Entrapment and Condensation of DNA in Neutral Reverse Micelles

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ABSTRACT DNA condensation and compaction is induced by a variety of condensing agents such as polycations. The present study analyzed the structure of plasmid DNA (DNA) in the small inner space of reverse micelles formed from nonionic surfactants (isotropic phase). Spectroscopic studies indicated that DNA was dissolved in an organic solvent in the presence of a neutral detergent. Fluorescent quenching of ethidium bromide and of rhodamine covalently attached to DNA suggested that the DNA within neutral, reverse micelles was condensed. Circular dichroism indicated that the DNA structure was C form (member of B family) and not the dehydrated A form. Concordantly, NMR experiments indicated that the reverse micelles contained a pool of free water, even at a ratio of water to surfactant (Wo) of 3.75. Electron microscopic analysis also indicated that the DNA was in a ring-like structure, probably toroids. Atomic force microscopic images also revealed small, compact particles after the condensed DNA structures were preserved using an innovative cross-linking strategy. In the lamellar phase, the DNA was configured in long strands that were 20 nm in diameter. Interestingly, such DNA structures, reminiscent of "nanowires," have apparently not been previously observed.

INTRODUCTION

DNA condensing agents cause, by a variety of mechanisms, DNA molecules to collapse and aggregate into ordered, highly condensed states (Bloomfield, 1996). Oligocations and polycations condense DNA most likely by neutralization of phosphate charge and lessened repulsion among DNA segments. DNA condensation occurs in solvents such as ethanol by making DNA–solvent interactions less favorable. Neutral or anionic polymers condense DNA by packing the DNA due to excluded volume. Furthermore, concentrated DNA solutions (>20 mg/mL for large DNA) containing physiological salt concentrations (150 mM) spontaneously form several different types of liquid crystalline phases (Strzelecka and Rill 1990a,b). In these cases, self-assembly is associated with locally or macroscopically crowded DNA solutions.

It would therefore be expected that DNA, after insertion into small water-pool cavities, would tend to form monomolecular or oligomolecular compact structures. Such a situation can be realized by inserting DNA into reverse micelles with sizes comparable to that of DNA. Reverse micelles are spherical water droplets surrounded by a monolayer of closely packed surfactant molecules dispersed in a solvent of low polarity. Only a few studies have been published on DNA or other polynucleotides in reverse micelles. Negatively-charged, reverse micelles are one type of micelle into which nucleic acids have been incorporated (Battistel et al., 1989; Imre and Luisi, 1982; Luisi and Magid, 1986). RNA and DNA with molecular masses of 30, 250, or 2700 kDa were solubilized in isooctane solution with the anionic surfactant AOT (sodium (*bis*-(2-ethylhexyl)sulfosuccinate)). Incorporation of 250-kDa DNA into anionic micelles led to a hypochromic effect, a red shift of the adsorption maximum and changes in the circular dichroism (CD) spectra reminiscent of the condensed Ψ-form. Even a plasmid 2700 kDa in size could be solubilized in reverse micelles. The adsorption properties of the larger size DNA were the same in the anionic reverse micelles as in water, but the CD spectra still demonstrated the condensed multimolecular form of DNA (Ψ-form) (Imre and Luisi, 1982; Luisi and Magid, 1986).

The solubilization of DNA–cationic detergent complexes in hydrophobic solvents was first reported by Ijiro and Okahata (1992). They demonstrated with the use of CD spectroscopy that the double-stranded, helical structure of DNA remains undisturbed after introduction of the complexes into chloroform. More recently, the transfer of DNA–cationic detergent complexes to nonpolar organic solvents was comprehensively studied (Mel'nikov and Lindman, 1999; Sergeyev et al., 1999a,b). DNA–cationic surfactant complexes in organic solvents existed as monomolecular, double-stranded, compacted macromolecules (Sergeyev et al., 1999b). The above cationic lipid–DNA complexes were formed by drying them from aqueous solutions and then resuspending them in organic solvents. Alternatively, polydeoxyadenylic-thymidylic acid (900–1900 kDa) was dissolved in a water-in-oil (w/o) microemulsion that contained the cationic detergent cetyltrimethylammonium bromide (Airoldi et al., 2000; Balestrieri et al., 1999). In low salt concentration, no changes were observed in the ultraviolet (UV) or CD spectra. Dissolution of poly[dA-dT] in a buffer with a high NaCl concentration resulted in condensation of the DNA (Ψ form) and destabilization of microemulsion.

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For both anionic and cationic reverse micelles, the effects on DNA structure could be not only affected by the restricted water volume, but also by strong Coulombic interactions that are often held solely responsible for DNA condensation. The present study analyzed the structure of plasmid DNA (DNA) in the small inner space of reverse micelles formed from nonionic surfactants. Nonionic surfactants have very low affinity toward polyelectrolytes (Lindman and Thalberg, 1993). The nonionic surfactant tetraethylenglycol dodecyl ether ($C_{12}E_4$) was used in alkanes, in which no cosurfactant was required for solubilization of water (Kunieda et al., 1991). Previously, a brief report (within a review article) noted that 250-kDa DNA can be solubilized in reverse micelles formed by $C_{12}E_4$ in isooctane in a Wo range of 4–9 (Battistel et al., 1989), where Wo is the molar ratio of water to surfactant. No change in DNA structure was observed. The present study now reports a more extensive study in which DNA condensation was observed in neutral, reverse micelles.

METHODS

Sample preparation and materials

The nonionic surfactant tetraethyleneglycol dodecyl ether ($C_{12}E_4$) (MW = 362.6, density = 0.95 g cm^{-3}) and anhydrous 2,2,4-trimethylpentane (TMP) (>99% pure) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethidium bromide (EtBr) and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol 6000 was purchased from Fluka Chemie AG (Buchs, Switzerland). A 5865-kb plasmid DNA (DNA) pCILuc was used as the closed, circular form in all the studies (Budker et al., 1996).

