ABSTRACT Plasmids are currently an indispensable molecular tool in life science research and a central asset for the modern biotechnology industry, supporting its mission to produce pharmaceutical proteins, antibodies, vaccines, industrial enzymes, and molecular diagnostics, to name a few key products. Furthermore, plasmids have gradually stepped up in the past 20 years as useful biopharmaceuticals in the context of gene therapy and DNA vaccination interventions. This review provides a concise coverage of the scientific progress that has been made since the emergence of what are called today plasmid biopharmaceuticals. The most relevant topics are discussed to provide researchers with an updated overview of the field. A brief outline of the initial breakthroughs and innovations is followed by a discussion of the motivation behind the medical uses of plasmids in the context of therapeutic and prophylactic interventions. The molecular characteristics and rationale underlying the design of plasmid vectors as gene transfer agents are described and a description of the most important methods used to deliver plasmid biopharmaceuticals in vivo (gene gun, electroporation, cationic lipids and polymers, and micro- and nanoparticles) is provided. The major safety issues (integration and autoimmunity) surrounding the use of plasmid biopharmaceuticals is discussed next. Aspects related to the large-scale manufacturing are also covered, and reference is made to the plasmid products that have received marketing authorization as of today.

INTRODUCTION
The contributions of plasmids to biology and their impact in biotechnology and discovery have been immense. Together with restriction enzymes, plasmids were one of the key molecular tools at the heart of the invention and development of DNA cloning and recombinant DNA by Hebert Boyer and Stanley Cohen (1, 2). These fundamental technologies shaped molecular biology and paved the way to the development of the modern, multibillion dollar biotechnology industry (2, 3). The ability to produce unlimited amounts of proteins via the cloning of the corresponding gene into a plasmid and subsequent transformation of a microbial host made it possible to develop a range of medically and industrially relevant products and applications. The development of molecular diagnostics and protein biopharmaceuticals, for example, would have been impossible without plasmids. However, few would have suspected in the earlier years of recombinant DNA that plasmids could one day assume the role of biopharmaceuticals themselves (4).

The breakthrough that sparked the development of plasmid biopharmaceuticals came in 1990, when Wolff and colleagues injected saline solutions of plasmids containing genes for chloramphenicol acetyltransferase, luciferase, and β-galactosidase into the skeletal muscle of live mice (Fig. 1) (5). The authors found that the reporter transgenes encoded in such a “naked” plasmid DNA molecule were expressed within the muscle cells and concomitantly envisaged the use of plasmid-mediated gene transfer into human muscle as a means of improving the effects of genetic diseases of muscle. Transfection by direct injection of naked DNA was subsequently found in tissues other than skeletal muscle, like liver (6), heart (7), and brain (8), and in species as varied as fish (9), chicken (10), and cattle (11). The proximate discovery that mice could elicit antibodies
Plasmid DNA molecules encoding an antigen showed that, in principle, plasmids could also be used to immunize animals against pathogens. This innovative approach, later termed DNA vaccination, constituted a radical departure from conventional immunization methodologies, which relied on the industrial production of the vaccinating antigens before their administration.

The seminal discoveries of the early 1990s that opened up the possibility of using plasmids as biopharmaceuticals for therapy (5) or prophylaxis (12, 13) were followed by 10 years of major innovations (Fig. 1) (14). The following are noteworthy examples of those milestones: the delivery of plasmid DNA by particle bombardment (15), the application of electroporation for in vivo delivery (16), the coexpression of cytokines alongside with the target genes (17), the addition of immunostimulatory CpG motifs to plasmid backbones (18), prime (with DNA vaccine)-boost (with non-DNA vaccine) vaccination (19), the use of targeting sequences to enhance the immunogenicity of DNA vaccines (20), the encapsulation of plasmids in microparticles (21), the design of minimal plasmids (so-called minicircles) containing only the functional elements required for expression of the transgene (22), the compaction of single molecules of DNA into minimally sized nanoparticles (23), and the systemic in vivo administration of plasmid DNA by rapid injection of large volumes of solution (24). These formative years (Fig. 1) were followed by intensive research efforts directed toward the adaptation of the major concepts developed earlier to new applications and to the expansion and accumulation of the scientific know-how related to the mechanisms of action of plasmid biopharmaceuticals in vivo, via laboratorial, preclinical, and clinical experimentation. Major innovations also took place on the “process” side as the industry sensed the increase in the maturity of the product prototypes and concepts and prepared for clinical development and manufacturing (14).

PLASMIDS IN DISEASE MANAGEMENT
Plasmids versus Viral Vectors
The use of plasmids as carriers of medically relevant genes is usually considered on par with viral vectors. Viral vectors are very effective at transferring genes because of their natural ability to deliver and express genes, while avoiding the different defense barriers of the host organism and cells. For this reason, recombinant viral vectors are the gene carriers of choice in more than 65% of the clinical trials of gene therapy registered as of January 2014. (Data were extracted from The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide website, http://www.wiley.co.uk/genmed/clinical [last accessed on 4 February 2014].) However, even though recombinant viruses used in gene transfer are designed to minimize the toxicity and immunogenicity of their natural counterparts, safety concerns associated with the use of viral vectors remain high as a result of a number of incidents and serious adverse events recorded during a number of gene therapy clinical trials (25). Plasmids, on the other hand, are characterized by an excellent safety profile (see below). For this reason, close to 20% of the gene therapy clinical trials recorded
up to 2014 had used plasmid DNA as a carrier of the therapeutic/prophylactic transgenes (The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide website mentioned above).

