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Analytical tools in *minicircle* production

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Analytical tools in minicircle production

Anja Rischmüller¹,², Martina Viefhues², Mareike Dieding², Markus Blaesen¹, Marco Schmeer¹, Ruth Baier¹, Dario Anselmetti², and Martin Schleef¹*

¹ PlasmidFactory GmbH & Co. KG, Meisenstr. 96, D-33607 Bielefeld, Germany
² Experimental Biophysics and Applied Nanoscience, Bielefeld University, D-33615 Bielefeld, Germany

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1. **Introduction**

Clinical application of DNA in either the human medical or veterinary field including DNA vaccination is – after a period of drawbacks and doubts – again expected to be successful. The applications in the field of veterinary vaccination require extremely large amounts of DNA and hence require a process technology that is productive enough to fulfil this. As summarized earlier [1, 2], this is generally possible but still at a quality level that ten years ago was not at all sufficient to meet regulatory requirements. The new types of vector systems summarized within this chapter are, not only due to the purification technology, able to now fulfil these requirements, especially because the systems themselves avoid high contamination levels.

After such tools have been evaluated to (a) fulfil the quality requirements and (b) regulatory needs, it is still an issue to be able to produce large amounts of DNA – particularly special types of DNA such as minicircle (MC) DNA.

1.1 **Gene transfer for therapy, vaccination, stem cells**

Recent applications of DNA in gene or cell therapy, vaccination or transfection of specific cell lines were summarized recently [3]. Mainly the applications for gene transfer into producing eukaryotic cells are an issue, since these lead to highly complex pharmaceutical products that can be produced economically and as closely as possible like in native situations. While earlier approaches tried to generate stable cell lines to produce proteins (e.g. antibodies, enzymes, etc.) or viruses (e.g. gene therapy vectors), it turned out that these approaches are time consuming and in parallel expensive. Only a small amount of DNA is required for this manipulation while the so-called transient transfections require larger amounts of DNA (up to grams for the transfection of cells within e.g. 100 L cultivation). This sounds like a ‘higher cost of goods’-aspect but with respect to the time saved in just testing certain constructs, this way finally leads to an advantage in biopharmaceutical manufacturing, which is more important than the cost-of-good aspect: the costs for time-to-market.
1.2 Plasmids

So far plasmid DNA was used for all starting steps ending up with a gene vector. This vector might be a plasmid by itself or a viral vector, a RNA or – as shown later in this paper – a minicircle. By definition any DNA circle (even large ones) is a plasmid if it contains an origin of replication (ori) working within a bacterial cell. These sequence elements are necessary to allow the bacterial cell to (a) replicate the plasmid with the goal of increasing or maintaining the number of plasmids per cell, and (b) regulating this number of plasmids. Certain approaches in plasmid DNA manufacturing made use of this to increase in direct or indirect ways the number of plasmids – hence the productivity of manufacturing[4, 5].

However, the ori is not small and still a sequence of bacterial origin. This means that, for pharmaceutical approaches, it might be less critical than a sequence for the resistance against certain antibiotics, but still the ori is present. In consequence it takes “capacity” of the plasmid vector, which means that larger plasmids reduce the number of pharmaceutically active molecules within a defined mg amount of DNA (or a specific “dose” in pharmaceutical applications). Additionally, it is more difficult to obtain high quality and productivity of large plasmids. Since the quality aspect is even more important after observing a suspected “toxicity of plasmids” in in vivo applications (turning out later as an aspect of contamination bacterial chromosomal DNA within kit-grade standard procedures) [6, 7], the type of purification is even more important. Additionally, reduction of plasmid size, especially by removing the bacterial sequence motifs, leads to unwanted CpG motifs, which may lead to uncontrolled immune responses [8].

