CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION PART 2: NON-VIRAL

SPOTLIGHT

COMMENTARY

The supporting role of plasmids in gene & cell therapy

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The biopharmaceutical relevance of producing plasmid DNA at large scale has increased steadily over the years due to the development of a growing number of direct and indirect applications. Be it as biological drugs or as starting materials, plasmids are pervasive across the gene and cell therapy industry of today. With hundreds of biopharmaceutical companies using plasmids in the clinical development of their products, plasmid manufacturing is starting to emerge as a key bottleneck. This commentary provides an overview of the uses of plasmids, discusses manufacturing challenges, and hints at what the future may bring.

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PLASMID AS BIOLOGICAL DRUGS

The rapid growth of the gene and cell therapy industry of the last years dramatically increased the demand for plasmid DNA [1–3]. Plasmids are used to directly deliver genetic information or genes that code for therapeutic proteins, RNA, or antigens to the target cells of patients (Figure 1A and Table 1). Moreover, plasmids are also used as a vehicle to deliver the molecular components of gene editor systems (e.g., editing enzymes, RNA guides) including clustered regularly interspaced short palindromic repeat DNA sequences (CRISPR), zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) [4]. In such *in vivo* uses, the appropriate plasmids are combined with other components (e.g., adjuvants, lipids, etc.) to generate a medicinal product that is transferred to patients [2]. In these applications, plasmids are biological therapeutics, which must be manufactured under current good manufacturing practices (cGMP), regulated, tested, and controlled appropriately [2].

PLASMIDS AS STARTING MATERIALS

Apart from their role as biologicals, plasmids play a supporting role as sophisticated starting materials in the context of the

manufacturing of engineered cell products (Figure 1Bi & Table 1) or of other biologicals (Figure 1Bii & Table 1). For example, plasmids are used as an alternative to viral vectors to genetically modify cells extracted from the patient or donor *ex vivo*, in the context of chimeric antigen receptor T cell (CAR-T) therapies [5], genome editing approaches [4], or mesenchymal stem cells therapies [6]. The first case entails the transfection of a patient's T cells with a plasmid system (e.g., coding for CAR genes, transposases, etc.) with the goal of attaining stable gene transfer, integration, and expression of CARs [5]. In the second case, plasmids are used to deliver the molecular components of gene editors like CRISPR, TALEN, or ZFN [4]. In either case, the plasmid-modified cells are infused back into patients. Finally, plasmids are also used to modify mesenchymal stem cells, for example with the goal of enhancing their therapeutic function *in vivo* [6].

Plasmids are also required in the manufacturing of viral vectors and mRNA, which can then be used either as biologicals on their own (Figure 1Bii), or as reagents for the *ex vivo* modification of a patient´s cells (Figure 1C & Table 1). For example, many

FIGURE 1

Direct and indirect applications of plasmid DNA in gene and cell therapies. Plasmids can be used as (A) biological drugs, e.g., as DNA vaccines or as components of *in vivo* non-viral gene therapy/editing platforms, or as (B) starting materials for (Bi) the *ex vivo* genetic engineering of cells (e.g., CAR-T cells, CRISPR-edited cells, etc.) or for (Bii) the manufacturing of viral vectors, mRNA and eventually RNA biologicals. The later can then be used either as biologicals on their own (Bii), or (C) as starting materials for the *ex vivo* modification of a patient's cells.

