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An advanced method for the small-scale production of high-quality minicircle DNA

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ABSTRACT

Minicircle DNA is a promising tool in the field of gene therapy, whose products are increasingly gaining market access. Greater transfection efficiency and longer expression time as well as lower immunogenicity contrast with cost-intensive production, which also stands in the way of a broader use of the advantages of this technology in research. Starting from a commercial minicircle production kit a simple protocol for the cost-effective small-scale production of high-quality minicircle DNA to be used at a research scale has been developed by combining and improving procedures of various publications. An optimized size-exclusion chromatography method led to almost pure minicircle DNA with a superior proportion of the desired supercoiled plasmid conformation. The pharmaceutical potential of the produced minicircle DNA was investigated *in vitro* by real-time impedance assays in a tumor cell model in case of coded suicide genes as well as by ELISA of the translation product in case of coded human coagulation factor IX.

1. Introduction

Great hopes rest on somatic gene therapy. It is considered as a promising opportunity to treat severe chronic diseases causally. After decades of basic research in genetic engineering, the exploitation of the potential of gene-based therapeutic approaches is now gaining momentum. Thus, a plenty of gene therapy products is developed or clinically tested, many have been approved already (Shahryari et al., 2019).

The transfection or transduction, which means the gene transfer by an appropriate vector into a eukaryotic cell, represents one of the major challenges in the development of a safe and efficient gene therapy. Common vehicles for the transfer of the effective nucleic acids are viral vectors exploited for their natural high transduction efficiency. However, concerns related to safety of viral vectors boosted research toward the development of non-viral alternative approaches, which are typically based on the delivery of plasmid DNA.

Generally, plasmid vectors are not integrated into the eukaryotic genome and remain episomally. Therefore, the transferred gene is only transiently expressed. Drastic adverse side effects due to an unpredicted manipulation of the cells genome such as an insertional mutagenesis can be excluded. Transient expression is advantageous in particular cases, e. g. vaccination or cancer treatment with suicide genes. Non-integrating viral vectors are also suitable for such applications.

Chromosomal insertion is frequently valuable for the gene therapy of severe hereditary diseases. Reproductive retroviral vectors are used for this purpose. The disadvantage of using these integrating retroviruses is the possibility of insertional mutagenesis. A prominent example is the gene therapy of X-linked severe combined immune deficiency (SCID-X1) in two clinical trials in France and the United Kingdom with a total of 20 boys. Although the treatment was successful in general and most children exhibited long-term immune reconstitution, five of them developed leukemia (one of these died) due to insertional mutagenesis via activation of a T cell proto-oncogene (Herzog, 2010). Regarding this particular example, one should consider that children suffering from SCID-X1 usually die from infections in their first life year and the mortality rate of bone marrow transplantation (conventional therapy) is higher (min. 10% in case of matched family donor, approx. 30% with transplants from unrelated donors (DFG, 2006).

However, an interesting advancement of non-viral vectors is represented by the Minicircle (MC) technology. According to a method for the production of MC DNA published 2010 in “Nature Biotechnology” (Kay et al., 2010), it is produced in a biotechnological two-step procedure

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(growth and induction) from a special so-called "parental plasmid" carrying the gene to be transferred using a genetically modified *E. coli* strain that expresses two enzymes required for this purpose in an L-arabinose-induced manner (Fig. 1).

In this process both the antibiotic resistance gene (undesirable in gene therapy) (EMA Committee for Advanced Therapies, 2018) and the bacterial plasmid backbone (provokes innate immune response via activation of toll-like receptor 9 (TLR9) through unmethylated CpG motifs (Klinman, 2004)) are removed. The resulting minicircles are significantly smaller which is known to increase the transfection efficiency (Kreiss et al., 1999) (intracellular transport through the cytosol into the nucleus is facilitated (Lechardeur and Lukacs, 2006)). In addition, compared to conventional plasmid DNA, the duration of gene expression is extended, probably also due to the lack of prokaryotic sequences, not because of missing CpG motifs, but based on the disconnection of the bacterial backbone from the eukaryotic expression cassette (Chen et al., 2003, 2008). The expected long-lasting transgene expression, at least in tissues with low cell turnover rate (Chen et al., 2003) could enable the treatment of monogenetic diseases like hemophilia B. Besides, the nonviral generation of induced pluripotent stem cells through MC DNA was described (Jia et al., 2010). Finally, the lower safety requirements compared to working with viral vectors is a very practical advantage of MC DNA.

The obstacle for a broad use of MC DNA in clinic, but also in research is the cost-intensive production. High-priced contract manufacturing is offered, but especially in research a cost-effective production of various small batches is required. MC production kits are available, but they are at a very high price and allow only a few batches to be produced, while good results are not guaranteed. To enable the access to amounts of MC DNA sufficient for cell culture experiments, we offer a step-by-step protocol (Table S1) for a cost-effective production of various small batches of high-quality MC DNA.

We show the superiority of the applied size-exclusion chromatography (SEC) method for the isolation of supercoiled (sc) MC plasmid DNA over already published SEC methods. The produced MC DNA is of therapeutic potential (suicide genes for cancer treatment and hFIX gene for treatment of haemophilia B). Additionally, MC DNA carrying the GFP (green fluorescent protein) reporter gene was produced und used to evaluate the biological activity and transfection efficiency via flow cytometry. The efficacy of the produced MC DNA carrying a suicide gene or the hFIX gene was evaluated in the cell culture.

