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Research Paper

Moisture-Induced Aggregation of Lyophilized DNA and its Prevention

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Purpose. To investigate the moisture-induced aggregation (i.e., a loss of solubility in water) of DNA in a solid state and to develop rational strategies for its prevention.
Methods. Lyophilized calf thymus DNA was exposed to relative humidity (RH) levels from 11% to 96% at 55°C. Following a 24-h incubation under these stressed conditions, the solubility of DNA in different aqueous solutions and the water uptake of DNA were determined. The effects of solution pH and NaCl concentration and the presence of excipients (dextran and sucrose) on the subsequent moisture-induced aggregation of DNA were examined. The extent of this aggregation was compared with that of a supercoiled plasmid DNA.
Results. Upon a 24-h incubation at 55°C, calf thymus DNA underwent a major moisture-induced aggregation reaching a maximum at a 60% RH; in contrast, the single-stranded DNA exhibited the maximal aggregation at a 96% RH. Moisture uptake and aqueous solubility studies revealed that the aggregation was primarily due to formation of inter-strand hydrogen bonds. Aggregation of DNA also proceeded at 37°C, albeit at a slower rate. Solution pH and NaCl concentration affected DNA aggregation only at higher RH levels. This aggregation was markedly reduced by co-lyophilization with dextran or sucrose (but not with PEG). The aggregation pattern of a supercoiled plasmid DNA was similar to that of its linear calf thymus counterpart.
Conclusions. The moisture-induced aggregation of lyophilized DNA is caused mainly by non-covalent cross-links between disordered, single-stranded regions of DNA. At high RH levels, renaturation and aggregation of DNA compete with each other. The aggregation is minimized at low RH levels, at optimal solution pH and salt concentration prior to lyophilization, and by co-lyophilizing with excipients capable of forming multiple hydrogen bonds, e.g., dextran and sucrose.

KEY WORDS: aggregation; DNA; lyophilization; moisture; relative humidity; solid state.

INTRODUCTION

Several hundred gene therapy clinical trials aimed at curing acquired and inherited diseases are currently ongoing worldwide (1–4); nearly all of them utilize DNA as the therapeutic moiety, either in its naked form or incorporated along with a vector (1). In addition, the use of DNA in vaccine applications has been explored (5–7). However promising such applications may be, the potential utility of the putative DNA-based pharmaceuticals requires their sufficient stability during large-scale production, shipping, and storage.

Storage of DNA-based preparations in a solid (particularly lyophilized) form is preferable due to low volumes, as well as diminished degradation reactions compared to those in water. While DNA solution stability has been thoroughly investigated (8–10), analogous systematic studies in a solid state are lacking. Like proteins (11–13), DNA is potentially liable to a moisture-induced, solid-state aggregation upon storage. When the relative humidity (RH) is varied from 96% to zero (resulting in the corresponding decrease in DNA hydration in the solid state), the DNA's double helical structure changes from B to A forms and then to a disordered one (14–17). And yet, while irreversible changes in DNA upon lyophilization have been investigated (18), little is known about the tendency of lyophilized solid DNA to aggregate over time in the presence of moisture. In fact, we have found just a single report in the literature dealing with this phenomenon (19); this 1977 paper, however, lacks a detailed mechanistic analysis of a moisture-induced aggregation of DNA, nor does it address the question of its prevention.

In the present study, we have systematically examined the phenomenon of a moisture-induced aggregation of linear, both double-stranded (ds-) and single-stranded (ss-) calf thymus DNAs; moreover, key observations have been generalized using a supercoiled plasmid DNA. Based on the mechanistic insights gained, we have developed rational strategies for protection against this detrimental process.

MATERIALS AND METHODS

Materials

All buffer components, inorganic salts, and chemical reagents used herein were of the highest purity grade

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commercially available (mainly from Aldrich Chemical Co., St. Louis, MO) and used without further purification. Calf thymus DNA, Na salt, was obtained form Sigma Chemical Co. (St. Louis, MO). The plasmid DNA, gWiz Beta-gal (8,278 base pairs) encoding the β-galactosidase gene, was purchased from Aldevron (Fargo, ND) as a 5.0 mg/ml stock solution in water. De-ionized water was used to prepare all aqueous solutions.

Preparation and Lyophilization of DNA Solutions

Calf thymus DNA was dissolved (1.5 mg/ml) in a 5 mM citrate buffer, pH 6.0 (unless otherwise specified; this pH was selected because it was used in ref. (19)), containing 1 mM Na EDTA. When needed, the pH of a DNA solution was adjusted using 1.0 N NaOH or HCl. To investigate the effect of NaCl and such excipients as surfactants, poly(ethylene glycol), dextran, and sucrose, DNA was dissolved in the pH 6.0 aqueous buffer containing them in desired concentrations.