**Role of Transgene Products**
Generically speaking, plasmid biopharmaceuticals are used to transfer genes with the goal of managing disease in humans and animals. The rationale behind this approach is that, once expressed in the target cells/tissues, the products coded in the plasmid-borne transgenes will act in such a way as to tackle and resolve the specific disease or clinical condition under study. The different functions exerted by plasmid-borne transgene products can be broadly divided into five categories, as briefly described next (26).

**Boosting**
Plasmids can be used to increase the expression of a specific endogenous protein, whose level is otherwise normal, by adding more copies of the coding genes. This could contribute to accelerating the endogenous response generated by our bodies in the context of a specific disease (26). For example, an increase in the expression of vascular endothelial growth factor (VEGF) can accelerate the vascularization of ischemic tissue in arterial diseases (27), and an increase in the expression of the hepatocyte growth factor (HGF) gene may enhance the function of dopaminergic neurons in Parkinson’s disease (28).

**Replacement**
When a hereditary defect in a single gene prevents the body from functioning normally, e.g., as in cystic fibrosis (29) or Duchenne muscular dystrophy (30), regular levels of the normal protein can be supplemented by transferring the correct gene via plasmids (26). Replacement can also be explored to compensate for the deterioration of normal levels of a protein as a consequence of disease (e.g., insulin in type 1 diabetes mellitus [31], erythropoietin in anemia [32]).

**Immune stimulation**
DNA vaccines can be designed on the basis of plasmids that transfer genes whose products are able to recruit the immune system (26, 33). These vaccines can be administered to prevent future episodes of the target disease (prophylactic vaccines) or to motivate the immune system to fight cancer (therapeutic vaccines). In the first case, the DNA vaccine carries the gene that codes for a specific antigen of the causative infectious agent (e.g., AIDS [34], malaria [35], tuberculosis [36], influenza [37]), whereas, in the second case, genes that code for products that increase tumor immunogenicity and mobilize immune cells to fight cancer are used (38). Unlike in the case of traditional vaccines, antigens delivered by DNA vaccines are synthesized endogenously, and, thus, the process of antigen presentation that ensues may mimic natural infection more closely.

**Cytotoxicity**
The plasmid-mediated transfer of genes can be used to kill malignant cells. The therapeutic strategy is usually designed so that the gene product plays an intermediate role (e.g., by replacing a missing key protein, stimulating the immune system into recognizing harmful cells, or introducing a new functionality that contributes to kill cells) in a more complex network of events that ultimately result in the death of the target cells (26).

**Blocking**
The genetic information in a plasmid can also code for short hairpin RNAs (shRNAs), which once expressed will knock down the expression of the disease-related target genes via RNA interference pathway (39, 40, 41).

**Plasmids for Therapy**
Gene transfer via plasmid molecules has been studied as a possibility to treat both hereditary disorders that are characterized by deficiencies at the single-gene level and diseases that are caused by a combination of environmental factors and genetic predisposition.

**Hereditary disorders**
In this case, the expectation is that the plasmid-mediated delivery of the correct genes results in the restoration of normal levels of the faulty protein and hence in the halting of the course of the disease. The management of a genetic disorder by using plasmids will inevitably rely on chronic administration, since plasmids are typically cleared by the cell machinery after a certain amount of time has elapsed. The possibility of the development of autoimmune responses or immune tolerance is thus a cause for concern. Examples of genetic disorders that have been addressed by plasmid-based gene transfer include (i) hemophilia, a coagulation disorder associated with defects in factor VIII (hemophilia A) and factor IX (hemophilia B) (42, 43); (ii) cystic fibrosis, a multiorgan disease caused by an abnormal cystic fibrosis transmembrane regulator gene (29, 44); and (iii) Duchenne muscular dystrophy, a neuromuscular disorder associated with defects in the dystrophin gene (30, 45). A key
challenge in the management of these diseases is to ensure that the corrective genes are delivered to the proper cell. One of the strategies studied to achieve this targeting relies on the use of plasmid delivery vehicles modified with ligands for specific receptors of the target cells. In other situations, the relevant tissue can be targeted by direct administration of the plasmid to the relevant tissue (e.g., aerosol delivery of plasmids to the lungs in the case of cystic fibrosis [29], intramuscular injection in the case of muscular dystrophy [30]).

Multifactorial diseases

Clinical research on plasmid biopharmaceuticals has focused strongly on multifactorial diseases that result from a combination of environmental factors and genetic predisposition. For example, in the context of coronary and peripheral arterial diseases, plasmids have been used to deliver specific genes such as fibroblast growth factor (FGF), VEGF, HGF, and AGGF1 that promote the formation of new blood vessels and thus increase blood flow to the affected ischemic tissues (myocardium, limbs) (46, 47, 48, 49).