2. Materials and methods

2.1. Minicircle production kit

The Kit has been purchased from BioCat GmbH (Heidelberg, Germany), but was developed by System Biosciences (SBI): "MC-Easy minicircle DNA Production Kit with ZYCY10P3S2T *E. coli* minicircle Producer Strain" (5 preps). The suicide genes (dianthin and saporin) were initially located on a pET11d vector. The gene of interest was flanked with the cleavage sites of SalI and NheI via polymerase chain reaction (PCR) and cloned into a pJET1.2 vector via blunt end ligation (CloneJET PCR cloning kit, Thermo Fisher, Waltham, USA). Subsequently, the gene was cloned into the kit vector pMC.CMV-MCS-SV40polyA ("MN501A1") under exploitation of both cleavage sites. Saporin and Dianthin are plant-derived toxins from *Saponaria officinalis* L. and *Dianthus caryophyllus* L., which irreversibly inactivate the ribosomal protein machinery (Schrot et al., 2015). The pcDNA3-HLP-hFIX (pFIX-HLP) plasmid contains the human coding sequence of the coagulation factor IX protein (hFIX) under the expression of the hybrid liver-specific promoter (HLP). The coding hFIX cassette was PCR amplified to insert the SpeI and SalI restriction sites and directly cloned into the vector pMC.CMV-MCS-SV40polyA. Thus, the corresponding parental plasmids (PP) were obtained and used for MC production like described in 2.3. For the production of GFP minicircles a commercial PP pMC.CMV-GFP-SV40PolyA (MN601A1) was used (BioCat GmbH, Heidelberg, Germany).

2.2. Production of starting material

The first step was to produce the starting materials (parental plasmid precursor pMC.CMV-MCS-SV40polyA (MN501A1), modified *E. coli* ZYCY10P3S2T strain, growth and induction medium). The MN501A1 was simply propagated in *E. coli* Dh5α cells and prepared with a ZymoPURE Midiprep Kit (Zymo Research Europe GmbH, Freiburg, Germany). The special *E. coli* ZYCY10P3S2T strain was cultured in antibiotic-free lysogeny broth (LB) medium (Miller's Modification, Sigma-Aldrich, Steinheim, Germany) under sterile conditions and used for the generation of competent cells with the Roti®-Transform Kit (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.3. MC formation

After cloning the genes of interest into MN501A1 (see 2.1) the

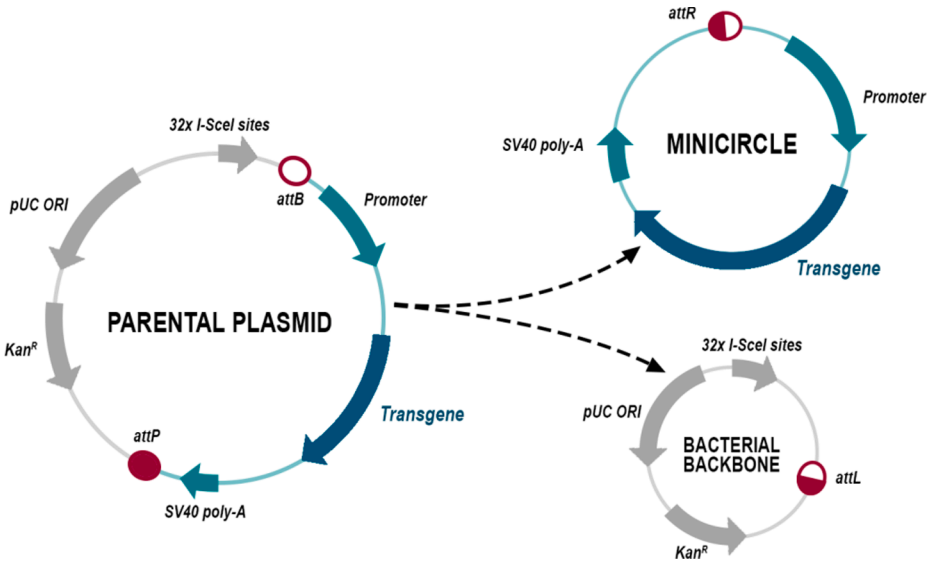

Fig. 1. Illustration of minicircle formation (according www.systembio.com). Addition of L-arabinose to a culture of the *E. coli* ZYCY10P3S2T strain induces the expression of the phage integrase PhiC31 and the homing endonuclease I-SceI. The integrase recognizes the bacterial attachment site (attB) as well as the phage attachment site (attP) and mediates their recombination resulting in minicircle (MC) and the bacterial backbone (also called miniplasmid). The latter is degraded by the I-SceI endonuclease (several recognition sites are spread over the bacterial backbone).

obtained parental plasmids were used to transform competent ZYCY10P3S2T cells, which were subsequently used to prepare glycerol stocks for later MC production. In principle the minicircle production and purification protocol (Table S1) follows the procedure published in 2010 (Kay et al., 2010). At first, the glycerol stock was plated out on an agar plate and incubated at 37 °C overnight. One CFU was slightly touched with a pipette tip to inoculate 2 mL pre-culture with it. After 1 h of incubation at 30 °C 50 μL of the pre-culture were used to inoculate the main culture (400 mL per approach). Modified terrific broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with Glycerol (4 mL/L) and kanamycin (50 μg/mL) was used as growth medium (400 mL per approach). The main culture was incubated in 2L culture flasks with chicanes at 30 °C and 100 rpm in a Thermo Scientific™ MaxQ™ 5000 Floor-Model Shaker overnight for 15.5 h. The induction medium composed of 384 mL LB broth, 16 mL 1 M NaOH and 400 μL 20% (m/v) L-arabinose for 400 mL overnight culture was added in order to reach a arabinose concentration of 0.01% in the final mixture. Induction was performed at 32 °C for additional 5 h.

2.4. MC pre-purification/SEC

Instead of using prep kits a primary purification and intermediate recovery of MC DNA based on existing publications (Alves et al., 2019; Horn et al., 1995) has been implemented (see step-by-step protocol in the Supplementary Information). For the following centrifugation steps in frame of the pre-purification of MC DNA an Avanti J-26XP high performance centrifuge from Beckman & Coulter was used.