The aforementioned solutions (typically 1.9–2.1 ml, based on the exact DNA concentration as determined by measuring its absorbance at 260 nm using an extinction coefficient of 20 $l \cdot g^{-1} \cdot cm^{-1}$) containing 3 mg of DNA were placed in 10-ml glass vials. These solutions were flash-frozen in liquid nitrogen and lyophilized in a Labconco Freeze Dryer 8 at 50 mTorr for 24 h.

Upon dissolution of this lyophilized DNA in pH 6.0 buffer and subsequent heating to 95°C or on raising the pH to 12.5, the absorbance at 260 nm increased by some 40% (the absorbance value of a 50 μg/ml solution rose from 0.7 to 1.0). Based on the extinction coefficients of ds-DNA and ss-DNA (20 and 30 $l \cdot g^{-1}\ cm^{-1}$, respectively), this indicated that the dissolved DNA was predominantly (over 80%) ds-calf thymus DNA (9).

For studies with ss-calf thymus DNA, the nucleic acid obtained from Sigma was dissolved in the pH 6.0 buffer and the resultant solution was heated at 95°C for 30 min, then immediately flash-frozen in liquid nitrogen, and subjected to lyophilization as described above. For studies with plasmid DNA, a 5 mg/ml nucleic acid solution in the pH 6.0 citrate buffer was diluted 3.3 fold with the same buffer; when the latter contained dextran, its ratio to DNA was 10:1 (w/w).

Solid-State Aggregation Studies at Various Relative Humidity Levels

Glass vials containing lyophilized DNA (3 mg in each) were placed in pre-equilibrated controlled humidity chambers maintained by using appropriate saturated salt solutions at 55°C, unless otherwise specified. The following salts were used to obtain the respective RH levels at 55°C (20): LiCl (11% RH), $MgCl_2 \cdot 6H_2O$ (33% RH), NaBr (50% RH), $NaNO_2$ (60% RH), KCl (82% RH), and K_2SO_4 (96% RH).

Following a 24-h incubation at various RH levels, the vials were removed, and 5× the original volume of the pH 6.0 buffer (with or without appropriate concentrations of additives like those used in solutions prior to lyophilization) was added to the DNA samples. The vials were gently shaken for 2 days (after which time no further dissolution took place) at 4°C to dissolve the still-soluble DNA and then centrifuged at 20,000 rpm in a Sorvall RC-5B centrifuge for 30 min. The resultant supernatant (0.11 ml) was removed and assayed for ss-DNA by diluting to 1.0 ml with aqueous NaOH (pH 12.5) and measuring the absorbance of this solution at 260 nm. Aggregation of DNA was quantified by comparing the amount of still-soluble DNA following incubation of lyophilized samples at various RHs with that in solution prior to lyophilization.

All measurements were carried out in triplicate, except for those involving the effect of solution pH and with plasmid DNA done in duplicate. For triplicate samples, the mean values and standard deviations are reported; for duplicate samples, the means of the data are given.

Water Content Analysis

Water contents of DNA samples following both lyophilization and subsequent incubation at various RH levels were analyzed using a Mettler DL18 autotitrator. Hydranal-titrant 5 and hydranal solvent were used as reagents for titration (11). Briefly, 2.0 ml of anhydrous methanol was added to the samples and, after a 30-s ultrasonication, 0.5 ml of the resulting dispersion was injected into the titrator. Anhydrous methanol was used as a blank. All water contents henceforth are reported in g/g of dry DNA. For each DNA sample and data point, the water content was calculated in triplicate, and the mean value and standard deviation were calculated. Unfortunately, the moisture uptake data obtained in the present study were not amenable to the classical Brunauer–Emmett–Teller (BET) gas adsorption equation (12) presumably because aggregated, denatured, and native DNAs at various RH levels ought to have different water adsorption behavior.

RESULTS AND DISCUSSION

Moisture-induced, solid-state aggregation of proteins is reasonably well understood (11–13). DNA shares with proteins its structural complexity (8). Furthermore, the double helix of DNA is stabilized by the same types of forces as those maintaining protein structures, namely, hydrophobic interactions (responsible for base stacking), inter-strand hydrogen bonding between the bases (responsible for complementary base pairing), and electrostatic repulsions (that in the phosphate backbone provide rigidity to the double helix) (21). Like proteins, DNA can undergo structural changes not only in solution but also in a dry state depending on the level of hydration which, in turn, is controlled by the relative humidity (RH) in its environment (14,17). Owing to these similarities, it seems plausible that DNA also can be liable to a moisture-induced, solid-state aggregation. This phenomenon, however, is obscure; in fact, there is only one, 30-year-old, Russian-language report describing a loss of aqueous solubility of dry DNA upon a long-term exposure to moisture at elevated temperatures (19). That seminal study proposed formation of inter-strand DNA cross-links as a reason for the aggregation but provided little detail. Given the emerging contemporary interest in stable dry DNA preparations for pharmaceutical purposes outlined in the Introduction, in the present work we systematically examined moisture-induced aggregation of lyophilized calf thymus DNA as a model nucleic acid and

then tested the generality of key aspects of this phenomenon with a plasmid DNA.

