Surfactant−DNA Gel Particles: Formation and Release Characteristics

M. Carmen Morán,*,† M. Graça Miguel,† and Björn Lindman†,‡

Chemistry Department, Coimbra University, 3004-535 Coimbra, Portugal, and Physical Chemistry 1, Lund University, P.O. Box 124, 22100 Lund, Sweden

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Aqueous mixtures of oppositely charged polyelectrolytes undergo associative phase separation, resulting in coacervation, gelation, or precipitation. This phenomenon has been exploited here to form DNA gel particles by interfacial diffusion. We report on the formation of DNA gel particles by mixing solutions of DNA (either single-stranded (ssDNA) or double-stranded (dsDNA)) with solutions of cationic surfactant cetyltrimetrylammonium bromide (CTAB). By using CTAB, the formation of DNA reservoir gel particles, without adding any kind of cross-linker or organic solvent, has been demonstrated. Particles have been characterized with respect to the degree of DNA entrapment, surface morphology, and secondary structure of DNA in the particles. The swelling/deswelling behavior and the DNA release have been investigated in response to salt additions. Analysis of the data has suggested a higher degree of interaction between ssDNA and the cationic surfactant, confirming the stronger amphiphilic character of the denatured DNA. Fluorescence microscopy studies have suggested that the formation of these particles is associated with a conservation of the secondary structure of DNA.

1. Introduction

Gene therapy offers the potential to cure a wide range of diseases by delivering a missing gene or a functional substitute of a defective gene.[1–6] The two most common methods for gene delivery use either viral[7,8] or synthetic vectors.[1–4,9,10]

A novel nonviral vector for gene therapy is recognized as successful if it is biocompatible, capable of interacting with DNA, able to form sufficiently small particles which can be formulated reproducibly, endocytosed, able to protect the complexed DNA from degradation during transport, and capable of delivering DNA to the target tissue in sufficient quantity.[11–13] Regarding this goal, some authors have developed a way to deliver DNA into the target cells by encapsulation in a controlled-release system. For this purpose, poly(lactic-coglycolic acid) (PLGA) microspheres have been used.[14–16] However, the problems encountered in reaching this goal were related not only to the microencapsulation technologies but also to the intrinsic nature of polyesters. Indeed, the PLGA microencapsulation technologies imply the use of organic solvents and high energy sources, thus leading to a significant degradation of the encapsulated macromolecule during the course of the PLGA hydrolysis. Following the identification of these problems, a number of strategies, aiming at either modifying the encapsulation techniques or using new encapsulation materials, were explored. Chitosan microspheres containing plasmid DNA were prepared by a complex coacervation method in the absence[17] or presence of a DNA condensing agent.[18]

Interestingly, interactions between oppositely charged surfactants and polyelectrolytes in aqueous solutions can lead to associative phase separation, where the concentrated phase assumes the form of a viscous liquid, gel, liquid crystal, or precipitate.[19] This behavior has been exploited to form gel particles, which have been prepared by dropwise addition of cellulose-based polycation solution (chitosan or *N*,*N*,*N*-trimethylammonium derivatized hydroxyethyl cellulose)[20–23] to anionic (sodium dodecyl sulfate, sodium perfluorooctanoate) and catanionic (cetyltrimethylammonium bromide/sodium perfluorooctanoate)[24] surfactant solutions.

Using this approach, we have recently developed a way to form DNA gel particles at water/water emulsion type interfaces by mixing DNA (either single-stranded (ssDNA) or double-stranded (dsDNA)) with the cationic surfactant cetyltrimetrylammonium bromide (CTAB) or the protein lysozyme.[25] Analysis of the data indicates a different mechanism of interaction of ssDNA and dsDNA for both surfactant and protein systems. The formation of a physical network in which surfactant micelles form polyanionic−multicationic electrostatic complexes as cross-link points seems to play an important role in the stabilization of the DNA particles.

Quaternary ammonium surfactants, despite their known cytotoxicity,[26] have already been used, in small amounts, to charge neutral liposomes, thereby improving their transfection efficiency; they have the advantage of lower cost when compared with other synthetic lipids.[26,27]

The goal of this study was to investigate the formation of DNA gel particles at water/water emulsion type interfaces by mixing DNA (either single-stranded (ssDNA) or double-stranded (dsDNA)) with the cationic surfactant cetyltrimetrylammonium bromide (CTAB). Particles were characterized with respect to DNA entrapment degree, surface morphology, and secondary structure of DNA in the particles. Swelling/deswelling behavior and DNA release were investigated in response to salt additions. The originality of this work consists in forming DNA reservoir gel particles without adding any kind of cross-linker or organic solvent.