Additionally, many clinical trials and a large body of scientific research have been directed at treating cancer with plasmids. Several therapeutic strategies can be devised to kill cancer cells on the basis of genes including tumor suppression, suicide gene therapy, antiangiogenesis, and immune stimulation. For example, cancer cells can be driven to commit suicide by delivering a missing or defective tumor suppressor gene like p53 (50, 51, 52, 53). The suicide gene therapy approach calls for plasmid-encoded proteins to convert a coadministered prodrug into an active, cytotoxic agent that kills the tumor cells. The coupling of the cytosine deaminase gene with the prodrug 5-fluorocytosine (54) and of the herpes simplex virus thymidine kinase gene with the antiviral drug ganciclovir (55) are two of the most researched enzyme-prodrug systems. In this case, the cytotoxicity is not limited to the transfected cells, since the active drug can diffuse and act on neighboring cells (bystander effect). Cancer can hypothetically be treated by inhibiting the formation of the network of blood vessels (i.e., angiogenesis) that supply nutrients and oxygen to tumoral cells. Plasmids have been used in this context to deliver genes that code for antiangiogenic proteins, such as the VEGF receptor sFLT147 (56), endostatin (41), or interleukin-12 to the affected tissues (57). Immune cells can be stimulated to fight cancer cells by using DNA vaccines that promote the expression of cell surface markers or cytokines. In the first case, plasmids are used to deliver the genes that code for tumor-associated antigens, like the prostate-specific antigen (58) in prostate cancer and gp75/tyrosinase-related proteins in melanoma (59). Once expressed, these antigenic markers are adequately processed and displayed, recruiting cytotoxic T lymphocytes (CTLs) that eventually kill the neoplastic cells (38, 60). In the second case, the regulatory role of interleukins, interferons, tumor necrosis factors, and colony-stimulating factors in the pathogenesis of cancer is explored as a means of generating potent antitumor responses (42, 61, 62).

Further examples of multifactorial diseases in which plasmid vectors have been used to deliver therapeutic genes include Alzheimer’s (63), anemia (32), arthritis (64), burn wounds (65), dental caries (66), diabetes mellitus (31), glaucoma (67), lupus (68), sepsis (69), spinal cord injury (70), and wound healing (71).

Plasmids for Prophylaxis

One of the most appealing applications of plasmid biopharmaceuticals is in the prevention of infectious diseases. In principle, DNA vaccines can be designed to immunize humans and animals against an enormous range of diseases caused by viruses, bacteria, protozoans, and fungi (33). Studies performed with animal models have demonstrated that the protection conferred by DNA vaccines against infectious agents occurs via the activation of the innate immune system and the induction of CTLs, T-helper (Th) cells, and neutralizing antibodies that are antigen specific (72). DNA vaccines from the earlier generation, in general, were poorly immunogenic (33). Over the past years, several strategies have been pursued to improve the immunogenicity of DNA vaccines that include the optimization of codons in the antigen gene (73), the coadministration of genes coding for immunostimulatory functions (e.g., cytokines [74]), the use of CpG motifs in plasmid backbones (75), the fusion of antigens with sequences that target specific major histocompatibility complex (MHC) pathways and Th-cell responses (76), the design of heterologous prime/boost immunization modalities (77), and the use of delivery methodologies such as the gene gun (78). DNA vaccine prototypes have been constructed by cloning genes coding for antigens associated with a range of diseases including AIDS (77), dengue (79), human papillomavirus (80), influenza (81), sleeping sickness (82), and tuberculosis (83).

Molecular Aspects

Basic Components

Plasmid-based gene therapy and DNA vaccination rely on an effective delivery and expression (in terms of level and duration) of the transgene to the target cells. The
typical plasmid vector (Fig. 2) is a covalently closed, double-stranded DNA molecule derived from natural plasmids, which is mostly found as a tightly twisted, supercoiled topoisomer (84). It contains a set of prokaryotic sequences necessary for plasmid amplification in a bacterial host (replication origin, antibiotic resistance gene), and an eukaryotic expression cassette, which includes the therapeutic gene and the regulatory elements required for expression in eukaryotic cells (e.g., promoter, polyadenylation sequence [Fig. 3]). The design of such a plasmid vector should take into consideration aspects such as the stability of plasmids in vivo, the profile of transgene expression, the impact of prokaryotic sequences, and the response of the host immune system to the vector.

**In Vivo Plasmid Stability**

The plasmid journey to the cell nucleus is hindered by several physical barriers, including the cytoplasmic membrane, the network of cytoskeleton proteins and organelles that overcrowd the cytoplasm, and the nucleus envelope (85). Moreover, the degradation of plasmid DNA by intra- and extracellular exo/endonucleases constitutes a major barrier to gene expression (86, 87). Only very small amounts (0.1%) of the plasmid molecules that enter cells reach the nucleus (86). The removal of secondary forming sequences (e.g., homopurine-rich and cruciforms) from the plasmid backbone (87, 88) and the coadministration of nuclease inhibitors like aurintricarboxylic acid (89) and DMI-2 (90) have both been advocated as a means to improve the resistance of plasmids to nucleases. Moreover, plasmids containing direct and inverted repeats, insertion sequences, and regions similar to genomic DNA can suffer genetic rearrangements, such as deletions, duplications, inversions, translocations, and insertions, albeit at very low frequencies (91, 92, 93, 94). These rearrangements can affect both plasmid production in *Escherichia coli* and the efficiency of transgene expression. These and other unstable regions should be removed from the vector or at least changed whenever possible. Removal of nonessential sequences from plasmids also reduces the size which per se decreases the number of intrinsic nuclease-susceptible or inhibitory regions and might increase the molecular stability.

**Transgene Expression**

Upon successful arrival of the transgene to the nucleus of the target cells, a reasonable amount of gene expression during a more or less extended time period is required to elicit the wanted therapeutic effect. The transgene expression profile can be modulated by judiciously selecting the best promoter and transcriptional regulators. A few promoters are currently used to drive transgene expression in the context of gene therapy and DNA vaccination. The cytomegalovirus (CMV) immediate early promoter is the most widely used in many vectors because of its high strength in many different tissues. The activity of the CMV promoter can be modulated by the presence of specific cytokines (95) or by other proteins like p53 and Mekk1 (96). The downregulation of the promoter activity might prevent transgene expression. Therefore, the use of alternative promoters is often considered, e.g., viral promoters (e.g., Rous sarcoma virus, simian virus 40 [SV40]) and some cellular promoters (e.g., human ubiquitin B [UbB], ubiquitin C, human elongation factor 1α), or chimeric promoters (e.g., CMV-chicken-β-actin, CMV-UbB) (97, 98, 99).