Reverse micelle solutions were prepared by the direct injection of different volumes of 25 mM HEPES pH 7.8, 0.5 mM EDTA buffer (HE buffer) or 20 mM MOPS pH 7.5, 0.1 mM EDTA (ME buffer) with different concentrations of DNA into a mixture of $C_{12}E_4$/TMP (12:88 v/v). The mixtures were agitated using a vortex stirrer until a transparent solution was obtained (usually 2 min).

Cysteine *Label* IT was prepared by amidation of the commercially available reagent amino *Label* IT (Mirus Corp., Madison WI) with *N*-Boc-S-trityl cysteine (Sigma) using dicyclohexylcarbodiimide (Aldrich) as the coupling agent. The product was purified by precipitation with diethyl ether. The trityl and Boc protecting groups were removed with trifluoroacetic acid. The resulting free thiol group was protected with Aldrithiol-2 (Aldrich) as the pyridyldithio mixed disulfide. This product was also purified by diethyl ether precipitation and confirmed by mass spectrometry (Sciex API 150EX, Applied Biosystems, Foster City, CA).

Spectroscopy

Inverse transmitted light in relation to Wo was measured using a UV/VIS spectrophotometer (Perkin-Elmer model Lambda 6) at 500 nm 10 min after sample preparation at 20°C. DNA UV absorption spectra from 220 to 300 nm were determined using the same spectrophotometer.

The micelles' size were measured by dynamic light scattering using a Zeta Plus photon correlation spectrometer equipped with 50 MW solid-state laser λ_{em} = 532 nm (Brookhaven Instruments Corp., Holtsville, NY). Two minutes after preparation of the micelles, the samples were spun 1 min at 12,000 rpm and size of micelles were measured for a period of 2 min.

For the CD measurements, 60 μg DNA in 20 and 60 μL of 10 mM potassium phosphate buffer pH 7.5 (used because of less optical activity than HEPES/MOPS) were injected into 1 mL $C_{12}E_4$/TMP. The CD measurements were carried out in a CD spectrometer (model 202, AVIV Instrument Inc., Lakewood, NJ) with cells of 0.5-cm path length equipped with a 30°C water-circulating bath. The ellipticity value for control samples that contained 20 and 60 μL of buffer in 1 mL of $C_{12}E_4$/TMP were subtracted from the ellipticity of experimental samples.

Nuclear magnetic resonance (^{1}H NMR spectra) of H_2O chemical shift was measured on a Bruker AC+250 spectrometer. The probe temperature was kept at 20°C. Deuterated acetone was used as an external lock in a capillary introduced into the NMR tube.

DNA condensation was assessed using the EtBr intercalation assay. Fluorescence (excitation and emission wavelength of 525 and 595 nm) was monitored using a Shimadzu RF 1501 Spectrophotometer. The EtBr (0.9 μg) was added to DNA solutions before their solubilization in TMP (2 μg of DNA in 3–67 μL of HE buffer in 0.7 mL TMP/$C_{12}E_4$.) The assays were performed after incubating 4 h at room temperature. The relative fluorescence values were determined as follows: $I_r = 100 * (I_{obs} - I_e)/(I_o - I_e)$ where I_{obs} is the measured fluorescence, I_e is the fluorescence of EtBr in absence of DNA, and I_o is fluorescence of DNA/EtBr in buffer. The fluorescence of free EtBr in buffer and in micelles was the same.

DNA condensation was also assessed using DNA covalently labeled with rhodamine (Rh-DNA) prepared as previously described (Trubetskoy et al., 1999) to a level of approximately one rhodamine group per 100 bases. Rhodamine fluorescence was monitored using an excitation and emission wavelength of 591 and 610 nm, respectively. 2.5 μg of Rh-DNA in different volumes of HE buffer were injected into 0.7 mL of TMP/$C_{12}E_4$. The relative fluorescence values were determined as $I_r = 100 * I_{obs}/I_o$, where I_{obs} is the measured fluorescence, and I_o is fluorescence of Rh-DNA in buffer.

Electron microscopy

A drop of poly-L-lysine (30–70 kDa, Sigma) solution in water (10 mg/mL) was put onto a glow-discharged Formvar-coated 200-mesh grid for 1 min. After the solution was removed and the grid was dried, a drop of TMP with different amounts of HE buffer with or without DNA (7 μg/1 mL TMP) was placed on the grid. After 5 min, the solution was removed and the grid was washed three times with TMP and one time with H_2O. Negative staining was performed with 1% uranyl acetate for 30 s. The samples were examined using a Jeol JEM 100S electron microscope with 100 kV accelerating voltage.

Atomic force microscopy

The DNA or cross-linked DNA was prepared at a concentration of 5 μg/mL in 10 mM HEPES, pH 7.5. $NiCl_2$ was added to the complexes at a final concentration of 0.2 mM. The complexes were immediately placed on freshly cleaved mica and incubated for 10 min at room temperature. Excess liquid was removed under a stream of nitrogen. The samples were viewed using a Digital Instruments Dimension 3100 atomic force microscopy instrument in tapping mode within 24 hrs after their preparation.

Cysteine DNA preparation and cross-linking

Thiol groups were introduced onto DNA (pCI Luc) by labeling with cysteine *Label* IT. Plasmid DNA (100 μg pCI Luc/mL) and different amounts of cysteine *Label* IT were combined in ME buffer. The labeling reaction was carried out at 37°C for 1 h. The labeled DNA was purified by ethanol precipitation. The purified DNA was reconstituted in ME buffer at a final concentration of 1 μg/μL. The level of cysteine reagent incorporation on DNA was estimated from the optical adsorption ratio of pyridine-2-thione (λ_{max} = 343 nm and extinction coefficient $E = 8.08 \times 10^3$) and DNA (λ_{max} = 260 nm and extinction $E = 6.6 \times 10^3$) after treatment of 15

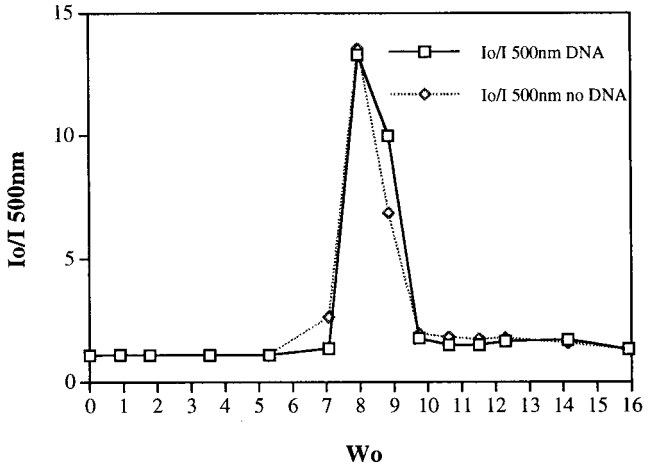

FIGURE 1 Inverse transmitted light (I_o/I where I_o is intensity of incident light and I is transmitted light) in relation to Wo. Light transmission was measured using a spectrophotometer at 500 nm of a w/o solution of $C_{12}E_4$ in 2,2,4-trimethylpentane at 20°C without DNA (◇) and in the presence of 11 μg/mL DNA (□).