In brief, bacteria were pelleted in 500 mL (JA-10 rotor) centrifuge bottles (6000 *g*, 4 °C, 20 min), suspended in 50 mL resuspension buffer (50 mM Tris base, 10 mM EDTA, pH 8.0) and lysed with lysis buffer (0.2 M NaOH, 1% SDS). After 5 min at room temperature 50 mL neutralization buffer (3 M potassium acetate, acetic acid, pH 5.5) was added. After 20 min incubation on ice the mixture was centrifuged (17,700 *g*, 4 °C, 60 min), the supernatant was transferred into new centrifuge bottles and 0.7 volumes of cold 2-propanol were added. After 2 h at -20 °C the nucleic acid precipitate was centrifuged (17,700 *g*, 4 °C, 30 min) and the supernatant was discarded. The air-dried pellet was dissolved in 20 mL TE buffer (10 mM Tris base, 1 mM EDTA, pH 7.4) and the solution was transferred into 50 mL (JA-25.50 rotor) centrifuge tubes already filled with 3.9 g ammonium acetate (to reach a final concentration of 2,5 M). After 15 min of incubation on ice and centrifugation (20,000 g, 4 °C, 30 min) the supernatant was transferred into new centrifuge tubes, mixed with 0.5 volumes of a 30% PEG/1.6 M NaCl solution and incubated in the refrigerator overnight. The precipitated enriched plasmid DNA was pelleted via centrifugation (22,000 *g*, 4 °C, 30 min) and residual PEG solution was removed carefully by pipetting. In order to reduce the load of small molecule contaminants onto the SEC column in the final purification a “no loss” dialysis with the Tube-O-DIALYZER™ Medi dialysis system (50 kDa MWCO, 0.2–2.5 mL sample volume, G-Biosciences, Geno Technology Inc., USA) was conducted against column buffer (TE with 0.1 M NaCl). In this way, the column had to be flushed less long for equilibration afterwards separation.

Eventually, according to the work of Horn (Horn et al., 1995) and Ferreira (Ferreira et al., 1997) the MC rich solution was subjected to SEC with Sephacryl™ S-1000 Superfine (GE Healthcare UK Limited, Buckinghamshire, UK) as stationary phase. An YMC ECO glass column (YMC Europe GmbH, Dinslaken, Germany) with 1000 mm length and an internal diameter of 25 mm was used. Bed height was 950 mm. The slurry was prepared as described in the Sephacryl™ S-1000 SF manual. The column was packed using an YMC coupling adapter under a flow of 1.72 mL/min as recommended. The sample (1 mL) was chromatographed with a flow rate of 0.3 mL/min at an old Merck Hitachi D-7000 HPLC device. TE/NaCl buffer was used (10 mM Tris base, 1 mM EDTA, 0.1 M NaCl) as mobile phase. The supercoiled MC DNA fraction (approx. 30 mL) was collected in a sterile and endotoxin-free 50 mL tube (Sarstedt AG & Co. KG, Nümbrecht, Germany). The final product has been obtained by concentration and desalination by ultrafiltration (UF) with Amicon ultra 15 tubes with 30 kDa MWCO (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, IRL) and UltraPure™ distilled water (Thermo Fisher, Darmstadt, Germany). Initially, a thinner YMC ECO glass column (I.D. = 10 mm) was used for optimization with a final flow rate of 0.1 mL/min.

2.5. Agarose gel electrophoresis and restriction analysis

For the optimization of the SEC (column with 10 mm I.D.) 150 μL fraction samples were concentrated by vacuum centrifugation to 20 μL mixed with 4 μL 6x loading dye, injected into a 1% agarose gel (50 mL with 0.2 μg/mL ethidium bromide) and ran at 110 V for 60 min with TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). For the final product control 150 to 200 ng MC DNA in 20 μL (native and digested/linearized with 0.5 μL Sal-I) and for comparison 700 to 800 ng feed solution (FS) nucleic acids in 20 μL were used and treated equally. DNA ladders 100 bp extended (Carl Roth GmbH, Karlsruhe, Germany) or 1 kb Plus (New England Biolabs, 240 County Road, Ipswich, Massachusetts 01938) were used. DNA concentrations and absorption ratios (A260/A280, A260/A230) were calculated with a Thermo Scientific™ Nanodrop™ UV/VIS spectrophotometer.

2.6. Evaluation of the biological activity of MC-Sap, MC-Dia, MC-GFP

2.6.1. Cell culture

The neuroblastoma cell line Neuro-2A (ATCC®CCL-131™) was cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Biochrom, Berlin, Germany), supplemented with stable glutamine and 10% of Fetal Bovine Serum (FBS). Cells were cultured at 37 °C and 5% carbon dioxide.

2.6.2. Transfection efficiency and biological activity

The MC DNA (D) was applied after nanoplexes (PD) had been formed with K16 lysine peptide (P) (GeneCust, Boynes, France) in a mass ratio of 4 to 1 (N/P ratio 10.85). These nanoplexes were tested in presence or absence of a particular saponin (AG1856) as transfection enhancer (Clochard et al., 2020). The formulation of nanoplexes (PD) and the transfections were performed as described by Sama (Sama et al., 2017) with the small modification that cell culture medium was used also for the transfection (instead of OptiMEM). The transfection efficiency was evaluated by flow cytometry (transfection with PD containing GFP-MC) and impedance based live cell imaging (transfection with PD with GFP- or Saporin- or Dianthin-MC) as described by Sama (Sama et al., 2017).

2.7. Cell culture and evaluation of FIX antigen levels

Human hepatocyte-derived carcinoma (Huh7) cells were cultured in DMEM supplemented with glutamine, penicillin and streptomycin antibiotics and with 10% of FBS. Cells were grown at 37 °C and 5% carbon dioxide conditions.