Within chapters 2, 3, and 4 of this book we summarized a new class of plasmids that were at least free of antibiotic resistance markers. They are more efficient due to their reduced size but require specific host strains that are either difficult to grow (by keeping quality and quantity) or not available free of charge and licenses or both. The development of DNA vectors lacking the bacterial backbone sequences is also important from a regulatory point of view, with respect to product safety.
The European authority for the evaluation of medical products (EMEA, now EMA) proposed within its guidelines for medical gene transfer products to avoid selection markers like resistances against antibiotics (CPMP/BWP/3088/99). Other national entities (e.g. Paul-Ehrlich-Institute in Germany, AFSSAPS in France and MCA in UK) agreed in this. Therefore, research institutes and companies like PlasmidFactory currently developed plasmid DNA without genes for the resistance against antibiotics and other selection markers. One elegant satisfying approach is the minicircle DNA, a homogenous circular derivative of plasmid DNA, consisting only of the active gene (cassette) of interest (GOI) and no ori nor any other not required DNA sequence.

1.3 Minicircle systems

Over the last years the idea of using a “circular gene of interest” and not having additional plasmid elements enclosed that are only needed to allow the selection, amplification and maintenance within bacterial producer cells was always of interest for the scientific community. Certain approaches were made as summarized within Chapter 1 of this book, but only two were able to overcome the critical borders of such systems. The general approach is to split a starting plasmid carrying sequences including the sequence of interest (e.g. with a gene expression cassette) into two parts: one being exclusively the sequence of interest with as little other sequences as necessary and a second part carrying all other sequences of the plasmid. These starting plasmids are the ancestors of the two split products and we call them “parental plasmids” (PP). The split products could in theory be generated by restriction digestion but then they are linear and do not have any supercoil structure. Hence, they are sensitive to nucleases due to their open end structure, even if in one approach [9] the spilt products are capped to protect them – but still without supercoiled structure. In our approach, both split products are circular instead, caused by an intramolecular recombination that does not release the split products without closing the circular DNA molecules again (see Fig. 1.3.1). Even more, both molecules are supercoiled (ccc). One of both contains the ori and – hence – is by definition a plasmid. We call it the miniplasmid (MP). Since the other one does not have this and also is not able to be further replicated
within the bacterial strain, it is not a plasmid anymore – we call it *minicircle* (MC). The original scientific development was patented and published by [10, 11]. Subsequently, other research teams started to further optimize the initial idea. A significant improvement was made in parallel by Bigger *et al.* [12] with the approach to get rid of the miniplasmid. They simply treated this with miniplasmid-specific digesting restriction enzymes, which results in a linear product (miniplasmid) and a supercoiled (not affected) circular product (*minicircle*). This mixture was easy to purify and all further work we performed could be started to deliver a *minicircle* product [13, 14].

2 Production of *minicircles*

2.1 The Parental plasmid (PP)

The parental plasmid (PP) carries a restriction site that is positioned between two recombination sequences later being used to perform the recombination. Any sequence of interest can be ligated into this restriction side. Between the same recombination sites but distal to the cloning position, all system-specific sequences can be placed, as we initially published [13].

2.2 Cultivation and induction

To evaluate if the parental plasmid is functional it is necessary to initially perform small scale cultivation and induction experiments followed by a large scale cultivation in a bioreactor. An overview on the *minicircle* production process is shown in Fig. 2.2.1.

For pre-culture, 300 mL shaking flasks with baffles and a filling volume of 30 mL in LB1/2-medium (10 g L\(^{-1}\) casein pepton, 5 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) NaCl) were used. The pre-culture was inoculated with a glycerol stock culture and cultivated at 30°C for approximately 15 hours. LB1/2-medium was supplemented with 20 g L\(^{-1}\) glucose to prevent early expression of the recombinase. The shaking flask cultivations were carried out under selective conditions by adding 0.45 mL of a kanamycine stock solution to obtain a final concentration of 150 mg L\(^{-1}\). For performing an induction experiment, 1000 mL
shaking flasks with baffles were used and inoculated with the over-night culture. The filling volume was 100 mL LB1/2-medium. The expression of the recombinase respectively the recombination process was started by adding arabinose to the medium. Thirty minutes after induction, the cultivation was stopped and the cells were harvested by centrifugation (7 min, 9000g), frozen and stored at -20°C before using alkaline lysis to obtain the recombination products. Fig. 2.2.2 shows an agarose gel electrophoresis image of parental plasmid pF0904pp prepared from the pre-culture taken shortly before induction as well as the recombination products (miniplasmid and minicircle) five minutes respectively thirty minutes after induction by adding of arabinose. Already within five minutes after induction of the recombinase expression almost all parental plasmid is split into minicircle and miniplasmid DNA.