μg of the modified DNA with 5 mM dithiothreitol (Sigma) for 1.5 h at 20°C to remove the pyridyldithio protecting group.

The labeled DNA was treated with 20 mM dithiothreitol (DTT, Sigma) for 1 h at 4°C to generate free thiols on the labeled plasmid. Reverse micelles were prepared as described above by dissolving 82 μL of 1 μg/μL Cys-DNA in 2.2 mL $C_{12}E_4$/TMP (Wo = 6.58). After formation of the micelles, sodium periodate was added to a final concentration of 2 mM with respect to the total aqueous portion. The samples were centrifuged for 1 min at 14,000 rpm to remove any aggregates. A control reaction was prepared following the same procedure using unlabeled DNA. The samples were incubated at 4°C for 2 h. The reverse micelle system was disrupted with the addition of 55 μL ethanol, 275 μL ME buffer, and 1.1 mL ethyl acetate. The reaction was vortexed and separated into two layers via centrifugation. The aqueous layer was washed twice with 2 mL ethyl acetate and once with 3 mL diethyl ether.

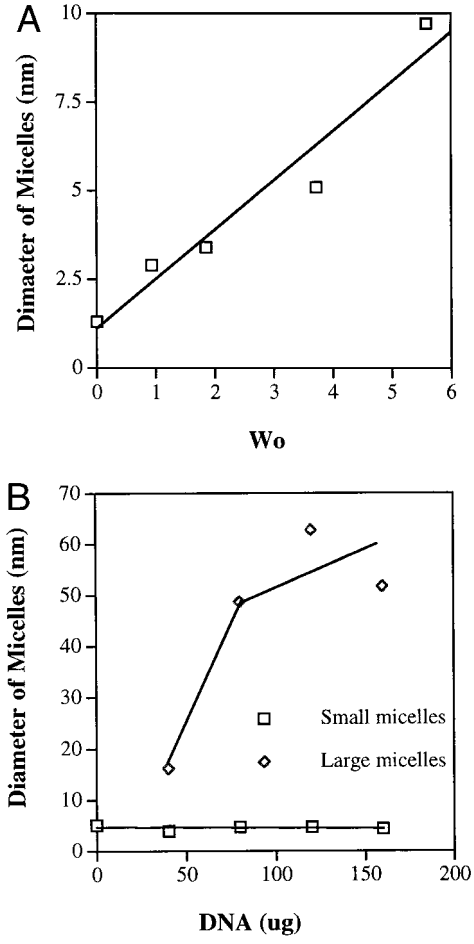

FIGURE 2 Size of w/o micelles using dynamic light scattering (particle sizer). (*A*) The dependence of the diameter of w/o micelles on water content without DNA. (*B*) At Wo = 3.54, the effect of DNA concentration on the diameters of small micelles (presumably empty) and large micelles (presumably containing DNA).

RESULTS

Solubility of DNA in reverse micelles

It has previously been shown that $C_{12}E_4$, dissolved in alkanes, forms isotropic solutions without liquid crystal structure (Kunieda et al., 1991). For $C_{12}E_4$ in dodecane, it was shown that, below a temperature of 29.2°C, a w/o microemulsion (isotropic phase) is present at a Wo less than 10, where Wo is the molar ratio of water to surfactant. With increasing water content, a two-phase and then a lamellar phase system are obtained. (Merdas et al., 1996).

In all experiments, a 12% solution of $C_{12}E_4$ in TMP (v/v) was used. Phase boundaries of the microemulsion mixtures at 20°C were determined by assaying transmitted light (Fig. 1). The isotropic phase existed up to Wo of 7. Low transmission (presumably a two-phase system) was seen for Wo between 7 and 9. At a Wo greater than 9.5, both the transmission of the mixture, and the viscosity increased, presumably due to the formation of a lamellar phase. These results are similar to the behavior of the w/o emulsion of $C_{12}E_4$ in dodecane (Merdas et al., 1996).

Solutions of 11 μg DNA in different volumes of HE buffer were added to $C_{12}E_4$/TMP and the light transmission determined (Fig. 1). The presence of the DNA (11 μg) did not substantially affect the inverse transmission profile at any of the various Wo values, indicating that DNA precipitation had not occurred, and that the DNA was dissolved in a nonpolar organic solvent in the presence of a neutral detergent. The DNA is present in either reverse micelles (Wo $<$ 7) or in a lamellar phase solution (Wo $>$ 9) (Fig. 1).

The size of micelles

The size of micelles with and without DNA were measured using a particle sizer (Brookhaven) (Fig. 2). Without DNA, the size of the micelles increased proportionally as the water content increased; going from 1.3 nm for "dry" micelles

(Wo = 0) to 9.7 nm for micelles with Wo = 5.6 (Fig. 2 *A*). These micelle sizes and growth trends agree with work by Zhu et al. (1992) and Vasilescu et al.(1995).

At Wo = 3.73, different amounts of DNA were dissolved in 1 mL of $C_{12}E_4$/TMP and the size of micelles was measured (Fig. 2 *B*). There were two types of micelles in all samples; presumably, "empty" small micelles and DNA-containing large micelles. The size of the small micelles did not depend upon the presence of DNA or its concentration (Fig. 2 *B, small micelle line*). However, the size of DNA-containing micelles did, reaching a maximum of 50–60 nm in diameter at a DNA concentration of ~80 μg/mL and no significant change was observed for samples at a concentration greater than 80 μg/mL (Fig. 2 *B, large micelle line*). It is important to note that the signal at the DNA concentration of 40 μg/mL was weak and therefore the size may not have been accurate.