Solid-State Aggregation of Calf Thymus DNA

Based on ref. (19), we started out by studying the time course of a solid-state aggregation of lyophilized calf thymus DNA at an "exacerbated" temperature of 55°C and two humidity levels, namely, a moderate RH of 60% and a high RH of 96%. Briefly, 3-mg lyophilized DNA samples in separate vials were placed in pre-equilibrated desiccators at these RH values in an oven maintained at 55°C; the samples were subsequently withdrawn at different times and analyzed for both DNA moisture content and remaining water-soluble DNA by incubating in a large excess of a buffered aqueous solution for 2 days.

Lyophilization by itself led to no appreciable (under 5%) loss of latent (i.e., potential) aqueous solubility of DNA; moreover, when stored in a desiccator at nearly 0% RH, no aggregation of the lyophilized DNA was observed (Fig. 1A, open squares). In contrast, at a 60% RH (Fig. 1A, closed circles) lyophilized DNA avidly aggregated as a function of time: for example, after a 24-h incubation some 99% of lyophilized DNA failed to subsequently re-dissolve in water. Somewhat counter-intuitively, much less aggregation of lyophilized DNA was observed at a 96% RH (Fig. 1A, open circles) than at 60%. When the pH of the supernatant containing the re-dissolved DNA following incubation at a 96% RH was adjusted to 12.5, some one-third rise in the 260 nm absorbance ensued (9). Since the extinction coefficient increases maximally by 50% upon a conversion from a 100% ds- to a complete ss-DNA (see Methods), this observation confirmed that the non-aggregated DNA remained predominantly (about 70%) in its native double-helical form.

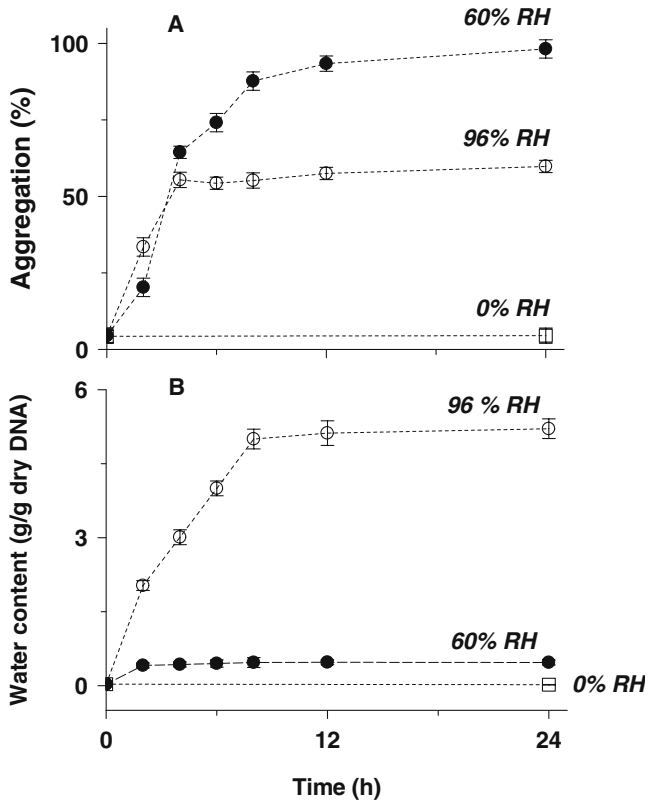

Fig. 1. The time-courses of (A) a moisture-induced aggregation of, and (B) a water uptake by, lyophilized calf thymus DNA at 55°C when stored at a 0% (□), 60% (●), and 96% (○) relative humidity levels. DNA (double-stranded in solution) was lyophilized from a 5 mM citrate buffer, pH 6.0, containing 1 mM EDTA (see Methods for details).

The water uptake behavior of lyophilized DNA also was found to strongly depend on RH values. No significant change in the water content was observed when DNA was stored under dry conditions (Fig. 1B, open squares). At a 60% RH (Fig. 1B, closed circles), the maximum water uptake of 0.52 g/g of dry DNA was reached within the first 2 h. In contrast, an order of magnitude greater water uptake (5.1 g/g of dry DNA) was observed at a 96% RH, but it was attained after a much longer period of time (Fig. 1B, open circles).