Large scale cultivations were carried out in a bioreactor (B. Braun BIO REACTOR, Diessel Biotech GmbH) with a total volume of 14 L operated with 10 L at an operating temperature of 37°C and a pH of 7.0. Adjustment of pH was carried out with 2 M sodium hydroxide solution and 2 M phosphoric acid. The flow rate of air was fixed at 1 vvm. The oxygen concentration (60%) was controlled by varying the stirrer speed in the range of 500 to 2000 min⁻¹. LB1/2-medium was used without addition of any antibiotics. All cultivations were performed as described earlier [13]. Before inoculation of the bioreactor the pre-culture was examined under the microscope. By adding arabinose to the culture the recombination was induced. After thirty minutes of further growth the cells were harvested by centrifugation for seven min at 9000g, filled in plastic bags, frozen and stored at -20°C before using for alkaline lysis to obtain the recombination products.

In Fig. 2.2.3 a time course of the induction at T=0 is shown. It is visible that no induction product is present and immediately after induction, the recombination starts being extremely efficient and is carried out to completion after not more than twenty minutes. The initial parental plasmid structure is transferred into two circular DNAs as visible after 2 min of incubation. By specific restriction digests the products can be distinguished and identified.
2.3 **Minicircle preparation**

The main approach is to separate the *minicircle* DNA from the non-desired miniplasmid DNA. We will show later that also other (contaminating) DNA can be removed by the affinity purification carried out subsequently. A specific *tag* sequence (*minicircle identification sequence*) is present on the *minicircle* molecule and this is specifically bound by a matrix for analytical or later large scale purification.

For isolation of the *minicircle* DNA from the recombination product, i.e. the mix of *minicircle* and miniplasmid, the *minicircle* DNA contains the *minicircle identification sequence* binding to a DNA binding protein attached to the surface of a solid matrix. The biotinylated DNA binding protein is specifically bound by a streptavidin covered solid matrix. The mixture of *minicircle* DNA and miniplasmid is added to the affinity chromatography material. In consequence, by means of this proprietary affinity chromatography, the *minicircle identification sequence* of the *minicircle* DNA binds to the DNA binding protein, the miniplasmid by lack of *tag* sequence does not bind. The non-binding miniplasmid is removed by washing. The eluate finally contains *minicircle* DNA only, which is further analyzed – e.g. by agarose gel electrophoresis (see Fig. 2.3.3).

3 **Analytics of minicircle production**

3.1 **In-process control**

Throughout the DNA manufacturing process certain analytical samples are taken to ensure the quality. For *minicircle* production it is especially important that the recombination does not start before this is required (see the use of a continuous microfluidic channel below). The amount of DNA within the cells growing, their integrity and all aspects of microbiological purity are carried out as published with respect to plasmid DNA manufacturing and are not explained again herein [15].
Instead, a technique first time used for the analysis of DNA from a minicircle system is shown here in detail: the atomic force microscopy (AFM). Additionally, we show results obtained by capillary gel electrophoresis (CGE) – a tool to identify and quantify the plasmid (here also the minicircle) topology. The CGE initially was established for plasmid DNA product analysis but found that there is a potential for the analysis of DNA structures in the recombination process of parental plasmids resulting in miniplasmid and minicircle as well [16]. Finally, the reliability of the non-existence of recombination product during cultivation is shown by use of a novel microfluidic channel.