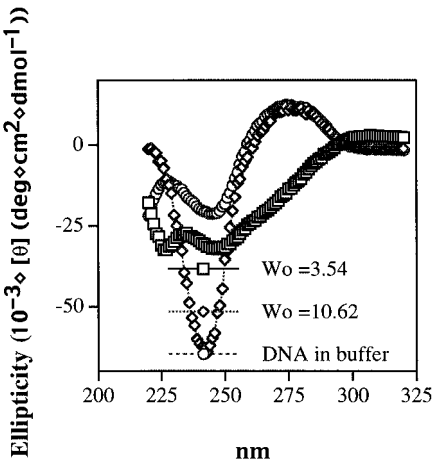

FIGURE 3 CD spectra of 16 μg DNA in 1 mL 10 mM K-phosphate buffer pH 7.5 (*circles*), in 20 μL phosphate buffer in 1 mL $C_{12}E_4$/TMP (Wo = 3.54, *squares*) or in 60 μL phosphate buffer in 1 mL $C_{12}E_4$/TMP (Wo = 10.62, *diamonds*).

Ultraviolet spectroscopy of DNA in w/o micelles

Solutions prepared from 36 μg of DNA in 10, 20, 30, and 50 μL HE buffer were injected into 1 mL $C_{12}E_4$/TMP and UV absorption spectra were obtained before and after a 5-min centrifugation at 15,000 rpm. As judged from the UV absorption spectrum record, DNA is fully dissolved, with minimal scattering at wavelengths longer than 230 nm (data not shown). Before centrifugation, the UV absorption spectrum indicated that the DNA was fully dissolved, with minimal scattering at wavelengths up to 230 nm (data not shown). Following centrifugation (5 min, 15,000 rpm), the UV spectrum indicated that there was no decrease in UV-absorption for the samples. Thus, it can be concluded that the micelles containing the DNA are small in size. Additionally, no hypochromic or hyperchromic effect was observed, because the UV spectra for the DNA in w/o micelles were the same as that determined for DNA in buffer (data not shown).

Circular dichroic assay

The conformation of DNA in reverse micelles was studied using CD spectroscopic analysis (Fig. 3). A solution of DNA in $C_{12}E_4$/TMP (Wo = 3.54) exhibited collapse of the 278 nm band, characteristic of the C form (i.e., the more highly wound B form with 10.2 instead of 10.4 base pairs per turn). Such change has been observed in lower dielectric solvents (e.g., methanol) (Girod et al., 1973), and in histone-DNA complexes (Baase and Johnson 1979). The CD spectra indicated no contribution from the A form conformation of DNA, suggesting that DNA was hydrated.

The CD spectra of DNA at Wo = 10.62, resembles the DNA spectra in buffer. One difference however, was an increase in the intensity of the negative band at 240 nm. This could be due to the presence of Ψ-DNA, in which the helices are oriented parallel to each other (Ghirlando et al., 1992). This effect has been observed in DNA complexes containing polylysine(Granados and Bello 1981; Shapiro et al., 1969), the cationic polymer 2-(dimethylamino) ethyl methacrylate (Cherng et al., 1999), and cationic liposomes (Akao et al., 1996).

It has been previously noted that the subtraction of the background CD spectrum of reverse micelles is problematic (Ricka et al., 1991). In these experiments, the background was recorded using unfilled reverse micelles. However, because the size of DNA-containing reverse micelles differs from that of unfilled micelles, the background spectra may be incorrect. This effect may be significant for samples with a high DNA concentration.

Free water determination

The changes observed in the CD spectra of DNA in reverse micelles may be the result of the hosted DNA being in the restricted space of the reverse micelles. Another possible explanation is that there is substantial dehydration of the DNA. At low concentrations of water, the DNA and the polar group of the detergent may compete for the available water, resulting in the DNA being strongly dehydrated. Previously, the ^{1}H NMR chemical shift of water has been used to determine the amount of bound water for microemulsions based on $C_{12}E_8$ (Waysbort et al., 1997). As such, the hydroxyl chemical shift as a function of water content was measured for $C_{12}E_4$/TMP microemulsions. Because only a single ^{1}H NMR signal was observed for the water resonance at various Wo values, a fast exchange condition was assumed between the bound and bulk water. Therefore, the chemical shift of water observed is proportional to the detergent water complex concentration [*DW*]. Fitting the

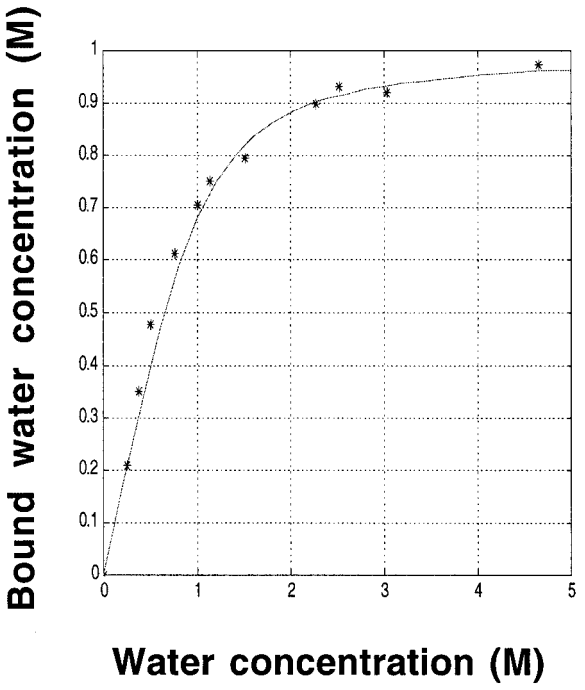

FIGURE 4 Concentration of bound water as a function of water concentration in 1 mL $C_{12}E_4$/TMP. The solid line was calculated for the equilibrium binding constant of water with detergent equal 0.15 M^{-1}. The points correspond to the value obtained from experiment.