Huh-7 cells were seeded on 12-well ($2x10^5$ cells/well) plates, transfected with Lipofectamine 2000 reagents (Thermo Scientific, Carlsbad, CA, USA) with different amount of pFIX-HLP or equimolar amount of MC-HLP-hFIX. Four hours post-transfection, medium was replaced with OptiMEM media supplemented with Vitamin K (5 μg/ml) to allow proper FIX maturation. Forty-eight hours post transfection, media were collected and FIX antigen was detected by ELISA assay (Affinity Biologicals, Ancaster, ON, Canada) using serial dilutions of human recombinant FIX as standard curve, as previously described (Scalet et al., 2019).

3. Results

3.1. Optimization of SEC & production of minicircles

The chromatographic resolution obtained with the thin column (I.D. 10 mm) was significantly improved compared to the results of Ferreira et al. (Ferreira et al., 1997) by decreasing the sample volume to a fortieth (0.2 mL) and the flow velocity to the half (7.64 cm/h instead of 14.93 cm/h). The chromatogram and agarose gel analysis of the collected fractions are shown in Fig. 2.

By using a thicker column (I.D. 25 mm) five times more sample volume could be applied (1 mL). The resolution was further improved by additional flow velocity reduction to the half (3.67 cm/h). Prepurified MC DNA out of 2 × 400 mL overnight culture was used as feed solution (FS) (see Table S1). An example for the resulting chromatogram is depicted in Fig. 3a. The maximum recording time for the chromatogram was 10 h, so the first 10 h are not depicted (only base line was seen).

Supercoiled (sc) MC-Saporin (2511 bp) was eluted approximately between 930 and 1080 min (15.5 and 18 h) directly after the open-circular (oc) conformation (with a little overhang) and before the RNA, which started to be eluted at the end of the chromatogram and resulted in the biggest peak by far (>1200 mV signal intensity) although most of it should be removed during prepurification. Initially, genomic (chromosomal) DNA followed by residual parental plasmid was eluted (see Fig. 2). To avoid big proportions of open-circular conformation the collection of the sc MC DNA peak was initialized in some distance to the first ascent (at 960 min in the chromatogram of Fig. 3a).

For the isolation of the 4.5 kb minicircle construct carrying the hFIX gene it was necessary to reduce flow rate and sample volume once more to obtain sufficient resolution (for adjustments see Table S3). The corresponding chromatogram and gel analysis are depicted in Fig. 4

3.2. Evaluation of purification protocol steps at the example of MC-Sap

Samples have been taken after different steps of the purification protocol in order to follow the purification progress in agarose gel electrophoresis (see Fig. 3b). For comparison, the column feed solution and the final isolate of supercoiled MC DNA were analyzed both native and digested.

3.3. Biological activity

3.3.1. Biological activity of MC-Sap, MC-Dia, MC-GFP

The functionality of the generated minicircles was verified in cell culture in two different ways: real-time cell analysis and flow cytometry.

The efficacy of the suicide gene carrying minicircles was evaluated with the iCelligence® system (ACEA Biosciences, Inc., San Diego, CA, USA), a real-time cell analysis device, which offers an online impedance measurement. Decreased impedance at the bottom of the cell culture vessel is usually a consequence of toxic effects like inhibition of cell proliferation or cell death. Different produced minicircles encoding for GFP or for a suicide gene (Dianthin or Saporin) were analyzed (see Fig. 5). Following application (after 48 h) the GFP construct resulted in a slightly decrease of the impedance signal compared to the negative control whereas the decrease was more prominent with the Saporin or Dianthin construct. The application of the latter constructs in presence of the saponin led to a remarkable drop of the impedance signal indicating a strong transfection enhancement (the saponin alone was not toxic). The MC-GFP construct did not result in such a drop and was therefore harmless.

A transfection efficiency close to 80% was determined via flow cytometry using nanoparticles containing MC-GFP in combination with saponin (see Fig. 6). Without saponin a tranfection efficiency of 10% was reached.

3.3.2. Biological activity of MC-HLP-hFIX

The ability of MC-HLP-hFIX minicircle to express hFIX protein was evaluated in human hepatic cell line to mimic the physiological site of FIX expression, the liver. Upon transfection of the hFIX coding plasmid or of the resulting minicircle, hFIX antigen levels were assessed in cellular media by ELISA assay and by exploiting serial dilution of human recombinant FIX as standard curve.

By keeping constant the amount of transfecting agent, the delivery of lower amount of pcDNA3-HLP-hFIX resulted in higher hFIX level due to higher transfection efficacy. The same effect was detected upon transfection of equimolar amount of the HLP-hFIX minicircle. Notably, the minicircle MC-HLP-hFIX performed better than the plasmid pcDNA3-HLP-hFIX, with an average 1,51-fold increase of antigen levels (see Fig. 7).

4. Discussion

The aim of this study was the development of a purification protocol (see Table S1) suitable for the typical amount of MC DNA, which can be generated by following the MC production protocol published by Kay et al. in 2010 (Kay et al., 2010), without using a Qiagen 2500 prep kit for the isolation. For the preparation via Qiagen prep kit Kay et al. used double the recommended buffer volume to ensure clean and complete recovery. Unfortunately, the MC DNA was always digested in the depicted agarose gels in their publication for which reason it is not possible to evaluate the proportion of the desired supercoiled conformation.