### 3.1.1 Atomic force microscopy (AFM)

DNA molecules can be visualized by atomic force microscopy (AFM). The atomic force microscope (Multimode AFM, Bruker, Santa Barbara, USA) is a versatile tool to map and measure surface structures with unprecedented resolution and accuracy at the nm-scale [17–19]. The basic setup consists of a force sensor (cantilever, Tap 300 Al-G, 300 kHz, 40 Nm⁻¹, Budget Sensors, USA) and a piezo scan tube on which the sample is placed (see Fig. 3.1.1). By scanning the cantilever tip over the surface of the sample the topography can be visualized. The interaction between the sample and the cantilever tip is transduced to minimal deflections of the force sensor that can be measured by the displacement of a laser beam. The laser beam is reflected from the back of the sensor and detected by a quadrant detector [20].

DNA molecules like parental plasmid DNA and minicircle DNA are immobilized directly on a mica wafer treated with Nickel(II) sulfate. The positively charged Ni²⁺ ions support the adsorption of the DNA molecules to the mica surface (both negatively charged). Mica has a minor surface roughness and is therefore an ideal substrate for DNA imaging [21]. According to Watson and Crick [22] the DNA chain has a diameter of 2 nm and one nucleotide unit distance is 0.34 nm. AFM measurements of parental plasmid, minicircle and miniplasmid are performed in AFM tapping mode.

Parental plasmid DNA (1.5 ng µL⁻¹) obtained from culture before the induction of recombination and minicircle and miniplasmid DNA (3.0 ng µL⁻¹) obtained
from samples after recombination are applied in these AFM measurements. Pure minicircle DNA, acquired after purification of the recombination product, is also measured by AFM (data not shown). Figure 3.1.2 shows AFM pictures of the 6.8 kbp parental plasmid DNA pF0904pp (A and B) as well as the corresponding 2.3 kbp minicircle DNA MC0904 (C) and the 4.5 kbp miniplasmid DNA MP0904 (D). Due to methodical preparation prior to AFM measurement, the DNA primarily presents itself in open circle (oc) or linearly conformation. To determine the length, a DNA strand can be measured gradually, e.g. with adequate image analysis software (ImageJ).

### 3.1.2 Capillary gel electrophoresis (CGE)

Quantitative determination of plasmid and minicircle homogeneity (typically 99% ccc form) is an important tool for in-process controls (IPCs) and quality control and release of the final product. The different topologies of plasmid and minicircle DNA can be quantified by capillary gel electrophoresis (CGE) [23, 24]. In Figure 3.1.3 a schematic setup of the CGE unit is shown. The order of migration in the capillary used depends on the DNA topology and is – in contrast to agarose gel electrophoresis (AGE) – reproducible and always the same, independent from plasmid size. In addition, CGE offers high resolution and high sensitivity. The ratio of each plasmid topology can be calculated by standardized peak area, which correlates linearly to the DNA concentration of each plasmid topology.

All minicircle preparations are analyzed by capillary gel electrophoresis. The CGE analyses are performed using a P/AGE™ 2050 (Beckman Coulter, München) equipped with a LIF detector (488 nm/520 nm). Coated capillaries (DB-17; J&W Scientific, Folsom, CA) with an effective length of 30 cm, an inner diameter of 100 µm and a coating thickness of 0.1 µm are used. Just prior to analysis, the intercalating dye YOYO (Molecular Probes, Eugene, OR) is added. After pre-staining with YOYO, the plasmid samples are introduced hydrodynamically into the capillary and electrophoresis is carried out at 100 V cm⁻¹ and 30°C. In Figure 3.1.4, electropherograms of parental plasmid
pF0904pp as well as minicircle DNA MC0904 and miniplasmid MP0904 are shown.