The transcriptional inactivation of the promoters that regulate transgenes is expected during the normal homeostasis of the cells. A sustained and long-term expression depends on the promoter type that includes regulatory elements such as enhancers, boundary elements, and silencers (98, 99) and also on the cell type.

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**FIGURE 2** Basic physical characteristics of plasmid vectors. Data presented is for 2,000- to 10,000-bp plasmids with a typical degree of supercoiling (Prazeres, 2011). Image is reprinted with permission from reference 84 with permission from Wiley. Copyright 2011, John Wiley and Sons, Inc. doi:10.1128/microbiolspec.PLAS-0022-2014.f2

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<tr>
<td>Diffusion coefficient, D (cm²/s)</td>
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and physiological state at the time of transcription (96). For example, the Kozak sequence (gccRccAUGG), which includes the start codon, helps the transcript to bind to ribosome to start translation. Another important regulatory sequence is the polyadenylation site (AAUAAA) located at the 3′ end of the mRNA that allows termination of transcription and is important for the nuclear export, stability of mRNA, and, consequently, translation. Many eukaryotic expression vectors use the bovine growth hormone, SV40, or rabbit β-globin terminator sequences (88, 98, 99), or endogenous terminators that are downstream from the open reading frame of the gene of interest to ensure proper transcriptional termination.

**Immune Response to Plasmid Vectors**

The administration of plasmid vectors that contain bacterial sequences is likely to generate immune responses. The innate immune system is able to discriminate microbial components and self-components by identifying the pathogen-associated molecular patterns (PAMPs). Depending on their composition (lipopolysaccharides, nucleic acids, proteins, etc.), PAMPs are recognized by different pattern-recognition receptors (PRRs), triggering single, multiple, cooperative, or redundant specific signaling pathways that set up an immune response (100, 101). Toll-like receptors (TLRs), like others PRRs, are expressed in the cell surface or intracellularly (e.g., TLR9) by various immune cell types (e.g., macrophages, dendritic cells, B cells) and also by nonprofessional immune cells (e.g., fibroblasts, epithelial cells) (100, 101). Usually, sensing of PAMPs by PRRs upregulates the transcription of type I interferons and proinflammatory cytokines.

DNAs of bacterial origin show a high frequency of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides. In contrast, CpG motifs are infrequent in mammalian DNAs, and when present, are highly methylated (5mCpG). Overall, DNA of bacterial origin is a PAMP that is recognized by intracellular TLR9 and by stimulator of interferon genes (STING) proteins, activating innate immunity response (101, 102, 103). The TLR9 signaling cascade induces expression of type I interferons and inflammatory genes, mainly through activation of the transcription factors interferon regulatory factor 7 (IRF7) and NF-κB (101, 102). STINGs are endoplasmic reticulum translocon-associated transmembrane dimer proteins that are critical for regulating the production of interferon in response to cytoplasmic DNA (102, 104). STINGs bind to cytosolic double-stranded DNA without a requirement for accessory molecules (103), activating the TANK-binding kinase 1 (TBK1). STING appears essential for escorting TBK1 to endosomal compartments for activation of the transcription factors IRF3/IRF7 and NF-κB, leading to the expression of type I interferon and inflammatory cytokines (103).

Although this immunostimulatory property of CpG motifs is undesirable when plasmids are used for gene therapy purposes, it can be used favorably as an adjuvant in DNA vaccination (105). The idea is that the
type and the number of CpG motifs in a plasmid backbone can be designed to modulate the humoral immune response triggered by DNA vaccines (106, 107, 108, 109). Several human clinical trials have used such CpG adjuvants in the context of preventive and therapeutic vaccination (103, 110).

Optimized Vectors

Some of the functional elements (origin of replication, prokaryotic resistance marker) found in a plasmid are required only during the replication process that takes place during the growth of the prokaryotic production host. Once the cell culture is halted, those prokaryotic sequences are no longer needed and may actually decrease stability, uptake, and efficacy. Furthermore, even though the presence of some prokaryotic sequences in plasmids is approved by the FDA and European Medicines Agency, their use can be detrimental both from a clinical and environmental point of view. For example, concerns have been raised that a widely used selection marker like the kanamycin resistance gene may be horizontally transmitted to the recipient’s enteric bacteria (111, 112). Thus, and in line with the recommendations of regulatory agencies to eliminate antibiotic resistance markers from plasmid vectors, a number of antibiotic-free selection systems have been developed to produce safer and smaller (and eventually more efficient) plasmids (99, 112, 113). Noteworthy examples include plasmids with conditional origin of replication and plasmids free of antibiotic resistance vectors expressing small RNA-OUT antisense RNA (114), RNAI/RNAII-based plasmids containing ColE1-type origin (115), and operator-repressor titration systems (116). Additionally, the absence of antibiotic resistance genes from bacterial origin in these minimized plasmids may lead to reductions in innate immune responses and minimize the risk of silencing of transgene expression.

The backbone of a plasmid-based DNA vaccine can be designed with the amount of correct unmethylated CpG sequences that maximizes immune response. On the contrary, an effective plasmid vector for gene therapy can be designed by avoiding unmethylated CpGs or by adding CpG antagonists of TLR9 (e.g., containing a (5-methyl-dC)p(7-deaza-dG) or (5-methyl-dC)p(arabino-G) motif) (117). Another strategy involves the methylation of the plasmid before its administration. While several methylases (SssI, HpaII, or HaeIII) have been used in vitro, the costs associated with this strategy have prompted researchers to explore the possibility of performing methylation in vivo, for example, using SssI methylase. This approach has shown prolonged transgene expression by circumventing immune recognition (118).