2. Materials and Methods

Materials. The sodium salt of deoxyribonucleic acid (DNA) from salmon testes of an average degree of polymerization of about 2000 base pairs was purchased from Sigma and used as received. The DNA concentrations were determined spectrophotometrically considering that

* Corresponding author. E-mail: mcarmen@qui.uc.pt.
† Coimbra University.
‡ Lund University.

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for an absorbance of 1, at 260 nm, a solution of dsDNA has a concentration of 50 μg/mL and a solution of ssDNA has a concentration of 40 μg/mL.[28] All DNA concentrations are presented in molarity per phosphate group, i.e., molarity per negative charge. The ratios in absorbance at 260 and 280 nm of the stock solutions were found to be between 1.8 and 1.9, which suggested the absence of proteins.[29] Cetyltrimethylammonium bromide (CTAB), obtained from Sigma, was recrystallized with acetone and ethanol. Sodium bromide (NaBr), sodium chloride (NaCl), and Tris base, all from Sigma, were used as supplied. Acridine orange (AO) was supplied by Molecular Probes (Invitrogen). All experiments were performed using Millipore Milli-Q deionized water (18.2 MΩ cm resistivity).

Particle Preparation. dsDNA stock solutions were prepared in 10 mM NaBr in order to stabilize the DNA secondary structure in its native B-form conformation.[30] ssDNA stock solutions were prepared by thermal denaturation of dsDNA stock solution at 80 °C for 15 min and then immediately dipping into ice for fast cooling, to prevent renaturation. CTAB was dissolved in Milli-Q water. DNA solutions were added dropwise via a 22-gauge needle into gently agitated surfactant solutions (2 mL). Under optimal conditions, droplets from DNA solutions instantaneously gelled into discrete particles upon contact with the surfactant solution. Thereafter, the particles were left to equilibrate in the solutions for a period of 2 h. After this period, the formed particles were separated by filtration through a G2 filter and washed with 5 × 8 mL volumes of Milli-Q water to remove the excess of salt.

Determination of the Degree of DNA Entrapment. The entrapment degree was determined by quantifying both the nonbound DNA in the supernatant solution and the bound DNA in the gel particles. The entire quantity of supernatant surfactant solution containing the nonbound DNA was removed to be studied with a spectrophotometer. Thereafter, the particles were washed with Milli-Q water as described in the previous section. The particles were magnetically stirred in pH 7.6 10 mM Tris HCl buffer to promote swelling and breakup of the structure. The resulting mixture, containing skins of the particles, was filtered, and then, the filtrates were studied with a spectrophotometer. The amount of DNA present in the obtained skins was estimated considering the initial amount of DNA added. Loading capacity (LC) and loading efficiency (LE) were determined by the following equations:

$$\mathrm{LC}(\%) = [(\text{total amount of DNA} - \text{nonbound DNA})/\text{weight of particles}] \times 100$$

$$\mathrm{LE}(\%) = [(\text{total amount of DNA} - \text{nonbound DNA})/\text{total amount of DNA}] \times 100$$

Three batches of particles of each system were prepared, and the results are presented as average and standard deviations.

Swelling and Dissolution Behavior of DNA Gel Particles. Swelling and dissolution studies were performed in either Milli-Q water or aqueous solutions containing different electrolytes, namely NaCl, NaBr, and buffering salts (Tris). Particles (around 100 mg) were exposed to dissolution media (4 mL) at an agitation rate of 40 rpm using the ST 5 CAT shaking platform. At specific time intervals, the entire quantity of dissolution medium was removed and the particles placed in the container were weighed. Then, a new solution was added in order to maintain a clean environment. This procedure was repeated until particles were completely dissolved. The data were then transformed into the relative weight loss using the following equation:

$$\text{Relative weight ratio (RW)} = (W_i - W_t)/W_i$$

where W_i stands for the initial weight of the particles and W_t is for the weight of the particles at time t.

DNA Release Studies. Simultaneosly to the studies of swelling/dissolution behavior, DNA release studies were carried out. Hence, at defined time intervals, the supernatant was collected and particles were resuspended in fresh solution. DNA released into the supernatant solutions was quantified by measuring the absorbance at 260 nm with a spectrophotometer (UV/vis spectrophotometer V-530 JASCO).

Scanning Electron Microscopy Imaging. Scanning electron microscopy (SEM) (Philips XL30- TMP) was used to evaluate both the outer and the inner surface morphology of the particles. Prior to that, the particles were lyophilized overnight (−46 °C, 0.035 mbar). All the samples were kept under vacuum condition and taken out just before the SEM observation. The dried particles were viewed without sputter coating.