That aggregation of DNA is observed only upon exposure to a humid, but not dry, environment indicates that this process is indeed caused by moisture. The lack of noticeable water absorption by lyophilized DNA exposed to a dry environment is consistent with this conclusion.

Interestingly, as revealed by Fig. 1, at a 60% RH DNA continued to aggregate well beyond 2 h even though the equilibrium water content no longer rose. This discrepancy suggests that even a relatively low water content (some 0.5 g/g of dry DNA) can enable enough molecular mobility in the lyophilized DNA for the aggregation to proceed. On the other hand, although the moisture content of DNA was far higher after a 96% RH incubation, the aggregation was nowhere near the level reached at a 60% RH, indicating that the loss of DNA solubility does not directly correlate with the water uptake.

The question arises as to why a much more severe aggregation is observed at a 60% than 96% (Fig. 1A). It is well established that the conformation of solid DNA depends on the degree of hydration and hence on the humidity of the surrounding atmosphere (14–17). At RH values over 90%, DNA is present in the B-form at room temperature. At somewhat lower RH levels, DNA tends to acquire a variety of forms (A, C, and D) depending on its sequence, salt concentration, and counter-ion type. Below a certain RH level, typically 60%, DNA acquires a disordered form characterized by a loss of base stacking and the presence of regions resembling ss-DNA (14,15).

Based on this information, we hypothesized that below some 60% RH lyophilized DNA aggregated due to formation of random, inter-strand, non-covalent cross-links [as proposed in ref. (19)]. However, at higher humidities, owing to plentiful hydration, renaturation of DNA to form a double helix competes with a random cross-linking forming insoluble DNA aggregates. To test this hypothesis, we examined in more detail the effect of RH on DNA's tendency to irreversibly aggregate.

Closed circles in Fig. 2A depict aggregation of lyophilized DNA incubated at various RH levels and 55°C for 24 h. It is seen that up to a 33% RH the loss of DNA's latent solubility in water increases slightly, then jumps dramatically at a 50% RH, and finally declines at RH levels above 60%. This aggregation profile correlates with the structural changes in DNA as a function of relative humidity reported

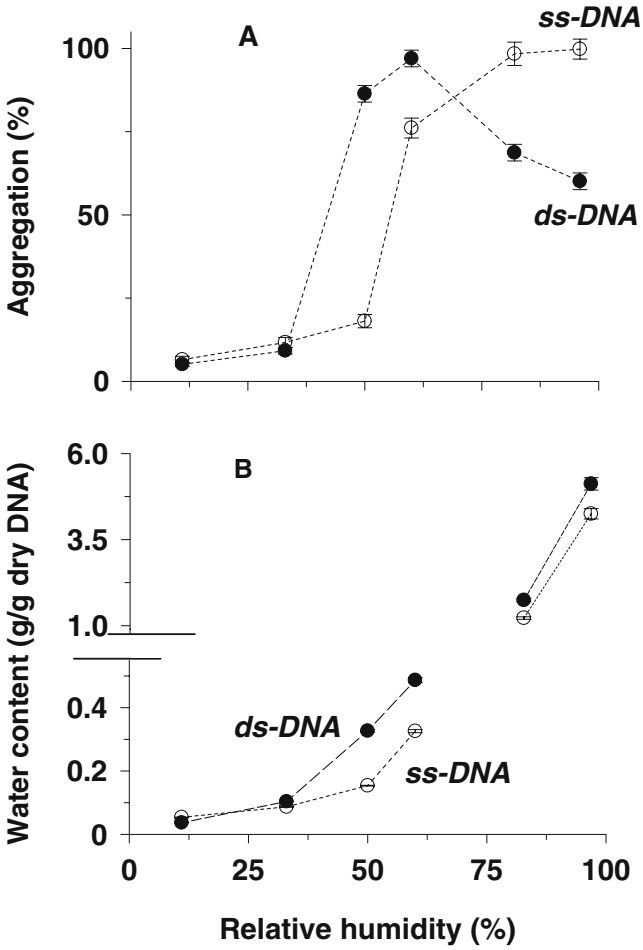

Fig. 2. The effect of relative humidity during a 24-h incubation at 55°C on (A) the extent of a solid-state aggregation of, and (B) the water content in, calf thymus DNA lyophilized from an aqueous solution containing the double-stranded (ds-, •) and single-stranded (ss-, ○) nucleic acid. The ss-DNA was produced by heating a solution of the ds- one in a 5 mM citrate buffer, pH 6.0, containing 1 mM EDTA at 90°C for 15 min, followed by flash-freezing in liquid nitrogen and lyophilization (see Methods for details).

in literature at room temperature (14,15), thus supporting the aforementioned hypothesis. Therefore, the rising aggregation up to some 60% RH apparently directly relates to the increase in the water content of DNA, which enhances the conformational mobility of DNA molecules, thereby resulting in more aggregation with still no appreciable renaturation. However, at higher RH levels the renaturation of DNA to the ordered double helical structures competes with the aggregation.