A large proportion of supercoiled plasmid conformation is desirable as this is the natural and physiological most active conformation. The U. S. FDA recommends a minimum specification of >80% supercoiled conformation for the industrial production of therapeutic plasmid DNA

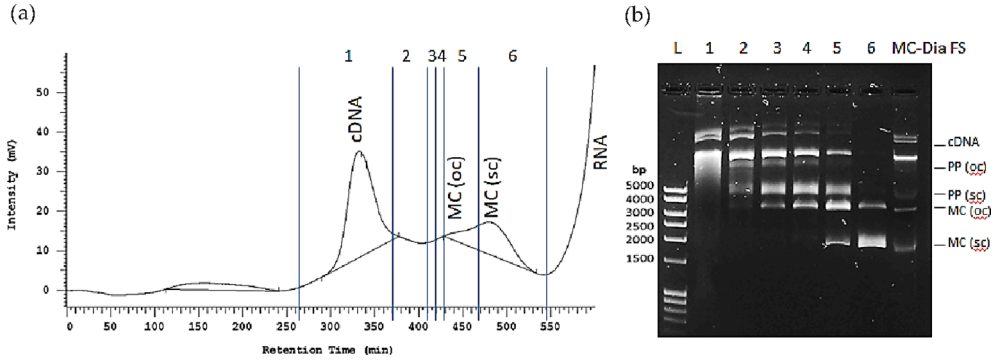

Fig. 2. Size-exclusion chromatography of MC-Dia FS with thin column. I.D. 10 mm; stationary phase: SephacrylTM S-1000 SF; mobile phase: TE buffer with 0.1 M NaCl; flow rate: 0.1 mL/min; sample volume: 0.2 mL. (a) Chromatogram with fractions 1 to 6 (detection λ: 210 nm). (b) Agarose gel electrophoresis of fractions 1 to 6 (lane 2 to 7) and MC-Dia FS (lane 8), DNA ladder 100 bp extended on lane 1; 1% agarose gel; 110 V for 1 h in TAE.

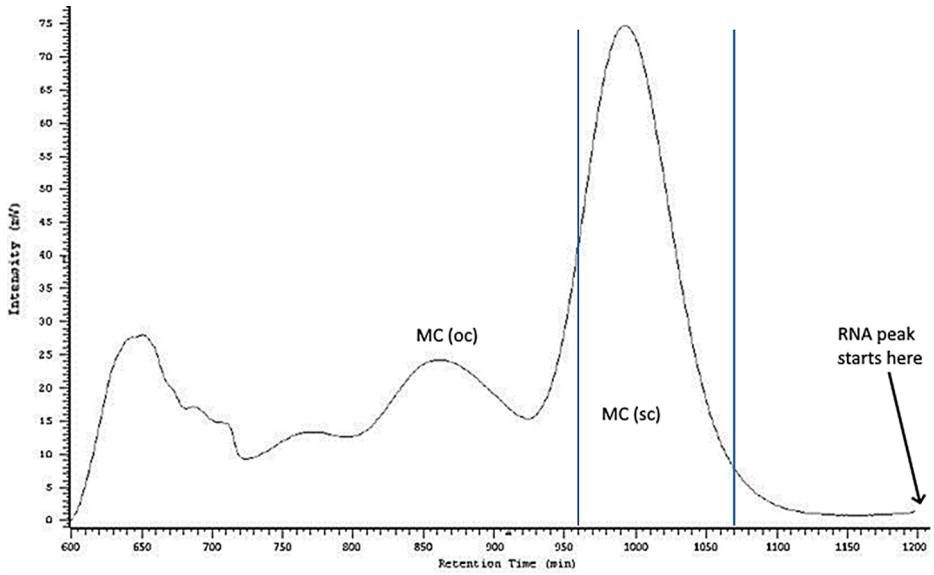

Fig. 3a. Chromatogram obtained from SEC of MC-Saporin (MC-Sap) feed solution obtained with thick column. Collection of supercoiled MC-Sap between 960 and 1070 min; column I.D. 25 mm; stationary phase: Sephacryl^{TM} S-1000 SF; mobile phase: TE buffer with 0.1 M NaCl; flow rate: 0.3 mL/min; sample volume: 1 mL; detection λ: 210 nm.

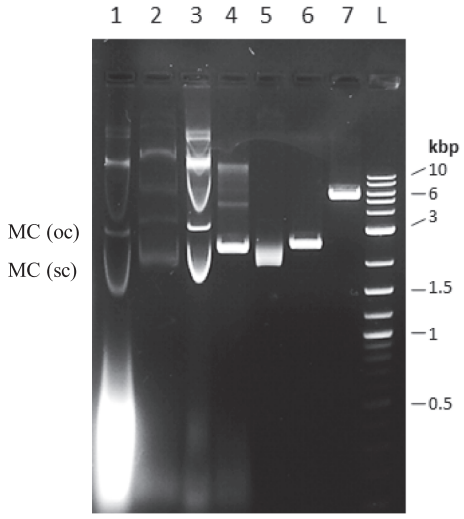

Fig. 3b. Agarose gel electrophoresis for analysis of the corresponding pre-purification steps. 1% agarose gel; 110 V for 1 h in TAE; lane 1: after 2-propanol precipitation; lane 2: after ammonium acetate precipitation; lane 3: column FS undigested; lane 4: column FS (digested with SalI); lane 5: final product undigested (sc MC-Sap); lane 6: final product (digested with SalI); lane 7: PP digested with SalI (6553 bp); lane 8: 1 kb Plus DNA Ladder (NEB).

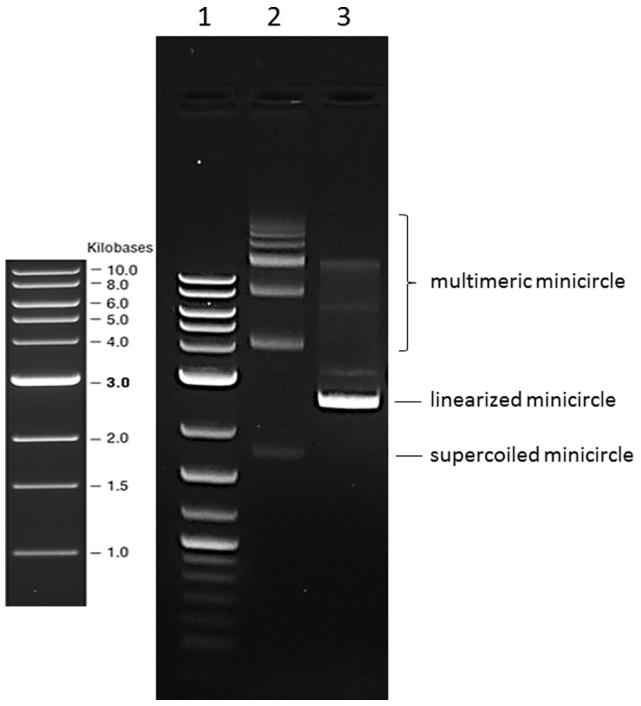

Fig. 3c. For comparison, agarose gel analysis of a MC-Sap prep with the MC preparation kit (SBI). 1% agarose gel; 110 V for 1 h in TAE; lane 1: DNA ladder 1 kb Plus (NEB); lane 2: MC kit prep (undigested); lane 3: MC kit prep (digested with SalI).