data to different equilibrium constants for this reaction, $D + W \leftrightarrow DW$, where D and W are free detergent and free water concentration respectively, the best fit for the equilibrium binding constant of water with detergent was determined to be $\sim$ 0.15 M^{-1} (assuming that, when the chemical shift reaches a constant value, the concentration of free detergent is negligible and the equation $D + W \leftrightarrow DW$ has been shifted to the right) (Fig. 4). The calculation was done assuming that, at a maximum, five molecules of H_2O could bind one detergent molecule (one water per each oxygen in the detergent). The Scatchard plot was linear, indicating the absence of cooperativity. A similar analysis was conducted for samples prepared from DNA and $C_{12}E_4$/TMP with Wo of 3.54 and 10.62. The results indicate 36% and 72%, respectively, of the water is free. The free water concentration was 0.4 and 2.4 M with a DNA base concentration of 0.18 mM in the CD experiment, more than the amount necessary for DNA hydration. For Wo of 3.54 and 10.62, the above results and calculation indicate that 80% and 86% of water is free, respectively. Both these amounts of free water are sufficient for DNA hydration.

DNA condensation

To determine the level of DNA condensation within the reverse micelles, the amount of EtBr fluorescence of the DNA in the reverse micelles was assessed at various water

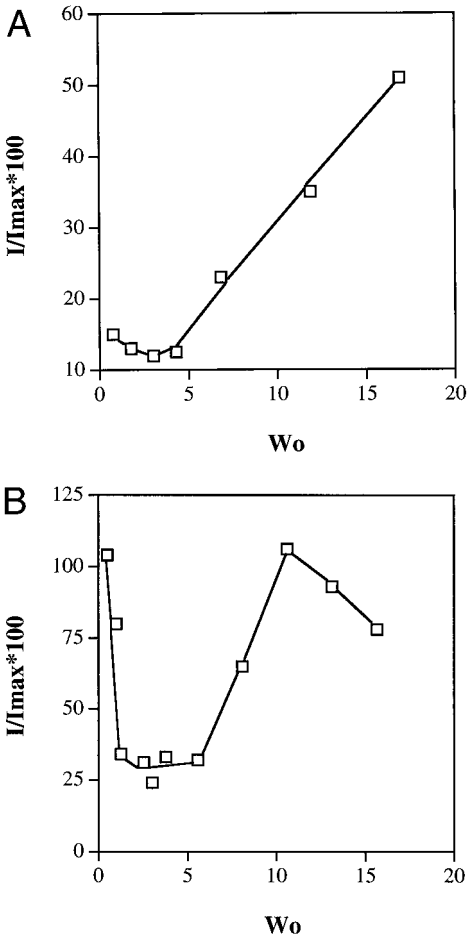

FIGURE 5 DNA condensation in reverse micelles at different amounts of water using either (*A*) ethidium bromide or (*B*) rhodamine-DNA techniques.

concentrations. Preparations of 2 μg of DNA and 0.9 μg of EtBr in different volumes of HE buffer were dissolved in 0.7 mL of TMP/$C_{12}E_4$. The resulting solutions had Wo varying from 0.76 to 16.92 (Fig. 5 *A*). At low concentrations of water (up to Wo = 4), the fluorescence intensity did not exceed 10% of DNA fluorescence intensity in HE. Fluorescence intensities gradually increased with increasing Wo, indicating that the DNA was becoming less condensed. Overall, the DNA in reverse micelles is condensed in micellular solutions with Wo $\leq$ 4, although some level of condensation is present in micellular solutions having Wo up to 16 (Fig. 5 *A*). For comparison, poly-L-lysine-mediated DNA condensation reduced EtBr fluorescence to $\sim$15% of the uncondensed value.

Rh-DNA was also used for determination of DNA condensation in w/o emulsions. The method is based upon the self-quenching that occurs when the rhodamine groups are brought closer together upon DNA condensation. As in the ethidium bromide assay, the rhodamine fluorescence intensity of DNA in reverse micelles was assessed at varying

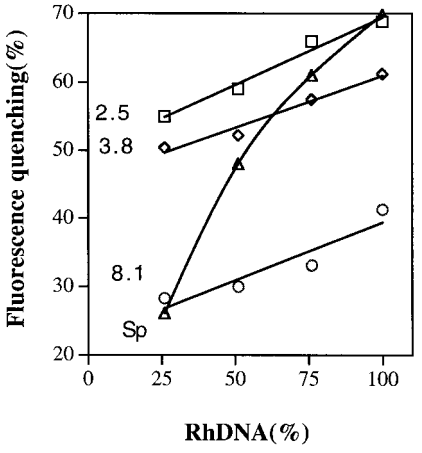

FIGURE 6 Fluorescence of 2.5 μg of Rh-DNA mixed with varying amounts of unlabelled DNA (percentage of Rh-DNA indicated on *x* axis) dissolved in $C_{12}E_4$/TMP reverse micelles with Wo = 2.5 (*square*), 3.8 (*diamond*), and 8.1 (*circle*). The DNA in 25 mM HEPES pH 7.8, 0.5 mM was injected into 0.7 mL $C_{12}E_4$/TMP. For control purposes, the same Rh-DNA mixtures were condensed with 2 mM spermine in HE buffer (*triangle*).

values of Wo (Fig. 5 *B*). The results indicate that DNA in reverse micelles was condensed at Wo values between 1.26 and 5.56 (Fig. 5 *B*). It should be noted that, around Wo = 10, turbidity contributed significantly to the fluorescence intensity. Interestingly, at very low water concentrations, the DNA appeared not to be condensed according to rhodamine fluorescence.

By mixing Rh-DNA with unlabelled DNA, it is possible to obtain some information on the number of DNA molecules that are condensed conjointly (locally together). If the local condensation state is unimolecular, then the extent of rhodamine quenching is unaffected by mixing labeled and unlabeled DNA. However, when condensation is multimolecular (more than one DNA molecules intertwined and condensed closely together), the rhodamine on the labeled DNA is separated by the unlabelled DNA and therefore the extent of quenching is reduced.