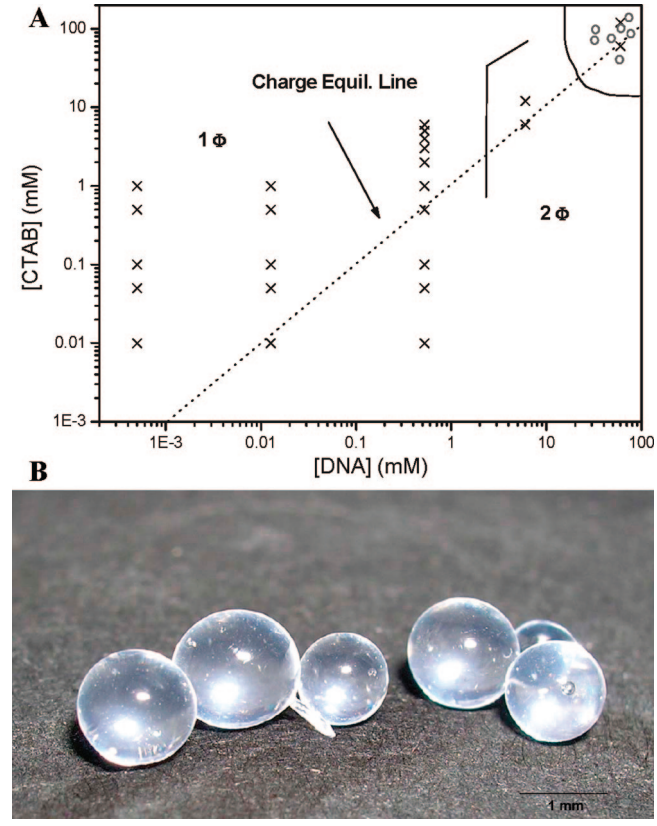

Figure 1. Formation of DNA gel particles. (A) Phase-map of the CTAB/dsDNA/water mixture at 25 °C, where 1ϕ and 2ϕ indicate the one and two phase regions, respectively: (×) studied compositions and (○) area where gel particles were observed (adapted from ref 25). (B) Representative morphology of CTAB−dsDNA particles.

Fluorescence Microscopy Imaging. Acridine orange staining was used to differentiate between different secondary structures of DNA in the particles. Freshly prepared particles were stained for 10 min with acridine orange (0.3 mg/mL), washed in distilled water, and immediately examined with a Zeiss AxioPhot epifluorescence microscope equipped with 460–480 nm excitation and 510 nm dicroic and 510 nm barrier filters. To capture images, a digital camera (Olympus, model DP70) was used.

3. Results and Discussion

Particle Preparation. The formation of the DNA gel particles was initialy studied using mixtures of dsDNA and CTAB. The obtained phase map is depicted in Figure 1A. As we described recently,[25] single-phase (1ϕ) solutions are observed in the polymer-poor and surfactant-poor regions of the phase map. On the polymer-rich side of the phase map, once charge equilibration was achieved, associative phase separation occurred (2ϕ). Finally, in the limit of very high polymer concentrations, a solid gel-like material forms. The size of the resulting particles reflects the size of the parent drop and varies between 1 and 2 mm (Figure 1B). A similar behavior was observed when particles were formed using denatured DNA.

Determination of the Degree of DNA Entrapment. Determinations of loading capacity (LC) and loading efficiency (LE) for the different formulations used do not give evidence for

Table 1. Characterization of the CTAB−DNA Gel Particles with Respect to DNA Loading Efficiency (LE) and Loading Capacity (LC)[a]

system	LE (% ± SD)	LC (% ± SD)	DNA released (% ± SD)	DNA complexed (% ± SD)
CTAB−dsDNA	99.80 ± 0.05	2.12 ± 0.14	47 ± 10	52 ± 10
CTAB−ssDNA	99.88 ± 0.05	2.15 ± 0.17	21 ± 10	79 ± 10

[a] DNA released and DNA complexed are related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, respectively, after particles were magnetically stirred overnight. All values were measured in triplicate and are presented as average and standard deviations.

differences as a function of the DNA conformation. The characteristics of the different systems are summarized in Table 1. In both cases, LE values were higher than 99%, confirming the effectiveness of DNA entrapment in the surfactant solution. Similar results were obtained in the determination of the entrapped DNA as a function of the weight of the particles (LC values).

Nevertheless, significative differences were observed in the delivery of the DNA once the breakup of the particles was promoted. Determination of the DNA in both the supernatant solutions and the skins derived from the particles is also summarized in Table 1. The obtained results indicate a different distribution of the DNA in the particles for different initial secondary structures.