Avoiding the presence of CpG sequences within regulatory sequences can also increase the level and the duration of expression (97, 119). Long-term gene expression (at least 19 months) has been reported by Wolff et al. (120), suggesting that plasmid DNA could stably persist and be expressed in nondividing muscle cells. However, strategies like chromosomal integration or episomal replication are usually required to obtain long expression periods. Interestingly, the Kay’s group (121) has shown that the administration of plasmid DNA containing CpG motifs (methylated or not) to the mouse liver will only lead to transgene silencing and innate immune responses if those sequences are covalently linked to the transgene. These authors hypothesized that the absence of DNA sequences devoid of transcriptional enhancers that maintain an active transcription state are prone to form repressive heterochromatin on the plasmid DNA backbone, which then spreads and inactivates the transgene in cis, but not in trans (121). Kay’s group also suggests that it is the length (>1 kb) and not the sequence of the extragenic DNA flanking the transgene expression cassette that leads to transgene silencing (122). This is a very complex subject from which much more insights are needed to understand the complex nature of regulation of gene expression.

The presence of bacterial sequences triggers an association with inactive forms of chromatin. Episomal DNA constructs with persistent expression have a slight greater abundance of histone H3 lysine 4 dimethylated, while unexpressed constructs showed enrichment in histone H3 lysine 9 trimethylated (123, 124, 125). Interestingly, AT-rich scaffold or matrix attachment regions, which facilitate opening and maintenance of euchromatin, can be incorporated near promoters to allow enhanced and persistent transgene expression (126).

Other plasmid derivatives that are devoid of bacterial sequences have been designed and tested successfully. Minicircles, for example, are double-stranded and supercoiled expression eukaryotic vectors devoid of bacterial sequences such as the origin of replication and the antibiotic resistant marker (22). Minicircles are produced in E. coli by excising the desired expression cassette from a parental plasmid. This excision takes place by promoting the in vivo recombination between two recombinase target sites strategically located in the parental plasmid backbone (Fig. 3). Several recombinases acting under the regulation of inducible promoters have been used to catalyze this excision, including λ-integrase, Cre recombinase, φC31 integrase, and Par resolvase (112, 113). The recombination event generates two products, a replication-deficient minicircle, which
contains the mammalian expression cassette, and a mini-
plasmid, which contains the undesired antibiotic resis-
tance gene and the bacterial origin of replication (Fig. 3).
The selection of the recombination system should be
aimed at providing the best balance between recombi-
nation efficiency and the yield of supercoiled minicircle
species. Since both products will coexist inside E. coli
cells once the process is terminated, adequate method-
ologies must be developed to isolate the therapeutically
useful minicircle from its counterpart (as well as from
the usual bacterial impurities). This separation consti-
tutes a challenge on its own owing to the similarity of
the physical-chemical characteristics of the two DNA
rings. One of the strategies devised to purify minicircles
relies on the placement of lactose operator sites in the
minicircle moiety and on the use of an affinity chroma-
tography matrix with bound lac repressor (LacI). When
a mixture of minicircles and miniplasmids is contacted
with this affinity column, minicircles will bind to LacI,
whereas miniplasmids are washed away in the flow-
through. Minicircles can subsequently be recovered by
eluting the column with isopropyl-β-D-1-thiogalacto-
pyranoside (127). Experimental evidence has shown that
adequately purified minicircles are able to generate a per-
sistent and high-level transgene expression in vivo (22).

The concept of transforming plasmids into minimal-
size gene transfer units also fostered the development of
short eukaryotic expression cassettes called Minimalistic
Immunogenically Defined Gene Expression (MIDGE)
vectors. Unlike the case of minicircles, however, the
final vector is a small, linear molecule that is covalently
closed at the extremities by two short hairpin oligonu-
cleotide sequences. The linear DNA fragments are gen-
erated either by restriction digestion of conventional
therapeutic vectors or by PCR-mediated amplifica-
tion (128, 129).

ADMINISTRATION AND DELIVERY
Barriers
The transportation of plasmids from the outside of the
body of a patient into the cell nuclei and the subsequent
expression of the gene cargo are critical for the success
of a plasmid-mediated gene transfer intervention (130).
However, this process is difficult to achieve because of
the existence of a series of barriers that DNA molecules
encounter during their journey across the entry route,
capillaries, interstitial spaces, tissues, body fluids, mem-
branes, and cells cytoplasm and that contribute to re-
duce the number of molecules arriving at the cell nucleus
(131). Examples of such barriers include mononuclear
phagocytes, blood components, low pH, plasma and
cellular endonucleases, cellular membranes, endosomes,
lysosomes, and narrow nuclear pore complexes (131,
132). The efficiency of plasmid vectors is critically de-
pendent of the use of delivery systems adequate to over-
come these barriers and guarantee that a significant
fraction of the administered pool of plasmid molecules
arrives safely and ready for transcription into the cell
nucleus (Fig. 4).

Plasmid biopharmaceuticals can be administered
through different routes depending on the disease and
therapeutic intervention planned. For example, intra-
tumoral administration is used in the treatment of solid
tumors (133), the airways are preferred when tackling
lung diseases (134), and muscular or skin tissues are
favored when administering prophylactic DNA vaccines
(72). Virtually all organs and tissues in the human body
have been used as entry points for plasmids (130). The
administration route, to a large extent, will constrain
the choice of the delivery system used to carry the plas-
mid from the vial on the shelf into the cell nucleus. Likewise, certain delivery systems and devices are spe-
cifically designed to serve defined entry routes.