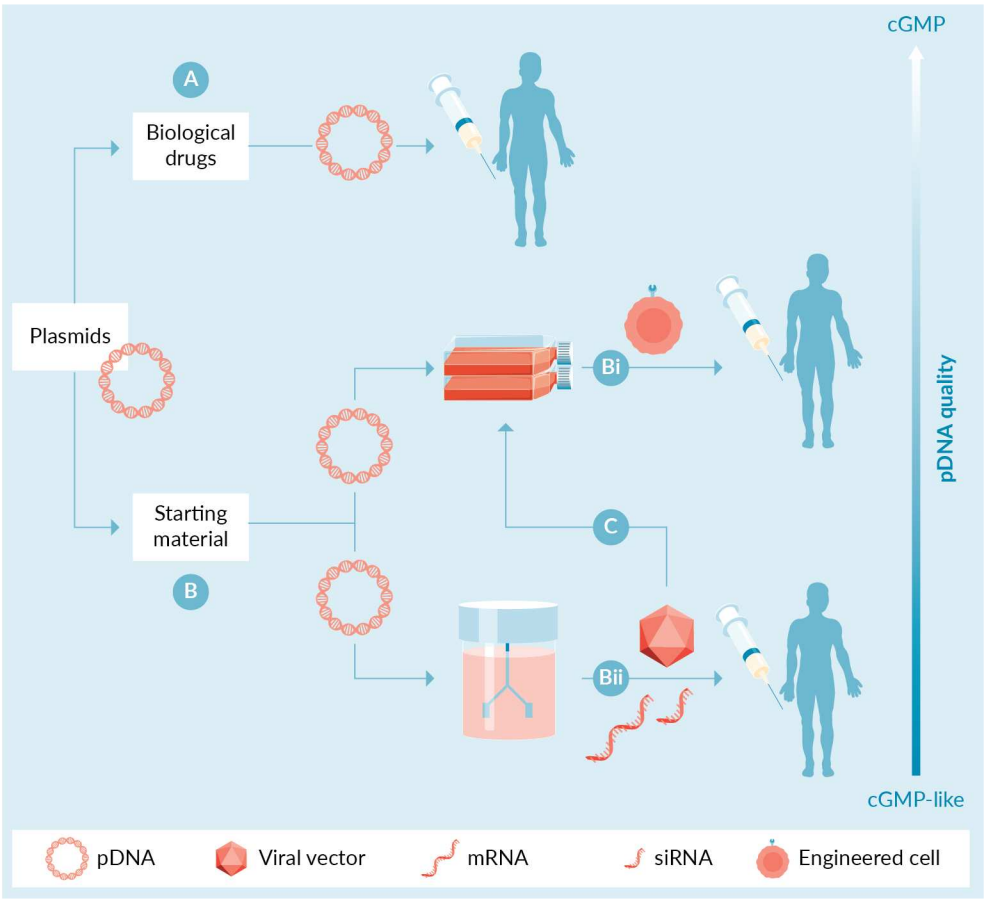

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TABLE 1

Overview of the uses of plasmids as biological drugs and starting materials* in gene and cell therapy.

Plasmid biologicals	Role of plasmids
Gene therapy, gene editing, and DNA vaccines	Plasmids carry genes that code for therapeutic proteins, mRNA, components of gene editing platforms (e.g., editing enzymes, RNA guides) or antigenic proteins. They are combined with other components (e.g., adjuvants, lipids, etc.) to generate a medicinal product that is directly administered to patients. cGMP grade is mandatory.
Plasmid starting materials	**Role of plasmids**
Ex vivo engineering of cells	Plasmids carrying genes that code for elements like proteins, components of gene editing platforms (e.g., editing enzymes, RNA guides) or mRNA are used to genetically engineer cells *ex vivo* (e.g., mesenchymal stem cells, CAR-T cells, CRISPR-edited cells, etc.), which are subsequently infused into patients. cGMP-like grade is required.
Manufacturing of biologicals	Plasmids carry genes that code for elements required to manufacture GMP-grade medicinal products like viral vectors (e.g., AAVs, LVs) or mRNA, which are subsequently administered to patients. cGMP-like grade is required.
Manufacturing of starting materials for *ex vivo* cell modification	Plasmids carry genes that code for elements which are required to manufacture starting materials like viral vectors, mRNA, minicircles, minivectors, and nanoplasmids. These starting materials are subsequently used to genetically engineer cells *ex vivo* (e.g., mesenchymal stem cells, CAR-T cells, CRISPR-edited cells, etc.), which are subsequently infused into patients. cGMP-like grade is required.

*Starting materials encompass all the materials from which active substances are manufactured.
AAVs: Adeno-associated viruses; CAR-T: Chimeric antigen receptor T cells; cGMP: Current good manufacturing practices; CRISP: Clustered regularly interspaced short palindromic repeats; GMP: Good manufacturing practice; LVs: Lentiviruses.

adeno-associated viral (AAV) and lentiviral (LV) vectors are produced by using multiple plasmids to transiently transfect producer cells such as human embryonic kidney (HEK) 293T cells [7]. As a case in point, the manufacturing of AAV particles rely on the use of three different plasmids—an AAV transfer plasmid with the gene of interest flanked by two inverted terminal repeats (ITRs), one plasmid containing AAV genes, and a helper plasmid encoding adenovirus helper genes [7,8]. Likewise, LV manufacturing by transient transfection of cells also requires the use of three or four distinct plasmids [9,10]. The resulting viral vectors can then be administered to patients or used to transduce cells *ex vivo*.

