

the presence of a nonreducing disaccharide (i.e., trehalose) [182]. Furthermore, storage studies conducted for longer periods of time in trehalose have been performed in our laboratory and similarly, we observed a progressive strand breakage (i.e., loss of SC content) over a period of 6 months in purified plasmid DNA formulations [82]. Particularly surprising was the observation that chemical damage took place at a very-low-storage temperature (e.g.,  $-20^{\circ}\text{C}$ ;  $T_{\text{g}} - T_{\text{storage}} > 50^{\circ}\text{C}$ ) despite samples being lyophilized in the presence of highly purified trehalose (at a higher ratio of sugar to DNA, 1000 w/w) and optimal glassy-state conditions (high  $T_{\text{g}}$ ,  $113^{\circ}\text{C}$  and low moisture levels of  $\sim 0.5\%$ , [82] and Molina M.—unpublished data). We also observed significant degradation (lost of SC) at room temperature, and, not surprisingly, at higher temperatures. The observation that nonreducing sugars fail to stabilize naked DNA during storage strongly suggests that other degradation mechanisms such as oxidative damage (formation of reactive oxygen species; ROS) are active in the dried cake. Considering that biomolecules are immobilized in a glassy-excipient phase where reactions requiring positional specificity would be considerably inhibited in the dried state [127], our recent findings show that degradation can still occur at significant rates despite maintenance of a glassy phase (e.g., in trehalose). In fact, our validated fluorescent-based method [115] has been used consistently to detect the formation of ROS in lyophilized naked DNA, lipid alone, as well as lipid/DNA complexes, and demonstrated that ROS are still active in the solid state [115, 178, 179, 183]. Furthermore, our findings suggest that trace metal contamination ( $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Fe}^{3+}$ ) is a prominent factor affecting the stability of these biomolecules, particularly when an unsaturated lipid is present in the formulation composition (e.g., dried lipid/DNA complexes, as described in corresponding section of this chapter) [179, 183]. It should also be noted that even very small quantities (parts per billion, ppb) of transition metals (e.g., ferrous iron  $-\text{Fe}^{2+}$ ) can mediate generation of ROS in the dried state [115]. Furthermore, trace amounts of metal contaminants are present even in the cleanest materials available commercially (e.g., clinical-grade DNA and GMP-grade sugars) [103, 115, 183]. It is generally recognized that free radical oxidation is a major chemical degradation pathway for DNA-based pharmaceuticals [61, 62, 64, 184] and that it is substantially stimulated by molecular oxygen [185, 186]. In an attempt to prevent oxidative damage and extend the shelf life of lyophilized DNA-based formulations in the dried state, our laboratory has evaluated various strategies including metal removal from reagents (demetalation, [61, 77, 187]), air displacement in the vial headspace [188], and fortification with chelator/antioxidant agents [62, 184]. We found that demetalation (Chelex 100 resin) and headspace oxygen displacement were insufficient to completely inhibit the formation of ROS in dried formulations [178]. Although removal of low levels of oxygen contamination from the gas stream in the vial headspace is a general practice in pharmaceutical manufacturing processes; however, it has been suggested that the low efficiency of a demetalation step to both eliminate metal contamination and prevent the formation of ROS in dried formulations is impracticable for large-scale purification and production [56]. Yet, undetectable metal levels (below detection limits,  $<5$  ppb) may induce damage during prolonged storage [61, 115, 183]. An effective strategy is to incorporate chelating agents into DNA preparations [82]. Indeed, our recent investigation has shown the great effectiveness of the ion-chelating agent DTPA to counteract the damaging effect of ROS (e.g., lost in SC) in lyophilized DNA-based formulations