxin concentration was still above 1000 EU mg⁻¹ and DNA was 5.5 (± 0.5) mg_{DNA} g_{polySia}⁻¹ [11].

3.3.2. Sodium hydroxide treatment

Sodium hydroxide is commonly used for endotoxin removal in purification processes for products produced in *E. coli* as Poly(3-Hydroxybutyrate) or polySia [36,37]. Treatment with sodium hydroxide at pH 13 showed no loss of polySia. The sample was neutralized to pH 7.4 after the treatment and further purified with anion exchange membrane adsorbers. As reference a sample without sodium hydroxide treatment was further purified to compare the obtained materials in terms of endotoxin concentration and chain length. Endotoxin was effectively removed with this method and maximal chain length showed no significant difference between the materials treated with sodium hydroxide and the control. Detailed information on the product purity and the chain length are shown in section “Purity”.

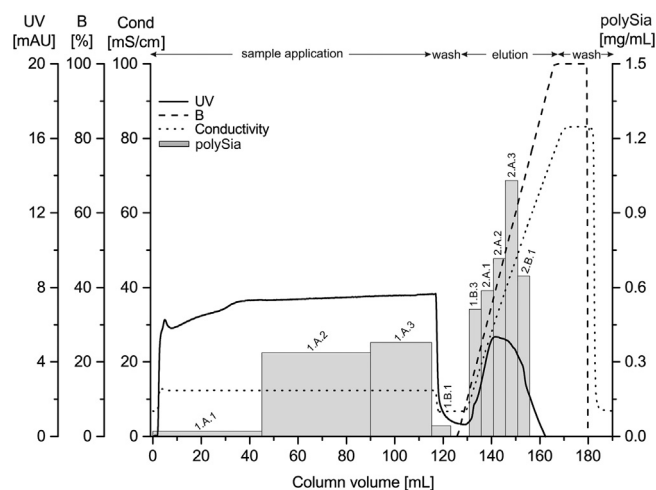


Fig. 2. Evaluation of the membrane adsorber in the FPLC system.

The membrane adsorbers Sartobind[®] Q 75 (membrane area: 75 cm², CV: 2.1 mL) were used for evaluation. Fractions of sample application (1.A.1–1.A.3) showed increasing content of polySia (grey bars) as maximal dynamic binding capacity of the membrane adsorber was reached. After sample application and washing NaCl content (B) (line: dash) was increased as also conductivity increases (line: dot). PolySia was mainly eluted between 0.1 M and 0.75 M NaCl. UV-signal (line: solid) showed only low intensity as protein content was reduced heavily before membrane adsorber evaluation.

3.3.3. Anion exchange membrane adsorbers for polySia purification

3.3.3.1. Evaluation of membrane adsorbers in FPLC system. Membrane adsorbers were tested in an FPLC system using Sartobind[®] Q 75 (membrane area: 75 cm², CV: 2.1 mL). PolySia binding capacity and elution profile were evaluated. The chromatogram comprising elution gradient, UV-signal, conductivity and polySia concentration in the collected fractions is shown in Fig. 2. The membrane adsorber was loaded with high amounts of long-chain polySia (maximal chain length ~98 DP). The maximal dynamic binding capacity was determined by the amount of adsorbed polySia after the binding capacity was exceeded and the adsorber was washed. The maximal dynamic binding capacity of the Q membrane adsorber was 0.25 (\pm 0.03) mg cm⁻².

After the loading step the column was washed with loading buffer to remove loosely bound polySia and other undesired components (e.g., endotoxin fragments). Afterwards polySia elution was performed with a linear salt gradient. Elution of polySia occurred between 0.1 M and 0.75 M NaCl. Eventually the membrane adsorber was washed with 1 M NaCl. As control polySia without previous sodium hydroxide treatment was used. The control showed similar binding and elution behavior. Comparison of endotoxin content and product stability of the material and the control is shown in section “Purity”.

3.3.3.2. Upscale of the purification process using membrane adsorbers. After evaluation shown before an upscale of the purification process using Sartobind[®] Q 75 mL (membrane area: 2700 cm², CV: 75 mL) was performed. Instead of a NaCl gradient as reported above isocratic elution with 0.75 M NaCl was performed, because further increase of NaCl concentration showed no further polySia elution. The membrane adsorber was loaded after equilibration with maximal 600 mg polySia, due to the capacity measured during evaluation. Several loading and elution cycles were performed to avoid polySia loss due to limitation of membrane adsorber capacity. After this membrane purification step almost no polySia was lost.

