key contaminants: *Escherichia coli* genomic DNA, RNA, and protein (<1%), and endotoxin levels should not exceed 40 EU/mg plasmid [159, 160]. Briefly, to date, manufacturing processes (i.e., upstream, fermentation, and downstream processing steps; for in-depth discussion, the reader is referred to articles that offer an extensive and up-to-date review of the processes [136, 161–163]) involved in the production of cGMP-grade plasmid DNA (drug substance) for human clinical trials have been designed in order to be cost-effective, efficient, and capable of producing large-scale quantities of SC pharmaceutical-grade plasmid DNA (e.g., ≥200 mg for pilot and early clinical trials or gram-to-kilogram quantities at industrial scale [122, 140, 164–168]). Accordingly, these higher capacities allow the preparation of DNA gene therapy or DNA vaccine formulations for clinical trials at the higher concentrations required (≥0.5 mg/mL) [30, 169–171].

Furthermore, it is important to mention that plasmid DNA homogeneity (i.e., most plasmid is in its SC form) is a major concern to pharmaceutical manufacturers since efficacy and stability depend, in part, on the topology of DNA (i.e., comparative topology in the relative amounts of SC; open circular, OC; and linear forms) [110, 140, 172–174]. It is generally recognized that via single-strand break, the SC form (used as a standard measure of DNA quality [160]) can convert to OC form, and further degradation can produce undesirable amounts of the linear form that has been associated with lower levels of expression once transfected [174, 175]. Typically, the different isoforms of plasmid DNA (i.e., relative amount of each of the forms—SC, OC, and linear—in a given sample) are determined by agarose gel electrophoresis [176]. This method has been routinely applied in the industry to analyze clinical-grade plasmid DNA. Unfortunately, the undesirable complications associated with gel staining (e.g., ethidium bromide) such as background variability, nonlinearity in staining, and the requirement of corrections for more accurate determination of SC content due to the distinct intercalating affinities of ethidium bromide for the different species [176] have encouraged the development of more accurate chromatographic methods: ultra performed liquid chromatography (UPLC; [110]) and anion-exchange high-pressure liquid chromatography (AE-HPLC; [173]). For instance, UPLC has proven to be a valuable tool to monitor the distribution of plasmid isoforms in dried samples subjected to extreme conditions such as metal contamination and storage under accelerated conditions [110]. Certainly, maintenance of plasmid DNA integrity (i.e., ≥80% SC as recommended by FDA, [160]) is an important structural characteristic that will have an impact not only on bulk production but also long-term stability (e.g., in the dried state), efficacy, and eventually clinical approval.

As indicated above, the plasmid DNA-containing formulation must be sufficiently stable (e.g., 18–24 months) against physical and chemical pathways of degradation. To this end, the formulation scientist must consider a series of relevant approaches (e.g., raising pH, addition of chelators and antioxidants, alone or in combination) to improve the stability of clinical-grade plasmid DNA during storage either in solution to minimize acid-catalyzed degradation pathways and oxidation (aside from nuclease-related contamination) [61, 64, 177] or in a dehydrated form to inhibit mainly free radical-mediated oxidative damage [82, 178], as described below. Plasmid DNA is typically manufactured in low-salt conditions (2–10 mM),