### 3.1.3 Continuous flow separation in microfluidic channels

The intramolecular recombination of parental plasmid DNA results in two circular DNA molecules: the minicircle DNA and the miniplasmid DNA (see Fig. 1.3.1). Under optimal conditions the parental plasmid is partitioned totally into minicircle and miniplasmid DNA. Prior to induction of recombination, exclusively parental plasmid DNA is present. A premature recombination of parental plasmid DNA into minicircle and miniplasmid DNA does not occur within this system. In case this would happen, the miniplasmid molecules easily could increase and even exceed the number of parental plasmid molecules.

To determine if parental plasmid DNA is exclusively present and to exclude the presence of any recombination products (minicircle and miniplasmid), several established techniques can be used, like polymerase chain reaction (PCR), DNA sequencing and agarose gel electrophoresis (AGE). In recent years microfluidics and so called Lab-on-a-chip devices became more important in analytical sciences [25–31]. In the following, a microfluidic device is presented that allows continuous flow detection and separation of minicircle DNA and its parental form. The separation is achieved by electrodeless dielectrophoresis in a microfluidic channel. With this approach, even single DNA molecules can be detected.

The continuous separation of biomolecules e.g. DNA molecules in microfluidic channels enables the implementation of measurements of small analytic volumes along the minicircle production. Thus, statements about state of process and purity of the probe can be made during the continuous measurement.

A spatial separation can be achieved when one component of the force stands vertical to the flow direction in the region of separation. Afterwards, the two analytic components may flow in parallel or can be led into different channels, this enables further downstream applications. The advantages of the separation
in a microfluidic channel are in particular the reduction of amount of analyte and time of separation to a few minutes. In comparison, the analysis by PCR and AGE would take a couple of hours.

Typical dimensions of microfluidic channels are in width and height of ten to a few hundred µm. In consequence, surface effects become dominating and the viscosity have a huge relevance. Whereas, in contrast to macroscopic scale, gravitation and inertia can be neglected [32–34]. Due to the small dimensions new effects occur. Especially for migration and separation phenomena a wide field is opened by microfluidics [29, 35–41]. Here, a microfluidic device is presented that allows continuous flow separation of minicircle DNA and its parental form by means of insulator based dielectrophoresis. Dielectrophoresis is the migration of a polarizable particle in an inhomogeneous electric field. An inhomogeneous electric field can be generated either by the use of microelectrodes within the microfluidic channel or by means of a non-conducting channel narrowing [42]. In the presented case the latter route was followed by using a bow-shaped barrier. The flow through height at the barrier is 180 nm by total heights of the channel of 5.5 µm. In length the channel is about 1 cm and the barrier has a length of 10 µm.

The device is produced by softlithography with polydimethylsiloxane (PDMS) [43]. Briefly, a masterwafer is produced by contact lithography [44, 45]. Afterwards, PDMS is poured over the structures and cured. The PDMS is peeled off, the structures are cut out and reservoir holes are punched at the ends of the channels. The chip is closed by PDMS coated cover slips after a plasmaoxidation [46].

By means of transverse course to the channel and the bow-shaped form of the barrier allows a continuous separation [45, 47]. The DNA molecules of different sizes migrate in the channel by electrophoresis in an electric direct current (dc) field, less than 15 V are applied at channels 1, 3 and 4, respectively (see Fig. 3.1.5 (A)). Reservoir 2 is grounded. A superimposed alternating (ac) voltage, 200 V at 350 Hz applied at channel 1, generates a dielectrophoretic potential at the barrier. For appropriate ac- and dc-voltages the DNA molecules are trapped, i.e. the dielectrophoretic force at the barrier is stronger than the
electrophoretic force and the Brownian motion. Since the depth of the
dielectrophoretic potential is proportional to the particles polarisability, depends
on object size and material, it becomes possible to separate two sorts of
particles [46, 48]. Here, the two sorts of DNA differ in size. Due to the different
polarisabilities minicircle DNA and parental DNA could be separated from each
other [48].