To further elucidate the role of moisture, the water content of the DNA samples after a 24-h incubation at 55°C at various RHs was determined by the Karl Fischer assay. As seen in Fig. 2B (closed circles), the DNA-water content climbs dramatically from 0.03 g/g at a 11% RH to 0.51 g/g at a 60% RH to as high as 5.1 g/g at a 96% RH. Thus up to a 60% RH level, the DNA aggregation appears to directly correlate with the water uptake. Note, however, that this rise in aggregation is not gradual, i.e., an abrupt jump in the extent of aggregation at a 50% RH, compared to that at 33%, is observed. This jump suggests that the aggregation process markedly accelerates after a certain level of moisture is reached in lyophilized DNA.

Calorimetric studies on denatured calf thymus DNA equilibrated at different RH levels reveal that its glass transition temperature, T_g, strongly depends on the water content (22). In particular, at a water content of under ~15% the T_g value of denatured DNA surges above 75°C, whereas at a water content of over 25% the T_g plummets below 40°C. These observations may explain the sudden jump in aggregation between 33% and 50% RH levels for ds-DNA observed by us (Fig. 2A). Since at a 33% RH (corresponding to some 10% water content of DNA), the T_g of the system exceeds 75°C, at 55°C (the incubation temperature in the present study) DNA exists as a glass with limited molecular mobility, thereby leading to less aggregation. At a 50% RH (corresponding to some 30% water content of DNA), however, the T_g of the system is under 40°C, indicating a viscous, liquid-like state of the hydrated system and hence a high mobility of DNA at 55°C. In other words, the transition of DNA from a glassy to rubbery state within this RH range apparently results in a greater conformational mobility and, consequently, a sharp acceleration of the aggregation.

To verify that the renaturation to form a double helical structure indeed competes with aggregation of DNA at higher RH levels, we extended the foregoing studies to ss-DNA. The latter was produced from ds-DNA by heating its solution at 90°C for 30 min and flash-freezing it in liquid nitrogen, followed by lyophilization. The dry ss-DNA samples were kept in desiccators with various RH levels at 55°C for 24 h, then incubated in an excess of aqueous buffer, centrifuged, and finally assayed for soluble ss-DNA by measuring the supernatant's absorbance at 260 nm. Separately, the solid DNA samples were also analyzed for the water content after exposure to various RH levels.

The ss-DNA molecules produced by this method should have highly disordered structures in a dry form. In the presence of abundant hydration (for example, at a 96% RH), these molecules would require drastic reorientation to renature and to form ds-DNA. This process differs from the renaturation from dry ds-DNA where, due to its retention of the overall orientation (despite some local disordering), upon rehydration at high RH levels the renaturation would have a lower energy barrier. This reasoning, combined with our hypothesis that at higher RH levels renaturation and aggregation compete against each other, suggests that the aggregation would be prevalent at higher RH levels in the case of ss-DNA. As seen in Fig. 2A (open circles), this is indeed the case. Evidently, the extent of aggregation of ss-DNA rises with atmospheric humidity, and the maximum occurs at a 96% RH. Moisture uptake studies indicate a lower water content in ss-, compared to ds-, DNA, presumably because of the exposed hydrophobic bases in the former; this behavior has been reported previously (23,24). As in ds-DNA, a certain water content is essential in dry ss-DNA, below which the rate of aggregation rapidly increases. The transition in the case of ss-DNA occurs below a 50% RH. Much less aggregation and lower water content is observed in ss-DNA at a 50% RH compared to ds-DNA (Fig. 2).

The foregoing studies point to the essential role of moisture in causing aggregation of dry DNA at lower to

Table 1. The Effect of Different Solution Conditions on the Dissolution of Aggregated Calf Thymus DNA Obtained After a 24-h Incubation of Lyophilized DNA at 55°C and a 60% Relative Humidity

Solution	Temperature (°C)	Incubation time in solution (days)[b]	Soluble DNA (%)
Buffer[a]	25	2	5 ± 2
	55	1	8 ± 2
	95	30 min	100 ± 3
1.0% (w/v) Polyoxyethylene stearate 100	25	2	5 ± 1
1.0% (w/v) Tween 80	25	2	4 ± 1
8 M Urea	25	1	18 ± 2
	55	1	80 ± 3
	65	1	99 ± 3
95% (v/v) Formamide	25	1	36 ± 2
	55	1	80 ± 3
Aqueous NaOH (pH 12.5)	25	1	98 ± 3

[a] Aqueous 5 mM citrate buffer, pH 6.0, containing 1 mM Na EDTA was used as the dissolution buffer and to prepare polyoxyethylene stearate 100, Tween 80, and urea solutions; 95% formamide and aqueous NaOH (pH adjusted to 12.5) were prepared using deionized water. [b] Unless otherwise specified.

intermediate RH levels and in initiating conformational changes in DNA — in competition with aggregation — at high RHs. Importantly, the relative rates of DNA renaturation and aggregation at higher RH levels strongly temperature-dependent. A likely explanation is that while DNA fully renatures at room temperature at high levels of hydration, at elevated temperatures (namely, 55°C) the rate of aggregation exceeds that of renaturation.