FIGURE 4 The intracellular barriers to plasmid-
based gene transfer. In their journey to the
nucleus, plasmids have to cross the phospho-
lipidic cell membrane through endocytosis (1),
escape entrapment and degradation in endo-
somes and lysosomes (2), survive degradation
by cytosolic nucleases, traffic the overcrowded
cytoplasm (3), and translocate across the nu-
PLAS-0022-2014.f4
Plasmid DNA

Plasmids can be delivered to cells of a living recipient via the needle injection of a saline solution of the plasmid into muscle (Fig. 5), as originally experimented by Wolff and coworkers (5). While this naked DNA approach is simple, easy to execute, and safe, in most cases, the efficiency of expression of transgenes in the skeletal muscle of nonhuman primates is inferior in comparison with alternative delivery methodologies (135). The key problem relies on the fact that plasmids are rapidly cleared from the injection site because of the action of endogenous nucleases. This means that only a fraction of the injected molecules will transfect cells and that this is essentially restricted to the injection site. The exact mechanism by which naked plasmids cross cell membranes is unclear, but suggestions have been made regarding a receptor-mediated formation of plasmid vesicles at the cell surface and subsequent formation of endosomes and then fusion to lysosomes (136, 137). Plasmids must then escape endosomes/lysosomes and travel through the cytoplasm toward the cell nucleus, most likely via a mechanism of active transport involving microtubule networks (138). The final crossing of the nuclear envelope is facilitated if cells are engaged in division, but in nondividing cells the nuclear pore complex (NPC) becomes the only gate to access the nucleus (138, 139). In the latter case, the presence of nuclear localization signals has been shown to increase the translocation of plasmids through NPCs to a certain extent (140).

Naked DNA can be delivered more effectively via the rapid intravenous injection of a large volume (8% to 10% of the body weight) of a plasmid-containing saline solution, a procedure that favors the transfection of hepatocytes (24, 141). However, this so-called hydrodynamic injection is an inherently invasive and complex procedure difficult to transfer to the clinic (142). Naked plasmid DNA can also be delivered via liquid jet injectors, devices that generate fine (~76 to 360 μm) high-pressure jets that puncture through the skin at high velocities (100 m s⁻¹) and deposit solutions in the tissue beneath (141, 143).

Gene Gun

Particle bombardment (aka biolistics) is one of the most effective ways to deliver plasmids to living cells and tissues (Fig. 5) (72, 144, 145). The method relies on a hand-held device (the gene gun) that uses pressurized gases such as helium to propel plasmid-coated nonporous metallic microparticles (0.1 to 5 μm). Cartridges must first be prepared with a dry powder of the plasmid-coated particles and then inserted into the gene gun. Gold has been the metal of choice for medical applications. When the device is triggered, a gas jet crosses the cartridge, releasing and accelerating the particles with a speed that allows penetration of target tissues or organs. Gene guns have been often used to obtain strong immune responses on delivery of DNA vaccines to the antigen-presenting cells residing in the top layers of skin. The microparticles ejected by the gene gun cross past the outermost layer of the epidermis and puncture through the membranes and across the cytoplasm and nuclei of cells (72, 144, 145). Following expression, the antigens encoded in the plasmid are then processed, eliciting primary cellular responses and fostering the production of antibodies (72, 145). A number of preclinical and human clinical trials have been conducted to study the outcome of gene-gun-delivered DNA vaccines in the context of immunization against infectious diseases. These studies have indicated that substantially smaller doses of DNA vaccine (~1 to 10 μg) are required to obtain immune responses (antibody titers and CD8+ T cells) in mice and primates in comparison with intramuscular or intradermal injections (72, 145, 146).

FIGURE 5 In vivo plasmid delivery. Plasmid DNA can be combined and formulated with buffers, stabilizers, and inorganic or organic matrices and molecules to produce: (i) a saline solution of plasmid, (ii) gold particles coated with plasmid, (iii) plasmids complexed with cationic lipids or polymers, (iv) polymeric microparticles with encapsulated or surface-adsorbed plasmid, or (v) nanoparticles of compacted plasmid. doi:10.1128/microbiolspec.PLAS-0022-2014.f5
Electroporation  
The low efficiency of gene expression that is associated with the injection of naked plasmid DNA can be improved with electric fields generated by high-energy pulses, i.e., via electroporation. Allegedly, such electric fields transiently increase the transmembrane potential, leading to the opening up of ephemeral (microseconds to seconds) transbilayer electropores (<10 nm), or to the creation of structural defects in the membranes (147, 148). Suggestions have also been made that electrophoretic effects are created that actively drive the negatively charged plasmids and foster their passage across pores and into the cell cytoplasm (147, 148). Other authors, however, advocate that transit across the destabilized membranes occurs by passive diffusion (149) or that charged plasmid vesicles or stable DNA/membrane complexes are formed and subsequently endocytized (150). Critical electric parameters that can be manipulated to control plasmid delivery by electroporation include the number, length (few microseconds to milliseconds), voltage (50 to 1500 V), and waveform (exponential decay or square wave) of the pulses (148). A few companies have designed and developed electroporation devices to meet the requirements for a safe and consistent clinical delivery of plasmid DNA. Such devices typically combine a system that delivers the required electric pulses with an injection needle for intramuscular administration (151, 152). In general, preclinical and clinical data have shown that in vivo uptake of plasmids by tissues like the skin and muscle and transgene expression can be increased by electroporation (153, 154). Negative aspects that have been associated with electroporation include muscle stimulation, patient discomfort, and tissue damage (148).