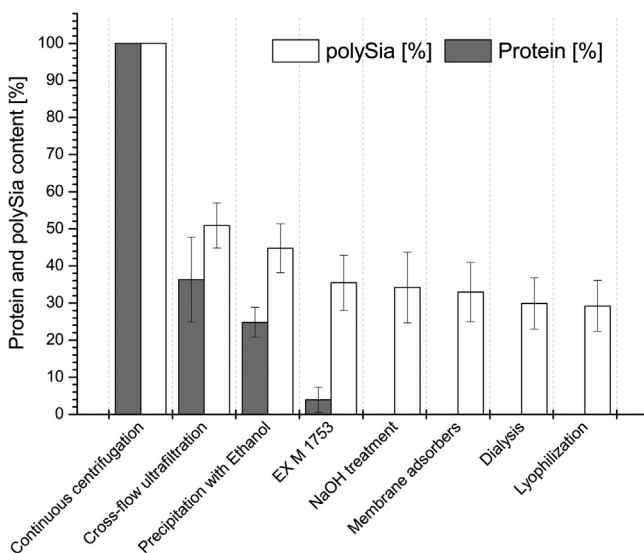


Fig. 3. Downstream processing.

Protein (grey bars) was reduced efficiently during downstream processing. After DSP no protein and DNA was detectable in the final product after lyophilization, due to implementation of NaOH treatment and membrane adsorbers. Endotoxin of the final product was below 3 EU mg⁻¹. The recovery yield of polySia (white bars) after DSP was 29 (\pm 7)%.

3.3.4. Dialysis and freeze drying

After purification with membrane adsorbers, dialysis and freeze drying steps of the product were performed as previously reported [11] to produce a tailor made product. The shown purification process had a recovery yield of 29 (\pm 7)%, as shown in Fig. 3. Compared to the easy to handle and time-saving previously reported process which focused only on the removal of protein [11], recovery yield was improved by 3% and endotoxin and DNA content was reduced heavily (data is shown in section “Purity”).

3.4. Analysis of the final product

3.4.1. Purity

Protein content of the produced polySia was below detection limit of the used method (Bradford method). Thus, protein content is comparable to the established purification methods [10–12]. The aim of the newly developed purification process implementing sodium hydroxide treatment and membrane adsorbers in the overall process was the reduction of the remaining DNA and endotoxin content.

Content of DNA in the final product was below detection limit measured by absorbance measurement at 260 nm and gel

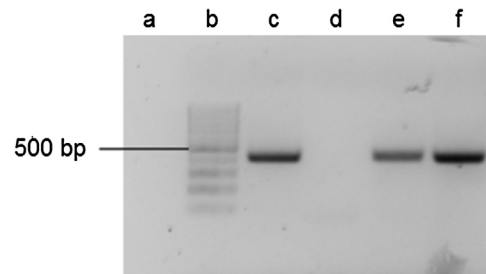


Fig. 4. Agarose gel electrophoresis for *E. coli* DNA analytic after polymerase chain reaction.

The final polySia product (d) and the negative control (water, a) show no signal for amplified DNA. The positive control (*E. coli* K12 DNA, c), the supernatant of the cultivation (e) and the supernatant after cell disruption (f) show DNA contamination. The detected *E. coli* K12 DNA has a size of ~400 bp as estimated by the marker (GeneRuler[™] 100 bp Plus DNA Ladder, Thermo Scientific, b), which is in alignment with the expected size of 418 bp.

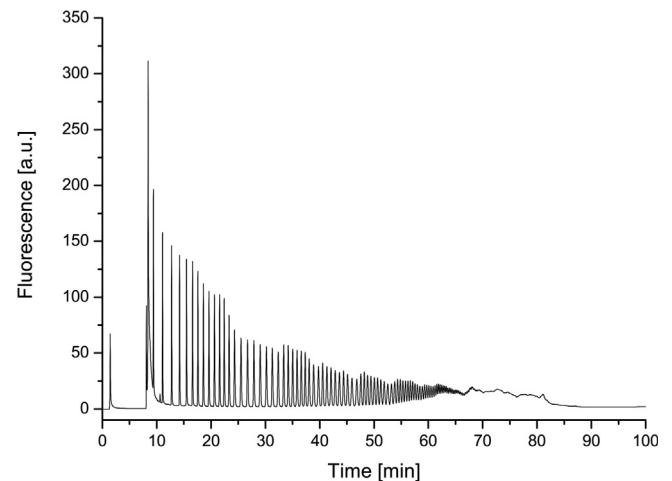


Fig. 5. Characterization of maximal polySia chain length.