The parental plasmid pF904pp used for this experiments has a size of 6.8 kbp,
the associated minicircle DNA MC0904 MC-CMV-GFP expressing GFP under
control of the CMV promoter has a size of 2.3 kbp (PlasmidFactory, Bielefeld,
Germany). The dominant conformation of the DNA molecules in this application
is covalently closed circle DNA (ccc DNA), which is verified via capillary gel
electrophoresis (CGE). To ensure a tracking of the DNA molecules these are
marked with intercalating fluorescence dye YOYO-1 in all on-chip-
measurements [49, 50]. To show the separation of parental plasmid DNA and
corresponding minicircle DNA these DNA molecules are adjusted to a ratio of
1:3 (see Fig. 3.1.5 (B)).

Experimentally, the mixture is continuously injected into the separation channel
2 towards the barrier. Therefore, the dc-voltages are varied first, such that the
DNA molecules occupy less than a third of the channel width. Afterwards, ac-
voltage and frequency are varied until two molecules bands occur behind the
barrier (see Fig. 3.1.5 (B)) indicating the parental DNA and the minicircle DNA.

In contrast to the performance with the mixture of parental plasmid and
minicircle DNA Fig. 3.1.5 (C) shows the application for a parental plasmid DNA
sample obtained from culture beforehand induction of recombination into the
microfluidic channel. The parameters are all the same. No minicircle DNA
molecules are detectable, indicating that the cultivation system is not leaky
before induction of recombination.

3.2 Finished product control

The quality control of a finished minicircle DNA is not different from the testing
carried out for regular plasmid DNA (see Tab. 3.2.1) [3]. The main
contaminants are deriving from the bacterial producer cell. These are LPS-Endotoxin, bacterial chromosomal DNA, RNA, Proteins, and potentially living microorganisms.

Lipopolysaccharides (endotoxins / LPS) are located on the outer membrane of gram-negative bacteria. The key effects of endotoxins are mediated by their interaction with specific receptors causing immune reaction and negatively influencing the vitality of the target cells, even in cell culture. The gram-negative bacterial endotoxin content in a plasmid DNA solution is determined by the kinetic chromogenic test. LPS cause the activation of a proenzyme in limulus amebocyte lysate (LAL). The activated enzyme in turn catalyses the splitting of p-nitroanilin (pNA) from a chromogenic synthetic substrate, which leads to a yellow staining. The freeing of the pNA is measured photometrically over a specified incubation period at 405 nm by a microtitre plate reader with the relevant software. The increase in yellow staining correlates with the concentration of endotoxins. The reference value is provided by the pure standard lipopolysaccharide of *E. coli* 055:B5. The concentration of endotoxins in the sample can be calculated from the absorption values of a dilution series of the standard. The test has a high sensitivity (0.005 EU/mL to 10.0 EU/mL) and linearity through the use of a chromogenic reagent.

Plasmid molecules are usually released from host cells by alkaline lysis. This process step is critical because the major contamination source of plasmid DNA productions is the bacterial chromosomal DNA (chrDNA) released at this step, too. If this high molecular material should be subject to degradation, either by enzymatic or physical matters (e.g. shear force), the resulting fragments will be present within the subsequent purification steps as well and need to be removed there. The presence of bacterial chromosomal DNA in plasmid preparation leads to immune stimulation in the target cells, e.g. in co-transfections, and to a systematic error because the total DNA amount determined by photometry means plasmid DNA plus chrDNA. As removal of these is a major task in plasmid manufacturing it is worth to avoid this by keeping the bacterial high-molecular DNA intact and remove it together with bacterial debris within the lysis separation and filtration.
To determine the proportion of bacterial chromosomal DNA in plasmid preparations, aliquots in suitable dilution are used as templates in quantitative real time PCRs (qRTPCRs) so that the number of gene copies of the ribosomal operons can be determined. The quantity of bacterial chromosomal DNA is calculated on this basis. The easiest way and minimal requirement to check for the absence of (bacterial) RNA is the visual inspection of an agarose gel scan. A digital image of an agarose gel is examined on a computer for the presence or absence of RNA contamination. Here, RNA can be identified as a "cloud-like form" in the agarose gel below 500 bp.