Solubilization of DNA Aggregates

Next, we investigated the nature of the DNA aggregates to shed light on the interactions that lead to their formation. To this end, the DNA aggregates formed after a 24-h incubation of ds-DNA at a 60% RH and 55°C were subjected to solubilization tests in different solutions. Based on a plausible assumption that the forces resulting in DNA aggregates are the same as those responsible for maintaining the helical structure of ds-DNA, i.e., hydrophobic interactions between bases and hydrogen bonds in specific base pairing, we used solution conditions known to denature ds-DNA. Table 1 depicts the relationship between the degree of solubilization of aggregated DNA and these solution conditions.

It is seen that under the conditions known to completely denature ds-DNA, such as 8 M urea or 95% formamide at 25°C (25,26), only marginal solubilization of the aggregated ds-DNA was observed. However, as the temperature was raised to 55°C, almost 80% of the DNA aggregates was solubilized by these denaturants, and at 65°C a complete solubilization was observed in 8 M urea. Urea and formamide denature ds-DNA to form ss-DNA primarily via disruption of hydrogen bonds involved in base pairing (25). That they fail to significantly solubilize DNA at 25°C and require higher temperatures to do so suggests that an extensive interstrand hydrogen bonding network, exceeding that involved in base pairing in ds-DNA, is responsible for the formation of the moisture-induced DNA aggregates. The latter also were insoluble in concentrated non-ionic surfactant solutions (Table 1, 2nd and 3rd entries) suggesting a minimal role for hydrophobic interactions in this DNA aggregation. This conclusion was further supported by our finding (data not shown) that adding the non-ionic surfactants to DNA solutions prior to lyophilization did not prevent moisture-induced aggregation of calf thymus DNA [this approach is used to prevent irreversible protein aggregation during and following lyophilization (27)].

As seen in Table 1, two other conditions known to denature ds-DNA (9), namely the temperature of 95°C (28,29) and the alkaline pH of 12.5 (30), were also able to completely solubilize aggregated DNA. These observations confirm a predominant role played by hydrogen bonding in the moisture-induced aggregation.

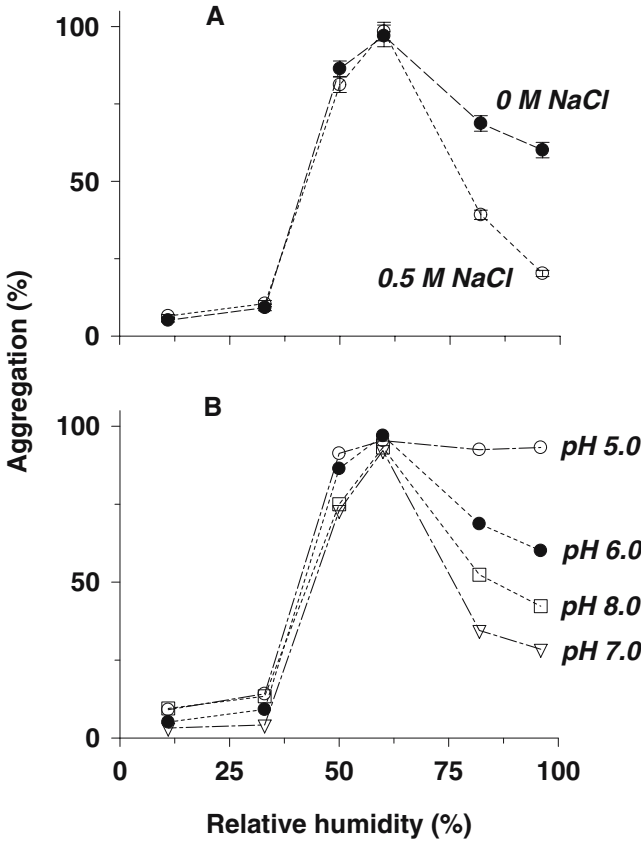

Fig. 3. The effect of solution (A) NaCl concentration [0 (●) and 0.5 M NaCl (○)] and (B) pH [5.0 (○), 6.0 (●), 7.0 (▽), and 8.0 (□)] prior to lyophilization on the extent of a solid-state aggregation of calf thymus DNA at various relative humidities. In the case of (A), a 5 mM citrate buffer, pH 6.0, containing 1 mM EDTA was used; in the case of (B), the pH of this buffer was adjusted prior to lyophilization using 1.0 N NaOH or HCl. For pH studies, the results are mean values of duplicate measurements.