The emergence of mRNA vaccines, which was spurred by the Covid19 crisis, also created a new utility and surge for plasmids [3]. In the context of mRNA technologies, plasmids are extensively used to generate the templates required for the *in vitro* transcription (IVT) reactions that generate mRNA [11,12]. Such templates are most often produced by enzymatic linearization of a purified plasmid or by amplification of the region of interest in that plasmid using PCR [12,13]. The mRNA products resulting from the IVT are then processed and purified further, up to a stage where they can be transferred to patients, e.g., in the context of mRNA vaccination or genome editing (Figure 1Bii and Table 1). Furthermore, mRNA products can be used to modify or edit cells *ex vivo* (Figure 1C and Table 1).

One can also foresee that plasmids and IVT strategies may come to play a more significant role in the manufacturing of small RNA molecules such as antisense oligonucleotides, RNA guides, or double-stranded RNA used in the context of siRNA products [14,15]. At present, solid-phase chemical synthesis, which can generate RNAs up to 50–100 nt in length, is the preferred method for the synthesis of most oligonucleotide-based drugs because of its cost-effectiveness, automated protocols, and remarkably short synthesis cycle times [16]. Nevertheless, IVT, which is widely used to synthesize RNA molecules for structural studies and basic RNA biology (e.g., splicing,

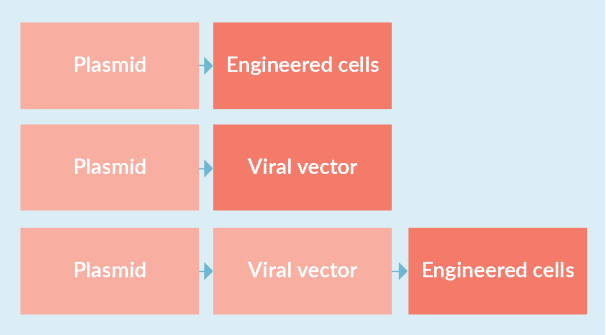
riboswitches, CRISPR, lncRNA), may become an attractive alternative in this context. If this ever comes to fruition, plasmids may well assume a critical role in small RNA manufacturing as they have in the case of mRNA vaccines, therapeutics, and reagents.

PLASMID GRADES

The indirect use of plasmid DNA as a starting material for viral vector or mRNA vaccine manufacturing requires the production of substantial amounts of material. For example, more than one kg of plasmid DNA is required to deliver one billion doses of mRNA vaccine [3]. Because plasmids are not intended to be present in the final medicinal products that are directly administered to patients but are rather used as starting materials for the cGMP manufacturing of other starting materials, biological drugs, or cell products, a cGMP grade is not strictly required. Nevertheless, although not all GMP aspects or a GMP certificate are required, the principles of GMP should be complied with during manufacturing since the starting material can end up in the finished medicinal product at residual levels and potentially impact its quality, safety, and efficacy. Ultimately, it will be up to the sponsor to perform an appropriate risk analysis to define the quality standards applicable to manufacture plasmid DNA suitable for further manufacturing of medicinal products under cGMP [17]. Relevant aspects to be duly considered will include, for example, the quality management system, documentation, raw materials, cell banks, production, specification, testing, and control and storage [17]. Thus, one can opt to produce a cGMP-like/high-quality grade plasmid DNA, which although falling short of all cGMP requirements, is still compliant with many regulatory recommendations [2,18]. Figure 2 highlights stages in the manufacturing of engineered cell products and viral vectors that are reliant on plasmid starting materials, where cGMP and cGMP principles should be applied (adapted from [17]).

FIGURE 2

Manufacturing of plasmids as starting materials for the manufacturing of engineered cell products and viral vectors. Manufacturing activities highlighted in light red should follow the principles of cGMP, whereas those highlighted in dark red should comply with full cGMP.



PLASMID MANUFACTURING AT SCALE

Although reasonably well established, manufacturing plasmid DNA at a large scale is not trivial, and manufacturers are constantly pressed to find ways to increase productivity without affecting quality [3,18]. This pressure to enhance manufacturing performance originates in part from the fact that the available capacity is not sufficient to respond to the increase in demand associated with the development of a growing number of applications of plasmids in a timely manner [3,18].

Currently, large-scale manufacturing of plasmid DNA depends exclusively on one platform host—*Escherichia coli* [1,2,19]. This preference is justified by the ability of *E. coli* to grow and divide rapidly under a range of conditions and to deliver high plasmid DNA yields. Further, many tools exist to support the molecular and microbial engineering of *E. coli*, including the creation of plasmid vectors and improved strains. Modified strains of *E. coli* are available that can be grown to densities of hundreds of grams per liter and produce up to 1–2 g plasmid DNA per liter of culture [20]. Efforts are also being directed towards the development of *E. coli* strains that may circumvent instability problems

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like the ones faced when dealing with ITR-containing plasmids such as those used in the context of the manufacturing of AAV transfer plasmids [8].