The quantification of the total protein content in a plasmid DNA solution is determined by the chromogenic test "BCA". The BCA test used is based on the Biuret reaction. This method permits the colorimetric verification of a bicinchoninic acid/Cu⁺-colour complex. Proteins reduce alkaline Cu(II) to Cu(I). Bicinchoninic acid forms an intensively violet complex with copper ions with an absorption maximum at 562 nm. The absorption is in turn directly proportional to the protein concentration. BSA Bovine serum albumin (BSA) at a concentration of 10-250 µg mL⁻¹ is used as a reference substance.

The standard test to determine the content/absence of contaminations by living organisms is the so-called bioburden assay.

According to Ph. Eur 2.6.12, two tests are performed with the total number of:

**aerobic bacteria:**

**Total aerobic microbial count (TAMC)**

Medium: casein-soya peptone (caso) agar medium

Incubation conditions: 3-5 days at 30°C to 35°C

**yeasts and moulds:** Total combined yeast/mould count (TYMC)

Medium: Sabouraud-dextrose agar

Incubation conditions: 5-7 days at 20°C to 25°C

Anaerobic bacteria are tested as well. The procedure is similar to the TAMC test but without oxygen, which is however not described in the Ph. Eur.
Plasmids exist in a covalently closed circular form (ccc form), which is negatively superspiralised. Individual strand ruptures caused enzymatically or through mechanical stress on the DNA cause a loosening of the molecule. The resulting form is described as an open circular form (oc form). Double-strand ruptures lead to linearization of the molecule. Errors in replication or homologous replication can also lead to multimeric plasmid forms. The plasmid topologies, which occur can be distinguished and quantified by capillary gel electrophoresis (CGE). These are divided in a gel-filled capillary in an electrical field and then detected optically.

4. Future goals

For first efficacy studies, minicircles with different genes to be used in various tissues, cells, animals and with different modes of administration (e.g. electro gene transfer, sonoporation, lipofection, jet injection etc.) have been produced and described by PlasmidFactory and in Chapters 10 and 12 of this book [51, 52]. Further studies are ongoing and will be published soon.

Here, in addition to their improved safety profile, minicircles have shown their potential to increase the efficacy of transgene expression. Hence, our minicircle production technology [14] facilitates the production of highly pure minicircle DNA for applications in gene therapy and vaccination as well as transient gene expression in any producer cells. The improved gene transfer and expression behavior of minicircle DNA compared to the corresponding plasmids offer new options for therapeutic non-viral gene transfer. Therefore, minicircles hold promise for potential clinical applications in different gene therapy and vaccine approaches, such as treating genetic defects, cancer immunotherapy, protective immunity against infectious diseases etc.

Our next steps, besides others, will be improvements in the minicircle production protocol as basis to produce minicircles at sufficient amounts (gram scale) and reproducibly high quality. This will be an essential requirement for using minicircles for in vivo applications, even in large animals and later on in human.
Additionally, we will establish *minicircle* DNA for production of viral vectors, i.e. helper / packaging and vector constructs. This will facilitate to overcome the potential risk of spreading antibiotic resistance genes by viral vectors as described by Chardeuf et al. [53] and most probably enhance the productivity in virus production runs. Furthermore, we will establish the potential of using *minicircle* DNA in plant biotechnology as well.
5 Acknowledgements

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### Tables

[Tab. 3.2.1 Quality controls *minicircle* DNA products.]