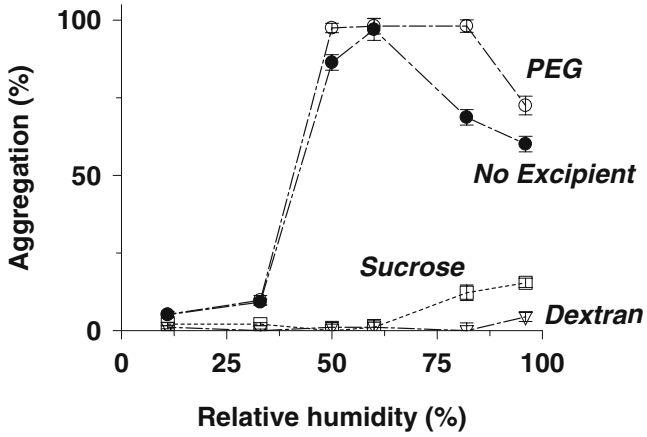

Fig. 4. The effect of excipients co-lyophilized with calf thymus DNA (present in a double-stranded form in solution prior to lyophilization) on the extent of a solid-state aggregation of DNA after a 24-h incubation at 55°C at various relative humidities. All excipients were present in a 10:1 (w/w) ratio to DNA in solution prior to lyophilization. Excipients: none (●); poly(ethylene glycol) (M_w=10,000) (○); dextran (M_w=142,000) (▽); and sucrose (□).

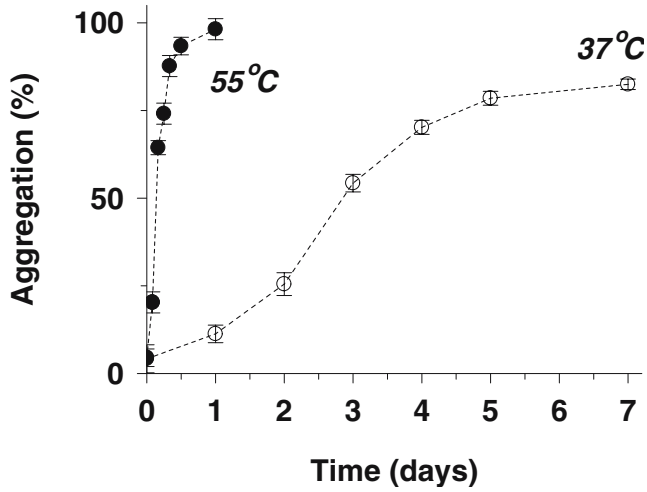

Fig. 5. The time course of a moisture-induced aggregation of lyophilized calf thymus DNA incubated at a 60% RH at 55°C (●) and at 37°C (○). The nucleic acid (double-stranded in solution) was lyophilized from a 5 mM citrate buffer, pH 6.0, containing 1 mM EDTA (see Methods for details).

Stabilization of DNA Against Moisture-Induced Aggregation

Based on the foregoing mechanistic insights, our next goal was to develop rational approaches to preventing the moisture-induced, solid-state aggregation of DNA. The crux of one such approach was to favor renaturation by promoting formation of ds-DNA. Since the presence of salts profoundly affects the conformation of DNA (17,31), we reasoned that even at relatively low RH values the double helical structure of DNA might be maintained in the presence of enough salt. To test this rationale, ds-DNA was lyophilized from a solution containing 0.5 M NaCl instead of none; the Na^+ concentration contributed by buffer species was 0.02 M. As seen in Fig. 3A, the moisture-induced aggregation of DNA lyophilized from the NaCl solution (open circles) was retarded only at high RH values of 82% and 96%. Thus 0.5 M NaCl does not seem to influence the solid-state DNA structure and to favor the renaturation *vs.* aggregation at lower RH levels. This observation is consistent with our hypothesis that only at higher RH values are the renaturation and aggregation concurrent and competitive processes and, therefore, the solutes that promote DNA renaturation also prevent DNA aggregation.

Another solution parameter that affects DNA renaturation is the pH (32,33). Fig. 3B shows the effect of solution pH prior to lyophilization on the moisture-induced aggregation of lyophilized DNA. One sees no profound pH-dependence of the extent of aggregation up to a 60% RH level. At higher humidities, however, the effect was significant, with the maximum aggregation observed at pH 5.0 and the minimum at pH 7.0. This pH-dependence is similar to that of DNA denaturation in solution (33), where ds-DNA tends to denature to ss-DNA as the pH is lowered below pH 6.0 or raised above pH 8.0. Hence, the pH that favors native ds-DNA should afford the greatest stability against aggregation at elevated RH levels where both renaturation and aggregation are important. Consistent with this mechanism, the maximal stability against the moisture-induced aggregation in Fig. 3B is observed at pH 7.0, and the stability decreases as the pH is shifted from there in either direction.