In the current experiments, Rh-DNA was mixed with different amounts of unlabeled DNA and condensed in reverse micelles at different Wo (Fig. 6). In the reverse micelles, dilution of Rh-DNA with unlabeled DNA had relatively little affect on the extent of quenching, suggesting that condensation is mainly unimolecular in reverse micelles. For comparison, the effect of 2 mM spermine on the fluorescence intensity of the same mixtures of Rh-DNA/unlabeled DNA was measured. In this case, the level of fluorescence quenching was proportional to the level of dilution of Rh-DNA by "cold," unlabeled DNA, which is an indication of multimolecular condensation. Previous studies have shown that spermine condensation involves the compaction of several DNA molecules into one toroidal structure (Bloomfield 1997).

Electron microscopy

For control purposes, samples that contained 20 or 60 μL of buffer (without DNA) in 1 mL TMP, and samples that contained 7 μg of DNA in 1 mL of buffer (no reverse micelles) were examined using transmission electron microscopy. No structures were detected (data not shown).

The DNA-containing reverse micelles consisted of 20 μL buffer in 1 mL $C_{12}E_4$/TMP (Wo = 3.53) and demonstrated ring-like structures that were 59.8 $\pm$ 12.5 nm in external diameter and 32.9 $\pm$ 12.1 nm in internal diameter (50 structures analyzed) (Fig. 7, *A* and *B*). The volume of the ring, *V*, was calculated using the formula for toroid volume, $(\pi^2/4)(R_{out} - R_{in})^2 * (R_{out} + R_{in})$, and thus equals 20.6 $*$ 10^3 nm^3. With consideration of DNA packing as a columnar hexagonal phase with interaxial distance of 3.2 nm (Podgornik et al., 1998), the monomer volume, $V_{mon} = (\sqrt{3}/4)^{1/2} * a^2 * l = 2.65$ nm^3, where *a* is the interaxial distance and *l* is length per nucleotide (0.34 nm). Therefore, every toroid structure contained 6832 bp or ~1.2 DNA molecules.

A sample of DNA in 60 μl buffer in $C_{12}E_4$/TMP (Wo = 10.61) demonstrated long threads that were 7–12 nm in diameter (Fig. 7 *C*). Many of the strands formed balls or bundles (Fig. 7 *D*). Also, round particles 10–20 nm in diameter were connected to or near the long threads (Fig. 7 *E*). A previous study observed the formation of calcium sulfate nanowires in w/o microemulsion of $C_{12}E_4$ in cyclohexane (Wo = 5) (Rees et al., 1999).

Direct cross-linking of DNA in reverse micelles

When DNA was extracted from the reverse micelles, its structure reverted back to native DNA according to dynamic light scattering and fluorescent quenching with Rh-DNA or EtBr. To preserve the DNA structure in reverse micelles, the DNA was covalently modified with sulfhydryl groups and oxidized in micelles to form disulfide bonds. The disulfide linkages cross-link the DNA molecule, essentially locking it into a condensed state (Fig. 8). DNA was labeled at the following ratios: (DNA wt: cysteine *Label* IT wt) 1:0.02 (0.12/100), 1:0.05 (0.3/100), 1:0.1 (0.6/100), 1:0.15 (0.9/100), and 1:0.2 (1.2/100) with the number of modifications per 100 base indicated in parentheses. After deprotection of the modified DNA, the thiol-containing DNA was packaged into reverse micelles and oxidized either by $NaIO_4$ or incubation for 12 h in the presence of oxygen. After isolation of the DNA from the micelles, the condensation was monitored by agarose gel (1%) electrophoresis (Fig. 8 *A*). All of the DNA was observed to remain in the loading well at labeling ratios above 1:0.1 (Fig. 8 *A*, *lanes 5–7*), whereas, at lower labeling ratios, DNA smears were also observed (Fig. 8 *A*, *lanes 3* and *4*). Migration of the DNA modified at 1:0.2 (1.2/100) and 1:0.5 (3/100) ratios did