(U.S. Food and Drug Administration, 2007). Size-exclusion chromatography with Sephacryl^{TM} S-1000 SF is a suitable method for the isolation of supercoiled plasmid DNA under mild conditions, even though a small proportion of open-circular conformation could remain when using the method presented. Additionally, the removal of genomic DNA, RNA, proteins and lipopolysaccharides (LPS) is performed simultaneously (Almeida et al., 2020; Horn et al., 1995). The removal of LPS is essential and a general challenge in providing pharmaceutical ingredients for parenteral application, especially when originated from *E. coli*, because cell wall debris of gram-negative bacteria is the main source of LPS (endotoxins).

In our experience, alkaline lysis is a delicate balance between bacterial quantity, buffer volume and exposure time. Uneven distribution of the lysis buffer easily leads to excessive pH values in individual areas of the preparation and, as a result, to an increased proportion of open-circular conformation of plasmid DNA. Excessive mixing, especially after addition of the neutralization buffer P3, easily leads to shearing of chromosomal DNA and contamination with it. Apart from this, traces of genomic DNA can hardly be excluded when using a plasmid preparation kit.

This was the reason why a size-exclusion chromatography method was adapted for the purification of supercoiled MC DNA after the

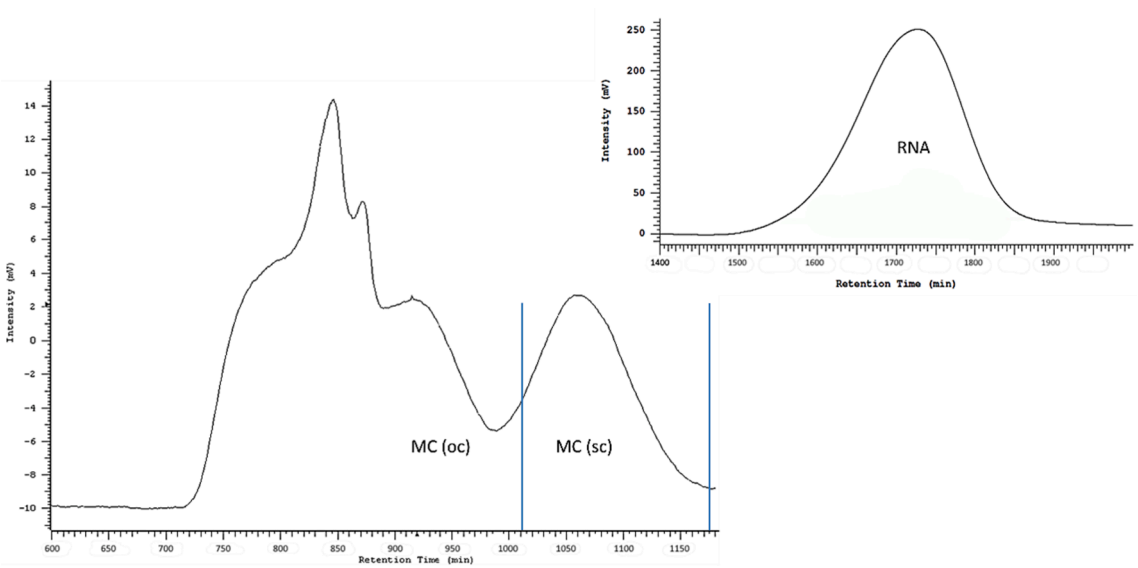

Fig. 4a. Chromatogram obtained from SEC of MC-HLP-hFIX feed solution with thick column (MC = minicircle, HLP = hybrid liver promoter, hFIX = human coagulation factor IX). Collection of supercoiled MC-HLP-hFIX between 1012 and 1176 min; column I.D. 25 mm; stationary phase: Sephacryl^{TM} S-1000 SF; mobile phase: TE buffer with 0.1 M NaCl; flow rate: 0.25 mL/min; sample volume: 0.25 mL; detection λ: 210 nm.

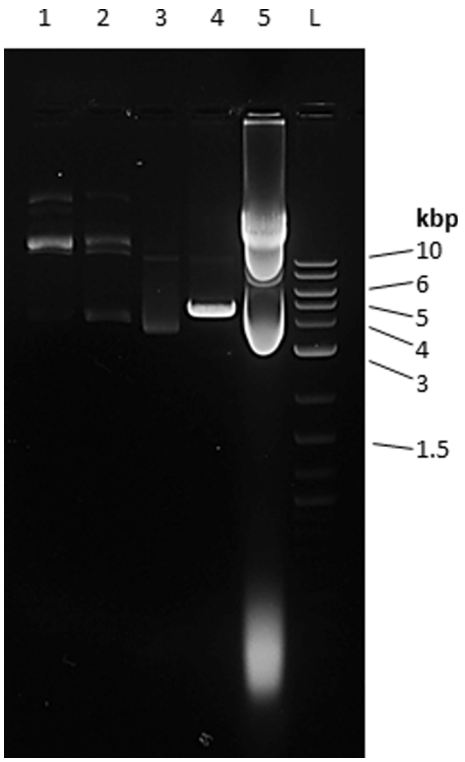

Fig. 4b. Agarose gel electrophoresis for analysis of the corresponding MC-HLP-hFIX purification result. 1% agarose gel; 110 V for 1 h in TAE; lane 1: fraction 945–990 min; lane 2: fraction 990–1012 min; lane 3: fraction 1012–1176 min = final product undigested (sc MC- HLP-hFIX); lane 4: final product (digested with SalI); lane 5: column FS undigested; lane 6: 1 kb Plus DNA Ladder (NEB).

alkaline lysis.