We next examined whether certain additives can stabilize DNA against the solid-state, moisture-induced aggregation. First, we tested two polymers, poly(ethylene glycol) (PEG, M_w=10,000) and dextran (M_w=142,000), in the hope that due to their ability to form a single amorphous phase with dry DNA (34–36) they can provide a physical barrier against inter-strand interactions. As seen in Fig. 4, PEG provided no protection (and neither did its 10-fold larger counterpart, data not shown), whereas dextran completely stabilized DNA against the moisture-induced aggregation, irrespective of the RH. Note that dextran was a potent stabilizer at 55°C even at RH levels of 80% and 96%, where

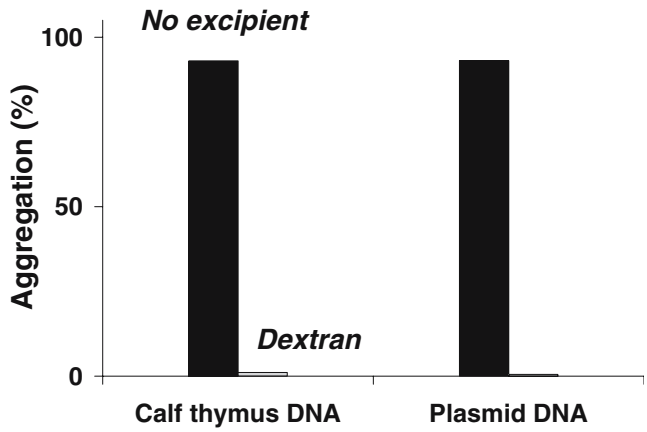

Fig. 6. Moisture-induced aggregations of a linear double-stranded calf thymus DNA and a supercoiled double-stranded plasmid DNA (Gwiz β-Gal encoding the expression of β-galactosidase), both lyophilized from an aqueous solution containing either no excipient (*the 1st and the 3rd bars*) or dextran (M_w=142,000) in a 10:1 (w/w) ratio to DNA. The results are mean values of duplicate measurements.

the system was presumably well hydrated and above its T_g value. Thus vitrification does not seem to be a requirement for the dextran's stabilization effect against the moisture-induced aggregation of DNA. Instead, this stabilization likely arises from dextran's ability to form extensive hydrogen bonding with DNA bases, thereby preventing hydrogen-bonded cross-links responsible for the aggregation. This view is consistent with the observed inability of PEG to prevent DNA aggregation because PEG has a far lesser H-bonding capability than dextran (although PEG's impotence due to its crystallization during lyophilization also cannot be ruled out).

To verify that hydrogen bonding is indeed the protective mechanism involved, we employed a low-molecular-weight excipient, sucrose, which can also readily form H-bonds. As seen in Fig. 4, it is almost as effective as dextran in stabilizing DNA against the moisture-induced aggregation, even at high (e.g., 96%) RH levels, where the DNA-sucrose co-lyophilizate is well hydrated and presumably above the T_g of the system. We conclude, therefore, that hydrogen-bonding excipients should be effective against moisture-induced aggregation of DNA in a solid state. In this regard, it is worth mentioning that polyplexes (i.e., DNA-polycation/lipid complexes) also have been protected by such excipients during lyophilization (37–39).

Effect of Temperature

The preceding aggregation studies of calf thymus DNA at various RH levels were performed at 55°C. We confirmed that such aggregation could also occur at more pharmaceutically relevant temperatures. As seen in Fig. 5, more than 80% of lyophilized DNA aggregated after 7 days at 37°C at a 60% RH (open circles); expectedly, this process, while very pronounced, was slower than at 55°C (closed circles in Fig. 5).

Moisture-Induced Aggregation of a Plasmid DNA

Finally, we examined the generality of the observed aggregation using a plasmid DNA extensively explored for gene therapy. To this end, we employed a ds-supercoiled gWiz β-gal plasmid DNA (8,278 base pairs) that encodes the expression of β-galactosidase. As seen in Fig. 6, after a 24-h incubation at 55°C and a 60% RH this plasmid DNA aggregates similarly to its calf thymus counterpart (the 3rd and 1st bars, respectively). Furthermore, dextran, in a 10:1 (w/w) ratio to DNA stabilizes the plasmid DNA against the moisture-induced aggregation as much as the linear, calf thymus nucleic acid.

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