The maximal chain length of the final polySia product was ~98 DP as measured by DMB-HPLC analysis. PolySia was labelled with DMB (1,2-diamino-4,5-methylene-dioxybenzene) and the different chain lengths can be detected with a detection threshold of 1.4 fmol [40]. Due to the partial hydrolysis of polySia during derivatization with DMB no quantitative analysis of the polySia chain length distribution was possible.

Table 1
Characteristics of the final product.

Characteristic	Content	Analytical method
Protein	Not detectable	Bradford method Absorbance measurement at 260 nm
DNA	Not detectable	Gel electrophoresis after polymerase chain reaction
Endotoxin	< 3EU mg ⁻¹	Based on LAL-test
Maximum chain length	~98 DP	DMB-HPLC analysis

electrophoresis after polymerase chain reaction as shown in Fig. 4. Thus, by implementation of sodium hydroxide treatment followed by anion exchange membrane adsorbers DNA was effectively reduced.

An important characteristic especially for products intended for medical application is the endotoxin content. In the previously reported process endotoxin content of the produced material was above 1000 EU mg⁻¹ [11]. Using a membrane adsorber based purification method endotoxin content was reduced to 14 EU mg⁻¹ [12]. With the downstream processing developed in this study a final product with endotoxin content less than 3 EU mg⁻¹ was produced. A control using only membrane adsorbers without prior sodium hydroxide treatment showed an endotoxin concentration of 641 (±139) EU mg⁻¹.

The overall reduction of the endotoxin content can be expressed with a log reduction value (LRV), which is the logarithmic quotient (log₁₀) of endotoxin concentration of the supernatant after cultivation divided by the endotoxin concentration after downstream processing. The endotoxin concentration after cultivation was >20*10⁶ EU mg_{polySia}⁻¹.

$$LRV = \log \frac{C_{\text{endotoxin_start}}}{C_{\text{endotoxin_end}}}$$

The LRV with sodium hydroxide treatment is ~7.0 and without sodium hydroxide treatment ~4.5. Thus, sodium hydroxide treatment is suitable to gain a product with low endotoxin contamination.

3.4.2. Chain length of the produced polySia

An important characteristic of the produced polySia is the chain length. Due to the optimized downstream processing using harsh treatment with sodium hydroxide stability of the polySia could be affected. In the following, the maximal chain length of the produced polySia was measured using DMB-HPLC analysis and compared to the control without sodium hydroxide treatment and to the already established process for polySia production in a disposable bag reactor [11].

Maximal chain length of the produced polySia was ~98 DP as shown in Fig. 5. The maximal chain length is comparable to the previously established processes [10–12]. The control without sodium hydroxide treatment showed no difference, neither in maximal chain length, nor in fluorescence intensity of the single peaks in the chromatogram. Thus, the sodium hydroxide treatment had no influence on the stability of the produced polySia.

All characteristics of the final product are shown in Table 1.

4. Conclusions

This study describes a complete production process based on single-use elements for production of polySia beginning with the cultivation of *E. coli* K1 in a disposable bag reactor and using downstream processing with sodium hydroxide treatment and disposable anion exchange membrane adsorbers to obtain a final product with high purity. Both, cultivation in a disposable bag reactor and DSP using membrane adsorbers are well accepted in

GMP-production. Further benefits of the used elements are the easy scale up to larger production capacities and flexibility.

The cultivation was optimized using defined preculture medium. Thus, only negligible amounts of complex media components (e.g., yeast extract) were transferred into the main bioreactor during inoculation. For quality assurance defined media are important during production processes [39]. Furthermore, due to the established maturation process higher product concentration of polySia in the supernatant was obtained.

After maturation cells were separated and DSP was developed based on the established protocol using ethanol precipitation and clay minerals [11], but also sodium hydroxide treatment and membrane adsorbers were implementing for further endotoxin and DNA depletion. With this DSP polySia with high purity was obtained, suitable for further applications (e.g., tissue engineering). Maximal chain length of polySia was comparable to the previously established purification processes [10–12] and was not affected by sodium hydroxide treatment.

Thus, the shown production process focusing on single-use elements well accepted in GMP-production is suitable for polySia production with low endotoxin content and protein and DNA contamination below the detection limits of the applied assay systems.

Conflict of interest

Ingo de Vries, Sarah Schreiber, Daniel Boßmann, Zawadi Hellmann and Dr. Sascha Beutel declare that they have no conflict of interest. Drs. Jens Kopatz and Harald Neumann are named inventors on patent applications related to the use of polysialic acid for neurodegenerative diseases filed by the Universities of Bonn and Cologne.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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