Cationic Lipids and Polymers  
The transfection ability of plasmid biopharmaceuticals can be improved by formulating the plasmids with specific molecules such as cationic lipids and soluble polymers (Fig. 5). The methodology relies on the electrostatic interaction between the polyanionic plasmids with a cationic lipid (e.g., DOTAP-1,2-dioleoyl-3-trimethylammonium propane [155]) or polymer (e.g., polyethyleneimine [PEI], polylsine [156]). As a result of this interaction, plasmids collapse and condense, acquiring dimensions which are substantially smaller than the size of the individual plasmids. This coating of the negatively charged plasmids with cationic “envelopes” facilitates the fusion of the complexes with the negatively charged cell membranes, thus favoring internalization by endocytosis, and also protects plasmids against the attack of lysosomal and cytosolic nucleases.

Micro- and Nanoparticles  
Gene delivery can also be accomplished by plasmid-loaded polymeric microparticles of a defined size (0.5 to 10 μm; Fig. 5). A key advantage of these microparticles is that they allow a more prolonged release of plasmids instead of the bolus type of delivery that is characteristic of the submicron plasmid/polymer complexes described above (21, 157, 158). Two of the most popular polymers used in this context are poly(DL-lactide-co-glycolide) and poly(DL-lactic acid) owing to their biocompatible and biodegradable nature. Plasmid molecules can be either encapsulated (21, 159, 160) or adsorbed to the surface of the microparticles (158). Plasmid-loaded microparticles can be administered via subcutaneous or intramuscular needle injection. Once in vivo, the particles are phagocytosed by professional antigen-presenting cells (macrophages, dendritic cells) and then transported to the lymph nodes where plasmids are gradually released (161). The usefulness of microparticles as in vivo plasmid delivery agents has been described in the context of several diseases, including cancer (162), hepatitis B (163), and tuberculosis (164). In general, plasmid/microparticle formulations are safe and able to increase gene expression and the immunogenicity of DNA vaccines (162, 163).

The lipid/polymer complexes and microparticles described above have sizes in the 200-nm to 5-μm range and typically contain several plasmid molecules. This means that some kind of disaggregation or dismantling process must take place in the cytoplasm for plasmid molecules to be able to pass through the 25-nm-wide nuclear pore complexes of the nuclear envelope of cells. One way to overcome this need for dismantling before nuclear entry is to produce plasmid nanoparticles with sizes smaller than 100 nm (Fig. 5). These nanoparticles can be prepared, for example, by using chitosan (165), peptide-polyethylene glycol conjugates (134), or protamine sulfate-calcium carbonate (166).

SAFETY ISSUES  
Like other biopharmaceuticals, plasmids hold in them the potential to injure recipients. The specific safety issues that have been raised in association with the clinical use of plasmids include (i) the potential of plasmids and derived fragments to integrate into the host genomic DNA (167) and (ii) the stimulation of anti-DNA antibodies and autoimmune reactions (168). These questions have been addressed during preclinical development by performing pharmacological and toxicological studies with adequate animal models in line with the
recommendations of regulatory bodies (169, 170). The goals of such studies include the definition of safe starting doses and escalation regimens and the identification of organs at risk and parameters to monitor toxicity.

The potential for integration in the genome is minimal since sequences that might drive homologous recombination and direct integration (e.g., insertion sequences, retroviral-like long terminal repeats, sequences homologous to the packaging sequences of retroviruses) are removed during the design of the plasmid molecule (171). So far, results show that the risk for integration of plasmid sequences is much lower than natural, random mutations (172, 173, 174, 175). Furthermore, biodistribution and persistence studies have indicated that most plasmids that are administered intramuscularly (e.g., by needle injection, needleless jet, or particle-mediated delivery) remain close to the injection site and are rapidly degraded by endogenous nuclease within the first minutes (174, 176, 177), reducing even further the likelihood of integration.

A number of animal experiments have been conducted to investigate whether the administration of plasmids and the concomitant expression of the encoded transgenes in vivo could generate and promote the development of autoimmunity and other deleterious immunological responses (168, 178, 179, 180). One of the specific safety concerns is whether plasmids can induce the production of anti-DNA antibodies. Such anti-DNA antibodies could form immune complexes with circulating DNA, damaging various tissues and blood vessels in critical areas of the body, as is characteristic of systemic lupus erythematosus (171). However, no link between plasmid administration and changes in clinical markers of autoimmunity has been found yet (33).

So far, none of the concerns highlighted above have materialized, with scientific and clinical studies indicating that plasmid biopharmaceuticals are in general well tolerated and safe (174, 176, 181, 182, 183). Another reason for the favorable appreciation surrounding plasmid biopharmaceuticals is related to the fact that they are, in the vast majority of cases, designed to promote transient expression of the encoded protein in the target human tissues.

**PLASMID MANUFACTURING**

**Overview**

The development of plasmid-manufacturing processes is an undertaking that must occur in parallel with product development, not only because it is required to generate material for preclinical and clinical trials, but also because the methodology that will ultimately produce the plasmid biopharmaceuticals for sale must be established before market approval is received (184). The manufacturing of a plasmid biopharmaceutical product will consist of a string of activities (Fig. 6) that are set up and carried out with the aim of consistently producing a defined amount (e.g., measured as biological activity or mass) of a product that is safe and efficacious (184). The preparation of cell banks containing the plasmid of interest and the selection and testing of raw materials are at the forefront of the activities. Cell culture and downstream processing unit operations are then selected, arranged, designed, and operated to manufacture unformulated (i.e., bulk) plasmid DNA (Fig. 6). This purified plasmid product must then be adequately formulated by considering aspects such as the method of delivery, the final product form, ingredients (excipients, adjuvants, stabilizers), dosage details, packaging, etc. After the “filling and finishing” stage, the product is ready for clinical testing or marketing (Fig. 6).

