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Development of a nicking endonuclease-assisted method for the purification of minicircles

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ABSTRACT

Minicircle (MC) DNA vectors are able to generate a high-level transgene expression *in vivo*, which is superior to the one afforded by conventional plasmids. MC vectors are produced by replicating a parental plasmid (PP) and promoting its recombination in *Escherichia coli*. This generates a MC with the expression cassette, and a miniplasmid (MP) with the replication segment. Unfortunately, wider use of MC vectors is hampered by difficulties in isolating the target MCs from their MP counterpart. In this proof-of-concept study, a reproducible process is described to improve the purification of supercoiled (sc) MCs that combines an *in vitro* enzymatic relaxation of sc MP impurities with topoisomer separation and RNA clearance by hydrophobic interaction chromatography (HIC) step. At the early stage of vector design, a site for the nicking endonuclease *Nb.B*bvCI was strategically placed in the MP part of the PP backbone. A process was then established that involves *E. coli* culture and recombination of PPs into target MC, cell harvesting and alkaline lysis, precipitation with isopropanol and ammonium sulfate and diafiltration/concentration by microfiltration. Next, an *in vitro* digestion step was carried out with *Nb.B*bvCI to nick of one of the strands of the MPs and of non-recombined PPs by *Nb.B*bvCI. As a result, sc MPs and non-recombined PPs were converted into the corresponding open circular (oc) forms whereas sc MCs remain unaffected. Finally, sc MC was isolated from oc DNA molecules (oc MPs, oc MC) and RNA by performing HIC with a phenyl-Sepharose column using a series of elution steps with decreasing ammonium sulfate concentrations. On the basis of agarose gel electrophoresis analysis, the sc MC-containing fractions were determined to be virtually free from nucleic acid impurities.

1. Introduction

Gene therapy relies on the use of vectors to deliver nucleic acids into cells with the aim of treating, curing or preventing a disorder by modification of endogenous gene expression [1,2]. The vectors developed with this objective should safely deliver the gene of interest into cells for transcription, while preserving it from degradation [3]. Comparatively to their viral counterparts, non-viral vectors present several advantages, especially in terms of safety [4]. Different delivery systems (*e.g.* cationic lipids and polymers, gene guns, nano and microparticles) have been used to guarantee that plasmids arrive safely and ready for transcription into the nucleus of target cells [5]. However, the entry of plasmids in cells is hampered by a series of barriers and thus transfection efficiency is usually inferior when compared with viral vectors [6]. Furthermore, the presence of bacterial regions in the backbone of plasmids (*e.g.* origin of replication, antibiotic resistance) can produce immunological reactions [7] and silence transgene expression [8]. Such problems can be solved by using smaller, plasmid-like minicircle (MC) vectors that contain only the expression cassette with the transgenes [9,10]. Experimental evidence has demonstrated that adequately purified MCs are able to generate a high-level transgene expression *in vivo* superior to the one afforded by conventional plasmids [9–13].

MCs are produced *in vivo* in *Escherichia coli* upon excision of the expression cassette via intramolecular site-specific recombination between two multimer resolution sites (MRS) strategically placed in a parental plasmid (PP) backbone. This generates a replication-deficient MC with the expression cassette, and a miniplasmid (MP) with the undesired prokaryotic replication segment [9,10,14,15]. In spite of their higher efficiency, a wider use of MC vectors has been hampered by difficulties in achieving high cell and recombination

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yields, and in purifying MCs from MPs [16]. In principle, higher MC productivities can be obtained by using rich culture media in controlled bioreactors and by inducing recombination at high cell concentrations near the end of growth. However, this typically results in cells that contain MCs and MPs, but also un-recombined PPs [17]. Moreover, efficient methods are needed to isolate the target MC from its MP counterpart. This is an extremely challenging separation since these two DNA molecules are usually very similar in terms of their size, topology and abundance in the process streams [18]. The first methods described for MC purification relied on the linearization of MPs by *in vitro* restriction with an adequate enzyme, followed by MC isolation using caesium chloride (CsCl) gradient centrifugation [9–11,19] or agarose gel electrophoresis [20]. However, CsCl centrifugation is not suitable for the large-scale manufacturing of clinical grade material [18] and is incompatible with the guidelines put forward by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [5]. Still, and although the technology required to produce pure MCs at large scale in order to run animal and clinical trials is still incipient, significant advances have been made in this area [15,21–23]. For example, Mayrhofer and co-workers developed a technique that explores the affinity interaction between lactose operator sites in the MC species with a solid matrix modified with the repressor of the lactose operon (LacI) [15]. When a mixture of MCs and MPs is contacted with a column packed with this matrix, MCs bind to LacI but MPs are washed away in the flowthrough. The recovery of bound MCs is subsequently accomplished by eluting the column with a buffer containing isopropyl β-*D*-1-thiogalactopyranoside [15]. Alternatively, Kay and co-workers resorted to an inducible *I-Sce*I nuclease that is embedded in the host bacterial cell and that specifically degrades MP species, which were engineered to contain an *I-Sce*I recognition site [21,22]. This allows the linearization and degradation of MPs in bacteria and thus facilitates subsequent isolation of MCs by routine plasmid purification procedures (*e.g.* anion-exchange mini-prep columns) [21,22].