<table>
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<td>Restriction digestion and agarose gel</td>
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<td>UV-Scan (220-320 nm)</td>
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<td>Presence of bacterial and fungal contaminants</td>
<td>Bioburden (TAMC, TYMC)</td>
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</table>
Fig. 1.3.1

[Scheme of minicircle and miniplasmid production for MC-CMV-GFP (MC0904).]
Fig. 2.2.1

[Process scheme of cultivation and harvest in minicircle production.]
Fig. 2.2.2

[Analysis of parental plasmid pF0904pp (500 ng DNA per lane) cultivated in *E. coli* before and after induction by agarose gel electrophoresis (0.8%, TAE buffer, 15 V cm⁻¹). DNA was strained with 1 mg L⁻¹ ethidium bromide for 15 min after electrophoresis. (M) DNA standard 1 kpb (PlasmidFactory, Bielefeld, Germany). Parental plasmid pF0904pp (6.8 kbp) with monomer, dimer and polymers (A) out of pre-culture before induction and (B) shortly before induction. Recombination product after induction with Arabinose with MC0904 (2.3 kbp) and MP0904 (4.5 kbp) in ccc conformation (C) after 5 min and (D) after 30 min.]
Fig. 2.2.3

[Analysis of parental plasmid pF0904pp (250 ng DNA per lane) cultivated in E. coli before and at different time points after induction by agarose gel electrophoresis (0.8%, TAE buffer, 15 V cm⁻¹). DNA was strained with 1 mg L⁻¹ ethidium bromide for 15 min after electrophoresis. (M) DNA standard 1 kpb (PlasmidFactory, Bielefeld, Germany). Before induction parental plasmid pF0904pp (6.8 kbp) as monomer, dimer and polymers is present, after induction the amount of minicircle DNA MC0904 (2.3 kbp) and miniplasmid DNA MP0904 (4.5 kbp) increases and the amount of parental plasmid pF0904pp decreases.]
Fig. 2.3.3

[Analysis of parental plasmid pF0904pp (500 ng DNA per lane) cultivated in *E. coli* before and after induction by agarose gel electrophoresis (0.8%, TAE buffer, 15 V cm⁻¹). DNA was strained with 1 mg L⁻¹ ethidium bromide for 15 min after electrophoresis. (M) DNA standard 1 kpb (PlasmidFactory, Bielefeld, Germany). (A) Recombination product after induction with Arabinose with *minicircle* DNA MC0904 (2.3 kbp) and miniplasmid DNA MP0904 (4.5 kbp) in ccc conformation after 30 min. (B) pure *minicircle* DNA MC0904 following chromatographic purification of *minicircle* DNA.]
Fig. 3.1.1

[Schematic setup of an atomic force microscope (AFM).]
Fig. 3.1.2

[Atomic force microscopy of parental plasmid pF0904pp (6.8 kbp) and its corresponding minicircle MC0904 (2.3 kbp) and miniplasmid MP0904 (4.5 kbp) DNA on mica. The imaging was performed in AFM tapping mode operation. (A) Parental plasmid DNA linear, (B) parental plasmid DNA oc, length of 2300 nm (C) minicircle DNA, oc, length of 780 nm (D) miniplasmid DNA, oc, length of 1500 nm.]
Fig. 3.1.3

[Schematic setup of capillary gel electrophoresis unit (CGE).]
Fig. 3.1.4

[CGE electropherograms of (A) recombination product with *minicircle* DNA MC0904 (2.3 kbp) and miniplasmid DNA MP0904 (4.5 kbp), (B) *minicircle* DNA MC0904 following chromatographic purification. Additional peaks are caused by air bubbles. The DNA samples were pre-strained in YOYO solution. CGE was performed in 30 cm capillary at 100 V cm\(^{-1}\).]
Fig. 3.1.5

[(A) Schematic diagram of a dielectrophoretic separation in a microfluidic channel (top view). The DNA molecules flew from left to right and undergo selective dielectrophoretic forces at the barrier. (B and C) Collages of]
fluorescence images of microfluidic experiments (with 200 V ac-voltage at 350 Hz). Single DNA-molecules (yellow spots) flow from left to right. (B) Separation of parental plasmid pF0904pp (6.8 kbp) and minicircle DNA MC0904 (2.3 kbp) mix (1:3). The parental plasmid DNA is trapped and deflected at the barrier, whereas the minicircle DNA overcomes the barrier unhindered. Thus, separation is achieved. (C) Total deflection of parental plasmid DNA, beforehand induction.