**Cell Culture**

Plasmids are produced by promoting replication in *E. coli*. Before routine culture, a strain has to be chosen (e.g., DH5α, JM109) or developed (e.g., GALG20 [185]). While the genetic background of these producer strains may vary, mutations in the *recA* and *endA* genes to minimize recombination events and plasmid DNA degradation,

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**FIGURE 6** An overview of the different activities and steps involved in the manufacturing of plasmid biopharmaceuticals. doi:10.1128/microbiolspec.PLAS-0022-2014.f6
respectively, are close to universal. Other important genetic traits are related to genetic modifications (e.g., pkyA, pkyF, pgi) that increase the metabolic flux toward the formation of nucleotide precursors (185). Once a strain has been selected and transformed with the target plasmid, high-production clones must be carefully selected and isolated and used to establish master and working cell banks with stocks of vials of the plasmid-bearing cells. Growth medium composition, bioreactor-operating variables, and cultivation strategy must then be selected that maximize plasmid production. By adequately combining these parameters with a high-copy-number plasmid, volumetric plasmid yields up to 2.0 g/l can be obtained (186).

Downstream Processing
The train of unit operations in the downstream processing section is designed to recover plasmid DNA and remove host impurities (genomic DNA, RNA, proteins, etc.) until a level of purity compatible with human use is met (184). The unit operations can be grouped into three stages: primary isolation, intermediate purification, and final purification. The starting point is typically a broth with high cell densities (>55 g of dry cell weight/l) and plasmid concentrations (1.6 to 2.0 g/l [186, 187]). In the first stage, cells are harvested (e.g., by microfiltration) and lysed (e.g., alkaline lysis, thermal lysis) to release plasmid DNA. In the subsequent intermediate purification stage, clarified lysates are processed by using operations such as tangential flow filtration, precipitation (188), adsorption (189), and aqueous two-phase systems (190) to reduce the impurity load and concentrate the plasmid. Final purification aims to remove the more recalcitrant impurities such as gDNA and pDNA variants. Traditional chromatographic modalities such as gel filtration, anion exchange, and hydrophobic interaction (191) have all been used, mostly in the fixed-bed mode, to purify plasmid. Efforts have also been made to develop chromatographic operations based on amino acid (192), thiophilic (193), and phenyl boronate (189) ligands. Although dominating, chromatography is faced with limitations (poor selectivity, coelution, low capacity, and slow internal diffusion) that are related to the structural nature of the stationary phases and molecules (molecular mass >10^6 gm mol^-1, D = 10^8 cm^2/s) involved (194, 195). Larger capacity and faster internal mass transfer can be achieved if chromatographic membranes and monoliths are used instead of beads (195). Once impurities have been reduced to levels below the specifications, corrections to the plasmid concentration and buffer exchange can be accomplished by operations like ultrafiltration and alcohol precipitation (196). The final step in the downstream processing train is usually sterile filtration with 0.22-μm filters (197).

THE ROAD TO THE MARKET
A handful of plasmid biopharmaceuticals have already found their way into the market (198). In 2005, a veterinary DNA vaccine designed and developed jointly by the CDC and Fort Dodge Animal Health (Fort Dodge, Iowa) to protect horses against West Nile virus was licensed by the Center for Veterinary Biologics of the U.S. Department of Agriculture, thus becoming the first DNA vaccine to be registered with a governmental regulatory body (199). The vaccine was subsequently launched in the market in December 2008, under the trade name West Nile Innovator DNA (200). In the same year, a DNA vaccine developed by Novartis Animal Health (Victoria, Canada) to protect farm-raised salmon against Infectious Hematopoietic Necrosis virus (Apex-1HIN) also obtained regulatory approval and license (201). In early 2007, the U.S. Department of Agriculture conditionally approved a therapeutic DNA vaccine that delivers the MHC gene to dog tumors to treat melanoma in dogs (202). The vaccine hit the market under the trade name Oncept (198). Finally, in 2008, an injectable plasmid DNA encoding for porcine Growth Hormone Releasing Hormone (GHRH) developed by VGX Animal Health, Inc. (The Woodlands, Texas) to decrease perinatal mortality and morbidity in pigs obtained market entrance approval from the Australian Pesticides and Veterinary Medicines Authority (203).

CONCLUDING REMARKS
Plasmid-mediated gene transfer has slowly materialized as a possible solution for the management of an entire constellation of veterinary and human diseases. The investment made during the past 20 years in the development of this new class of biopharmaceuticals has generated a substantial amount of scientific and technological knowledge. Furthermore, plasmid biopharmaceutical prototypes are currently at the clinical stage of development to tackle multifactorial diseases like cancer and cardiovascular disorders and to prevent the onset of infections like AIDS or influenza. So far, the data accumulated have shown that plasmids, in general, are well tolerated and safe. However, progress must be made to increase the potency and efficacy of plasmid molecules in vivo. Advances are clearly needed in the delivery methodologies used to increase the number of
administered plasmids that reach the cell nucleus. The manipulation of plasmids and plasmid-related molecules (e.g., minicircles, MIDGEs) is also likely to originate molecules better adapted to bypass cell barriers and to mediate the expression of therapeutic genes.

ACKNOWLEDGMENTS
Conflict of interest: We declare that we have no conflicts.

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Plasmid Biopharmaceuticals