In this work an alternative method is introduced for MC purification. *E. coli* strain BW2P is used as the producer system to drive the ParA resolvase-mediated recombination of PPs [14] from a single gene copy of *parA* stably inserted in the bacterial chromosome. The ParA resolvase expression is under the control of the arabinose inducible P_{BAD}/AraC promotor, which has an optimized ribosomal binding site [24]. As a first step, *E. coli* cells transformed with PPs are cultivated in flasks using an appropriate medium and MCs are generated by triggering recombination via arabinose induction. Next, alkaline lysis of cells, precipitation steps and dialysis are used to obtain clarified lysates with MCs, MPs and un-recombined PPs, alongside with other impurities. The purification of MCs from MPs is then tackled by exploring the *in vitro* enzymatic action of the nicking endonuclease *Nb.B*bvCI over a target site strategically placed in the MP part of the PP backbone (Fig. 1a). *Nb.B*bvCI is a nuclease obtained by mutation of the catalytic subunit R2 of the heterodimeric *Rb.B*bvCI from *Bacillus brevis*, which acts on DNA substrates within a seven-base pair asymmetric recognition sequence by introducing a single cut in one of the DNA chains (CCTCAGC/GCTGAGG → CCTCAGC/GCT̂GAGG) rather than cleaving the duplex [25]. Thus, *Nb.B*bvCI is able to relax the supercoiled (sc) structure of the MPs and of non-recombined PPs by nicking one of the DNA strands, while leaving the sc MCs intact (Fig. 1b). Hydrophobic interaction chromatography is finally used to purify sc MCs from the relaxed, open circular (oc) MPs, MCs and PPs (Fig. 1b). In this paper we present proof-of-concept experiments that demonstrate that such a process is able to deliver pure sc MCs free from nucleic acid impurities.

2. Materials and methods

2.1. Materials

Phenyl Sepharose 6 Fast Flow (High Sub) was obtained from GE Healthcare (Uppsala, Sweden). Restriction enzymes *Sac*II and *Xho*I were from Promega (Madison, Wisconsin). Restriction enzyme Bsp1407I (isoschizomer of *Bsr*GI) was from ThermoFisher Scientific (Waltham, MA). Nicking enzyme *Nb.B*bvCI was from New England Biolabs (Ipswich, MA). All salts used were of analytical grade.

2.2. Bacterial strain and plasmid

The producer *E. coli* strain BW2P was constructed by disrupting the *endA* gene in the genome of the *E. coli* strain BW27783 (The Coli Genetic Stock Center at Yale) via the insertion of a single copy of the P_{BAD}/araC-parA cassette [24]. The cassette contains the ParA resolvase gene under a P_{BAD} promoter with an optimized ribosome binding site and the AraC repressor gene in opposite direction [17]. After successful cassette insertion, the construction of BW2P was finalized by knocking out the *recA* gene [24]. The parental pMINILi-CVG (4,563 bp) was constructed as described previously [24]. Briefly, this plasmid contains: (i) an expression cassette with a green fluorescent protein (GFP)-vascular endothelial growth factor (VEGF) gene fusion and the human cytomegalovirus (CMV) immediate-early promoter, (ii) two 133 bp-long multimer resolution sites (MRS) flanking the eukaryotic expression cassette, (iii) a BGH polyadenylation sequence, (iv) a pMB1 origin of replication, (v) a kanamycin resistance gene for selection in *E. coli* and (vi) the recognition site for the nicking endonuclease *Nb.B*bvCI (Fig. 1a). Plasmid pMINILi-CVG was transformed by heat shock into the BW2P strain.

2.3. Parental plasmid and minicircle production

A pre-inoculum was prepared by inoculating a loop of frozen BW2P cells harboring pMINILi-CVG into 15 mL conical centrifuge tubes with 5 mL of LB medium (Sigma, St. Louis, MO) supplemented with 30 μg/mL kanamycin (Amresco, Solon, OH) and 0.5% (w/v) glucose (to repress P_{BAD}/araC-parA) and incubating overnight at 37 °C and 250 rpm. Next, an inoculum was prepared by seeding 30 mL of LB plus 30 μg/mL kanamycin and 0.5% (w/v) glucose in 100 mL shake flasks with the appropriate number of cells from the pre-inoculum to reach a starting optical density at 600 nm (OD_{600nm}) of 0.1. Cultures were incubated at 37 °C and 250 rpm until a final $OD_{600nm} \approx 2.5$ was reached.

For the production of PP, 2 L shake flasks with 250 mL of LB supplemented with 30 μg/mL of kanamycin and 0.5% (w/v) glucose were inoculated up to an OD_{600nm} of 0.1 with an inoculum prepared as described above. Cells were allowed to grow for about 5 h at 37 °C and 250 rpm until reaching the early stationary phase. The spent medium was then centrifuged for 15 min at 6,000 *g* and 4 °C and pellets were stored at −20 °C until further processing.

For the production of MC, 2 L shake flasks with 250 mL of LB supplemented with 30 μg/mL of kanamycin were inoculated up to an OD_{600nm} of 0.1 with an inoculum prepared as described above. Cells were incubated at 37 °C and 250 rpm for about 3 h until reaching the late exponential phase ($OD_{600nm} \approx 2.5$). At this moment, recombination of PP into MC and MP was induced by addition of 0.01% (w/v) L-(+) arabinose (Merck, Darmstadt, Germany) directly into the medium. Culture samples (2 mL) were collected at 0, 1 and 2 h of recombination, centrifuged (3 min at 6,000 *g* and 4 °C) and stored at −20 °C for further analysis of recombination efficiency. After 2 h of recombination, cell growth was suspended and the medium was