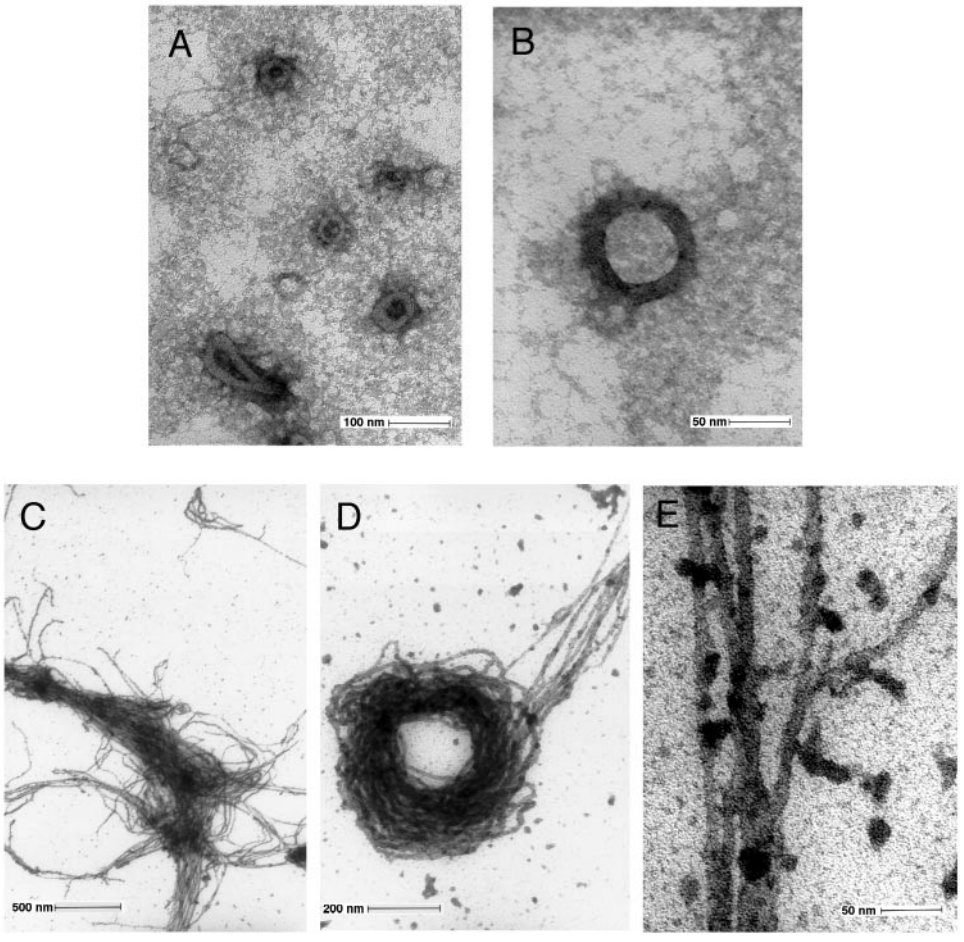

FIGURE 7 Transmission electron microscopy of DNA in $C_{12}E_4$/TMP reverse micelles with (*A–B*) Wo = 3.53 or (*C–E*) Wo = 10.61. Size bars with their respective representative lengths are in each figure.

not substantially change after oxidation in buffer alone (Fig. 8 *B*, *lanes 5* and *6*), indicating that inclusion into micelles was required for cross-linking. At the higher modification ratio of 1:1 (6/100), DNA was mainly in the well and presumably cross-linked or aggregated (Fig. 8 *B*, *lane 7*). In addition, the micellular reaction using thiol-containing DNA that was modified at ratio 1:0.2 was divided into two portions (Fig. 8 *C*). One portion was reduced for 1 h at 4°C with 50 mM dithiothreitol, but the other portion was not (as above). DNA in the nonreduced sample was mainly retained in the well (Fig. 8 *C*, *lane 2*), whereas the unlabeled control and the reduced sample migrated normally (Fig. 8 *C*, *lanes 1* and *3*).

Atomic force microscopic analysis indicated that DNA labeled with cysteine at a ratio of 1:0.2 and oxidized in reverse micelles was compacted, affording small particles with a diameter of 20–30 nm (Fig. 9 *A*). With an increased level of cysteine modification (1:1), the DNA was condensed into both small particles and some larger aggregated structures (Fig. 9 *B*).

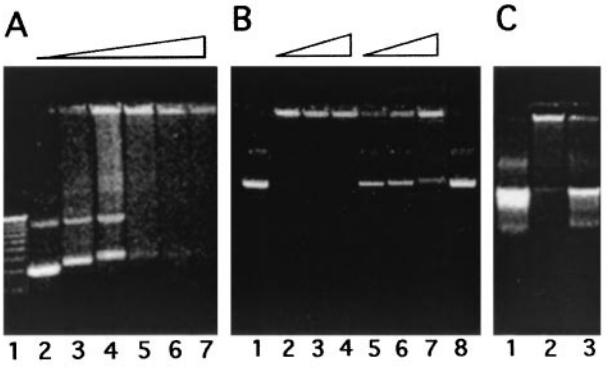

FIGURE 8 Agarose gel electrophoresis and EtBr staining of pCILuc cross-linked in $C_{12}E_4$/TMP reverse micelles with Wo = 6.58. (*A*) *Lane 1*, λ ladder; *lane 2*, unmodified DNA; *lane 3*, 1:0.02 labeled DNA; *lane 4*, 1:0.05 labeled DNA; *lane 5*, 1:0.1 labeled pCI luc; *lane 6*, 1:0.15 labeled DNA; *lane 7*, 1:0.2 labeled pCILuc. (*B*) *Lanes 1–4*, DNA oxidized in micelles; *lanes 5–7*, oxidized in water buffer; *line 1*, unmodified DNA; *lanes 2* and *5*, DNA modified at 1:0.2 ratio; *lanes 3* and *6*, DNA modified at 1:0.5 ratio; and *lanes 4* and I7, DNA modified at 1:1 ratio. (*C*) *Lane 1*, unlabeled control plasmid; *lane 2*, thiol DNA after micelle formation/oxidation; *lane 3*, thiol DNA after micelle formation/oxidation and reduction by 20 mM TCEP treatment 1 hr at 4°.

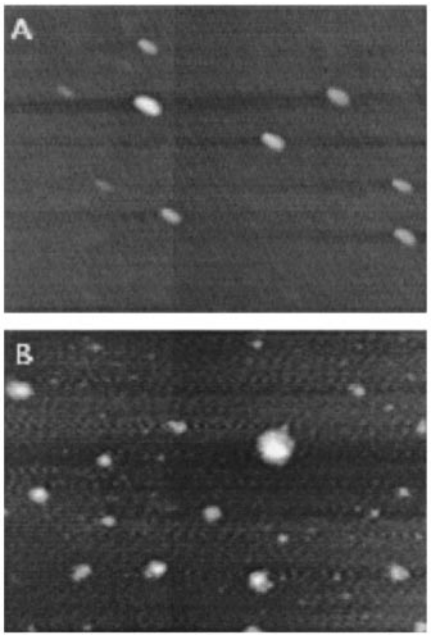

FIGURE 9 AFM images of cysteine-labeled DNA cross-linked in $C_{12}E_4$/TMP reverse micelles with Wo = 5. (*A*) Cysteine-labeled DNA at ratio of 1:0.2. (Field 0.5 × 0.37 μm). (*B*) Cysteine-labeled DNA at ratio of 1:1. (Field 2 × 1.47 μm).

DISCUSSION

Our studies indicated that DNA can be dissolved in an organic solvent in the presence of a neutral detergent. This conclusion was based on the observation that the reverse micelle DNA mixture had characteristic DNA ultraviolet absorption after centrifugation and minimal turbidity. The DNA-containing micelles had a maximum size of 50–60 nm in diameter as compared to the ~10-nm maximum size of empty micelles (Fig. 2 *B*). This increase in micelle size is consistent with several other studies that found that dissolving a water-soluble polymer in the water core of a reverse microemulsion changed the size of the droplets (Lianos et al., 1992; Papoutsi et al., 1993, 1994). Interestingly, the 50–60-nm size of the micelles is smaller than 6-kb, supercoiled DNA's diameter of gyration, which is ~200 nm (Fishman and Patterson 1996). Other large polymers could not be dissolved in smaller micelles. For example, the dissolving of polyethylene glycol (PEG) in w/o droplets depended on the ratio between the size of the water droplets (radius of droplet, R_w) and size of the polymer (radius of gyration, R_g). For $R_w/R_g < 1$, the solubility of PEG in w/o micelles was reported to be extremely low (Bellocq, 1998). Thus, the solubility of DNA in reverse micelles is not entirely predictable, suggesting that a special feature of the DNA, such as its ability to condense into a more compact structure, is required.