There are several publications available, which confirm the general suitability of SEC with Sephacryl^{TM} S-1000 SF for the purification of plasmid DNA, but the chromatographic resolution of the nucleic acid species - especially of the plasmid conformations - obtained with the SEC parameters applied by us is superior.

If you have a look in the work of Horn et al. (Horn et al., 1995) and compare the chromatogram (Fig. 3a in their publication) with the chromatograms presented in this study (Fig. 3a & Fig. 4a) one will recognize that they must had to have a large proportion of open-circular conformation (which elutes before the supercoiled form), although this is not really visible in the agarose gel (Fig. 3b, (Horn et al., 1995)). Maybe alkaline lysis was performed excessively.

Ferreira et al. (1997) applied a smaller bed height and flow rate, but a bigger flow velocity taking the smaller column diameter into account. With both methods too fast flow velocity was applied for the separation of macromolecules via SEC. Besides, the sample volumes were too large (8 and 10 mL). Consequently, the obtained resolution was not sufficient for the separation of the different plasmid conformations. For a better overview about SEC parameters of different published methods for the plasmid purification via SEC with Sephacryl^{TM} S-1000 SF have a look at Table S3.

More recently in 2020, a slightly improved SEC method for the isolation of supercoiled minicircle DNA was published. Almeida et al. (2020) investigated the influence of bed height, flow rate and sample volume on the separation efficiency and confirmed once more the suitability of this methodology for the removement of RNA, proteins, lipopolysaccharides and genomic DNA. Although they describe the isolation of pure supercoiled minicircle DNA, it is obvious in the agarose gel analyses that the oc and sc conformation elute simultaneously. Consequently, the isolation of pure sc plasmid DNA seems not possible with this method. The improved separation in comparison to the work of Horn et al. and Ferreira et al. is based on the reduction of flow velocity and especially of sample volume (see Table S3).

GE Healthcare recommends a flow velocity of 1–10 cm/h for SEC, the bigger the molecules the slower the flow should be chosen. Regarding the optimal sample volume 0.5–1% of the bed volume is an useful guiding value for SEC. Table S3 underlines the influence of the column dimensions. Taking the flow velocity [cm/h] and the ratio of sample volume to bed volume (as well as the size of the start zone) into consideration is helpful for comparing different methods and columns as well as for the optimization of a particular method.

We attribute our additional improvement in resolution compared to the results of Almeida et al. to a further flow velocity reduction in combination with a smaller sample/column bed proportion. As we have used a thicker column this is equal to a much smaller start zone.

The presented purification protocol is applicable if only small amounts (0.1–0.2 mg) of different minicircles are required and

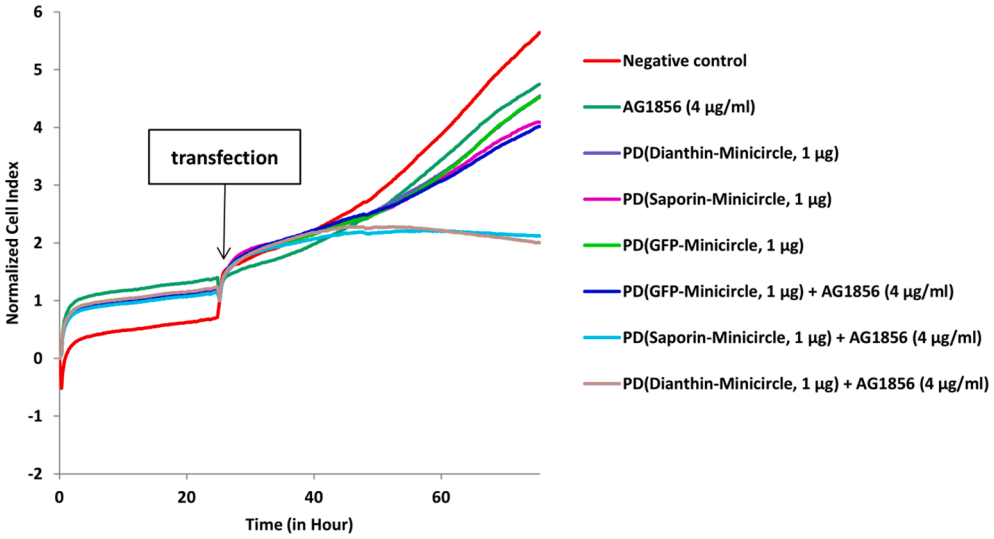

Fig. 5. Real-time cell analysis by impedance measurement of Neuro-2A cells after transfection with nanoplexes (PD = Peptide-DNA). Nanoplexes made from different minicircles (effector genes: Saporin, Dianthin, GFP) were applied with and without a novel saponin (AG1856) for transfection enhancement ("Sapofection"). Non-toxicity results in higher impedance. PD (Saporin-MC) and PD (Dianthin-MC) caused slightly reduced impedance; together with AG1856, the decrease was obvious. The influence of AG1856 alone or PD (GFP-MC) alone or both together (PD (GFP-Minicircle) + AG1856) on cell viability was marginal. Figure exemplary (shows software generated mean of both duplicates of one experiment) – each condition was tested in duplicates in two independent experiments.