One way to improve plasmid amounts generated during manufacturing, as well as to ease regulatory approval and improve plasmid biological functions, is to focus on the engineering of DNA backbones. Efforts have been directed towards the generation of plasmids and plasmid systems (e.g., minicircles, nanoplasmids, minivectors) that are smaller, free from antibiotic resistant genes, increase manufacturing yields and provide high transgene expression [21–25].

The isolation and purification of plasmids from *E. coli* biomass recovered at the end of fermentation is an engineering challenge that has been solved for the most part, especially at smaller scales. The train of unit operations used in the downstream processing of plasmids almost inevitably includes alkaline lysis, tangential flow filtration and chromatography steps [1]. Different combinations of operations are used that deliver plasmid DNA with residual amounts of host impurities (genomic DNA, RNA, proteins, lipopolysaccharides, etc.) that are compatible with regulatory requirements.

Critical issues that have not been solved to satisfaction include poor reproducibility of alkaline lysis, lack of capacity and isoform selectivity in chromatography and loss of supercoiled isoforms during processing due to shear [1,26]. The final sterile filtration with 0.22 µm filters may also be cumbersome when dealing with very large plasmids [27].

Once manufactured, the bulk purified plasmids obtained from each batch should be rigorously characterized. Release specifications of plasmids used as a starting material will essentially focus on the same attributes as those covered when manufacturing plasmids as biological drugs [28]. This means that assays for identity (e.g., sequence, homogeneity), potency (e.g., concentration, homogeneity) and purity (e.g., host impurities, bioburden, residual kanamycin) and the corresponding acceptance criteria must be in place [28].

TRANSLATION INSIGHT

The relevance of producing plasmids at large scale has surged over the last years, not only because of the development of plasmid biological drugs like the ones used in DNA vaccination, *in vivo* gene therapy and gene editing, but mostly due to the supporting role they are currently playing in the manufacturing of many gene and cell therapy products, including viral vectors, viral-vectored vaccines, mRNA vaccines, minicircles/minivectors/nanoplasmids, and engineered cells.

Apart from the current uses, one can anticipate that plasmids may come to play a significant role in the manufacturing of small RNA molecules (e.g., antisense oligonucleotides, RNA guides, siRNA products) by IVT. While at first one may question whether IVT will ever be able to compete with the very well-established chemical synthesis of oligonucleotides, one driver for a move in this direction may come from an unexpected field: agriculture. Specifically, the development of new pesticide tools based on the induction of gene silencing through RNAi in plant pathogens and other pests is pushing for the development of cost-efficient methods for large production of bulk amounts of dsRNA [29]. IVT is emerging as an alternative in this context given that chemical synthesis is most likely not amenable to the large-scale and low-cost manufacturing that is required to implement siRNA.

Looking forward in terms of plasmid manufacturing, a few developments can be envisioned that would facilitate or altogether change how plasmids are made today. For example, while the current performance of *E. coli* as a plasmid producer looks unbeatable, one may wonder if the high demand for plasmids could not justify a search for a bacterial host with characteristics more favorable for manufacturing. Gram positive bacteria would be advantageous as plasmid producers because

they lack lipopolysaccharides—one of the most troublesome impurities associated with plasmids isolated from *E. coli*. As was the case in the production of recombinant proteins, which saw an emergence of producer hosts other than *E. coli*, there might be other hosts waiting to be discovered and developed into plasmid producers.

Engineering of plasmids backbones is also likely to have an impact in the field. Further, one should look out for radical innovations like the use of minimal synthetic constructs such as 'doggybone' and dumbbell-shaped DNA vectors, which are manufactured enzymatically [30,31]. Nevertheless, although these represent important advancements in the field, the likelihood of plasmids becoming obsolete in the future is small. Further, minicircles, mini vectors, and other minimized vectors still depend upon plasmids for their manufacturing [24,25].

Single-use technologies [32], process analytical technologies [33], automation [34], digitalization [35], and continuous manufacturing [36] are industry trends that may change the way plasmids are manufactured in the future [18]. In the latter case, for example, the design of continuous cell lysis processes that are robust and able to consistently deliver intact plasmids is another advancement to look for. The additional coupling of a post-lysis, pre-purification step by tangential flow filtration would significantly improve the productivity of the first part of the downstream processing of plasmids.

In conclusion, the central role currently played by plasmids in the development and manufacturing of many gene and cell therapy products fully justifies that a significant increase in R&D efforts and investments is made towards improving their effectiveness and manufacturing.

BIOGRAPHY

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