Two methods indicated that the DNA within reverse micelles was, in fact, condensed. These included the fluorescent quenching of both Rh-DNA and EtBr complexed with DNA. Although the basis for the fluorescence quenching of Rh-DNA upon condensation within micelles is understandable (Trubetskoy et al., 1999), the basis for EtBr/DNA quenching in micelles requires the following discussion, given that its general mechanism is obtuse in the literature. When DNA is condensed into particles by interaction with cationic polymers or cationic amphiphiles, the ability of DNA to bind EtBr, a cationic compound, is inhibited, and quenching is observed (Gershon et al., 1993; Tang and Szoka 1997). Although decreased binding results, in part, from electrostatic competition between the cationic condensing agent and positively charged EtBr, other mechanisms are possible. According to alternative models, the release of bound EtBr is caused by DNA bending and condensation that is induced by polyamine binding above a critical concentration (Basu et al., 1990). DNA flexibility is required for EtBr intercalation (Sobell et al., 1978). Therefore, DNA condensation may decrease EtBr binding affinity independent of electrostatic competition. To verify this model, we determined whether EtBr fluorescence quenching occurs when DNA is condensed by a high concentration of PEG (Lerman 1971). Using 6 kDa PEG, EtBr fluorescence was completely quenched at a concentration of 40% PEG in HE buffer and at 30% PEG in 50 mM NaCl (data not shown). Thus, EtBr fluorescence can be quenched in condensed DNA without electrostatic competition and is consistent with the quenching observed in condensed DNA within neutral, reverse micelles. In addition, EtBr is insoluble in nonpolar solvents like alkanes, and its emission properties do not change in the presence of neutral micelles (Pal et al., 1998).

CD spectroscopic analysis was performed to further explore the DNA state in the neutral, reverse micelles. CD spectra indicated that the DNA structure was the C form (member of B family) and not the dehydrated A form, even though the micelles do not contain much water at Wo = 3.75, where Wo is the molar ratio of water to surfactant. It should be noted that CD spectroscopic analysis of micelles does have inherent difficulties, especially the subtraction of the background CD spectrum (Ricka et al., 1991). For these studies, the background CD spectrum was recorded for unfilled reverse micelles. However, because the size of DNA-containing reverse micelles differs from that of unfilled micelles, the background spectra may be incorrect, especially for samples with a high DNA concentration. However, for the purposes of this investigation, the CD spectra were obtained to determine whether the DNA existed in a hydrated (B or C form) or a dehydrated (A form). Because the effects of light scattering in the long wavelength (>260 nm) of the spectrum are minimal, we concluded that the DNA was not in the A form.

^{1}H NMR experiments indicated that the reverse micelles contained a pool of free water, even at a Wo = 3.75. Several other approaches, such as time-resolved fluorescence

Stokes shift and analysis of the compressibility of water in reverse micelles also found free water in $C_{12}E_4$/TMP micelles at Wo from 1.1 to 5 (Caldararu et al., 1994; Amararene et al., 1997; Waysbort et al., 1997; Pant and Levinger, 2000). Thus, both the CD spectra and NMR determination of free water content indicate that the DNA in neutral reverse micelles is not condensed simply from dehydration.

Furthermore, the structure of DNA in the micelles was dependent on the water content. As discussed above, DNA in reverse micelles in an isotropic phase (Wo = 2.5–7) was in the C form and completely condensed. Electron microscopic analysis also indicated that the DNA was in a ring-like structure, probably toroids. Interestingly, the size of the toroidal structures in micelles appeared to be different from the toroids obtained from oligocation condensation. DNA polymers over a wide range of lengths (i.e., <1–48 kb) have been shown to produce toroids of approximately 100 nm outside and 28 nm inside diameter (Arscott et al., 1990; Bloomfield 1991).

In a lamellar phase (Wo > 9), the CD spectra of DNA was closer to the B form, but with increased intensity of the negative band at 240 nm, suggesting parallel orientation of DNA strands. EtBr studies indicated that the DNA is partially condensed. Electron microscopic images indicated that the DNA was configured in long strands that were 20 nm in diameter, approximately the thickness of two supercoiled DNA strands. Interestingly, such DNA structures, reminiscent of "nanowires," have apparently not been previously observed. Comparing the DNA structures in both the micellar and lamellar phase, it appears that the shape of the water phase affects the shape of the micellar DNA aggregates. For inorganic material, a similar dependency of structure on water content in $C_{12}E_4$ micelles has been observed (Rees et al., 1999).

The Rh-DNA studies implied that the DNA condensed unimolecularly in micelles (Fig. 6). The EM studies also suggested that the condensed structures were mostly unimolecular. To obtain EM images of the structures, the micelles and organic solvent have to be removed, raising the question of whether the EM images are reflective of the actual structure in the micelles. To address this concern, we developed an innovative approach to "freeze" the structures in the micelles by chemically cross-linking the DNA (Fig. 9). Atomic force microscopy images of the DNA cross-linked within micelles revealed small particles with a diameter of 20 to 30 nm, consistent with a single DNA molecule within each micelle. Their small size (as compared to poly-L-lysine-condensed toroids) and lack of holes may be due to the effect of cross-linking.

In conclusion, this study demonstrates that DNA can be condensed into compact structures within neutral, reverse micelles and partially condensed into nanowires within the lamellar phase. These techniques will be useful for further studies into the mechanism of DNA condensation. In addition, reverse micelles could also be used to produce synthetic nonviral vectors for gene therapy (Wu et al., 2001; Wolff et al., 1997).

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