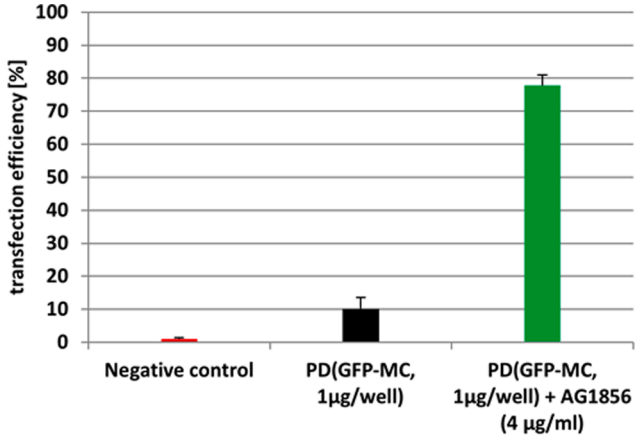

Fig. 6. Determination of transfection efficiency by flow cytometry. PD (GFP-MC) alone led to 10% transfected cells, PD (GFP-MC) in combination with AG1856 led to almost 80% transfected cells. Each condition was tested in duplicates and two independent experiments (n = 4). Transfection efficiency: Negative control 1.005 ± 0.3422%; PD (GFP-MC) 10.1075 ± 3.4632%; PD (GFP-MC) + AG1856 77.7825 ± 3.1697%.

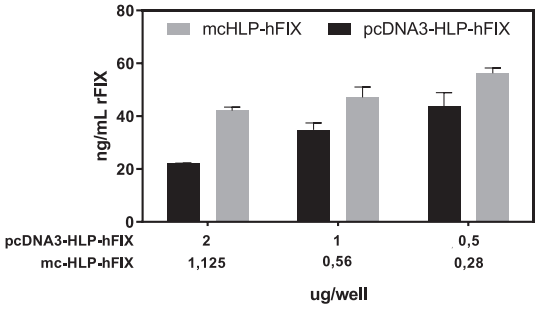

Fig. 7. Evaluation of hFIX expression by detection of hFIX antigen level by ELISA assay on culture medium collected after MC-HLP-hFIX and pcDNA3-HLP-hFIX transfection in Huh-7 (n = 2).

commercial prep kits should be waived. The latter can be advantageous in terms of cost-effectiveness as well as sustainability since a lot of waste can be avoided. If time should be saved, but the prep kit result is qualitative insufficient, a combination of Qiagen prep kit and subsequent size-exclusion column chromatography could be an option. The more preparations are performed the more cost-effective the presented method is over the MC production kit under assumption of the availability of the required laboratory equipment. Contract manufacturing is the most expensive option.

Initially, the MC production kit from SBI was used to produce MC-Sap. Unfortunately, the original user manual (Version 75/30/2017) was not optimized regarding the incubation time (4–6 h, correct is 1 h) and the needed volume of pre-culture for inoculating the overnight culture (0.5–1 mL to 200 mL growth medium, correct is 20 µL) which led to ruined batches in the beginning. If successful, the kit preparation might still require an additional DNAse digestion with subsequent recovery by alcohol precipitation to remove remaining chromosomal DNA (part of the kit); an additional step that includes the risk of decreased yield and contamination with protein. Finally, we recognized a major share of multimeric minicircle in the kit preparation, whereas the presented method leads to isolation of supercoiled monomeric minicircle (see Fig. 3c).

With the presented protocol, we were able to produce up to 200 µg high-quality supercoiled MC-Sap (2.5 kb) per 800 mL overnight culture for instance. The MC production protocol was tested several times by different users for the preparation of minicircles of various size. The final product was analyzed spectrophotometrically to determine the DNA concentration as well as to evaluate the quality of the preparation based on the absorption ratios A260/A280 and A260/A230). The obtained

Table 1
Spectrophotometric analysis of several produced MC batches.

MC batch	Size [bp]	Final product [µL]	DNA conc. [ng/µL]	DNA [µg]	A 260/280	A 260/230
MC production kit (SBI):						
MC-Sap 1	2511	250	195,8	49,0	2,31	2,65
Presented method:						
MC-GFP	1523	300	655,8	196,7	1,87	2,20
MC-Sap 2	2511	320	596,1	190,8	1,89	2,33
MC-Sap 3	2511	250	650,0	162,5	1,88	2,28
MC-X 1	2536	320	664,8	212,7	1,89	2,29
MC-X 2	2536	300	910,5	273,2	1,87	2,19
MC-Y	3324	350	599,9	210,0	1,89	2,42
MC-hFIX-HLP	4489	330	350,6	115,7	1,88	2,57

absorption ratios of the produced MC batches (see table 1) represent high DNA purity (A260/A280 $>$ 1.8, A260/A230 $\geq$ 2.0) as well as reproducibility (no significant change of the ratios between different preparations).

5. Conclusion

This study offers a step-by-step protocol for the production and purification of MC DNA with yields of some hundred micrograms. The final purification of the supercoiled minicircle conformation is accomplished by an advanced size-exclusion chromatography method. This method underlines the importance of a slow flow velocity and a small start zone in the size-exclusion chromatography of macromolecules such as DNA. The method was tested for MC constructs of 1.5 to 4.5 kbp. Bigger constructs (>4 kbp) might require a reduction of flow velocity and sample volume for comparable resolution (see Table S3, last column). The quality of the obtained minicircle DNA was evaluated spectrophotometrically as well as via agarose gel analysis; bioactivity was proven in the cell culture. Similar size-exclusion chromatography methods were already shown to be suitable to remove genomic DNA, RNA, proteins and lipopolysaccharides sufficiently but were less suitable for the separation of supercoiled and open-circular minicircle/plasmid conformation.

CRediT authorship contribution statement

Hardy Mitdank: Conceptualization, Investigation, Writing - original draft. **Simko Sama:** Investigation. **Meike Tröger:** Investigation. **Maria Francesca Testa:** Investigation. **Mattia Ferrarese:** Investigation. **Dario Balestra:** Conceptualization, Writing - original draft. **Mirko Pinotti:** Conceptualization, Writing - original draft. **Alexander Weng:** Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120830.

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