This trend in DNA distribution can be attributed to differences between the two secondary structures. From previous studies, both experimental and theoretical, on polyelectrolyte−surfactant systems, it is known that the linear charge density of the polyelectrolyte, its flexibility, and any amphiphilic character will play a significant role on corresponding interactions.[31–34] We note that the linear charge density of dsDNA (0.59 negative charges/Å) is considerably higher than for ssDNA (0.29 negative charges/Å), and from a simple electrostatic mechanism, dsDNA should interact more strongly with oppositely charged polyelectrolytes. Regarding chain flexibility, ssDNA has a much higher flexibility than dsDNA, which is quite rigid and characterized by a large persistence length (500 Å).[33,34] In simulations, the role of flexibility of the polyelectrolyte has been documented in some detail, and it was found that a flexible chain in general interacts more strongly with an oppositely charged macroion than a rigid one.[35] Precipitation studies of DNA solutions by the addition of dodecyl trimethylammonium (DTAB) demonstrated that for ssDNA solutions a lower amount of surfactant is necessary to induce phase separation than for dsDNA solutions,[36,37] probably reflecting a combination of flexibility and amphiphilicity effects.

Taking into account all these considerations, differences in the distribution would be related with the capacity to form stronger DNA−surfactant complexes. Thus, a higher amount of complexes would be expected in the case of ssDNA, thus decreasing the amount of noncomplexed DNA, which could be detected in solution.

Particle Swelling and Deswelling Kinetics. Gels are considered to have great potential as drug reservoirs. Loaded drugs would be released by diffusion from the gels or by erosion of them. Hence, the release mechanism can be controlled by swelling or dissolution of the gels.[38] When the gel particles described above are inserted in a medium, different responses are encountered: swelling or deswelling, dissolution, and release of DNA.

Molecular interactions in aqueous solution are affected to a greater or lesser extent by the presence of electrolytes, depending on the contribution of electrostatic forces to the binding. It has been shown that the addition of salt reduces the strength of electrostatic interactions between surfactants and polyelectrolytes, which results in a larger critical association concentration (CAC).[39–44] Moreover, the addition of a sufficiently large amount of salt can completely screen the electrostatic interaction and prevent the formation of polyelectrolyte/surfactant complexes.[45–48]

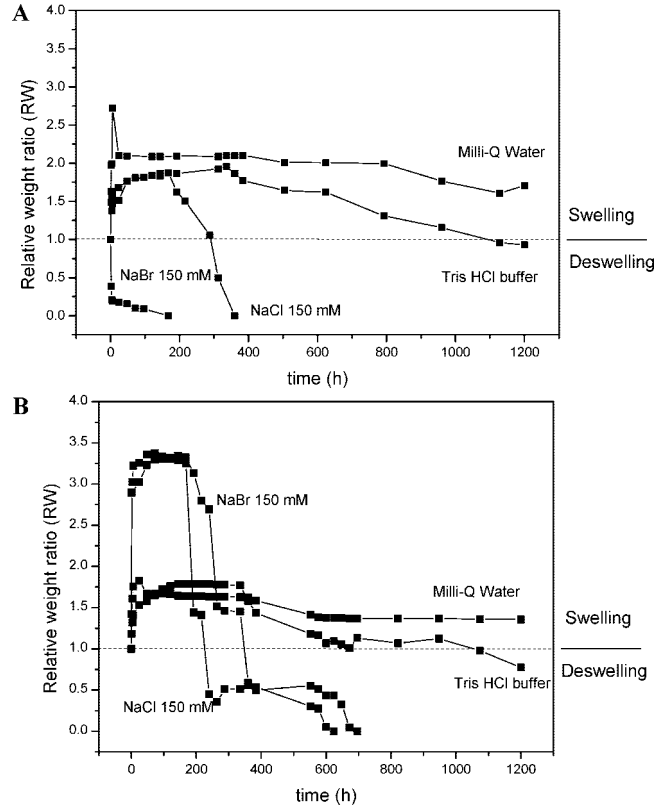

Figure 2. Relative weight ratio measurements performed on CTAB−dsDNA (A) and CTAB−ssDNA (B) particles after exposure to different solutions.

We modified the contribution of electrostatic interactions in CTAB−DNA binding by monitoring the effect of the medium ionic strength on the DNA release assays. CTAB−DNA particles were placed in either deionized water or aqueous solutions containing different electrolytes, namely, NaCl, NaBr, and buffering salts (Tris). Figure 2 depicts the effect of salt on the relative swelling ratio of CTAB−dsDNA and CTAB−ssDNA gels, respectively.

An analysis of the data shows two different types of behavior, independent of the secondary structure of the DNA. The observed trend reflects differences in salt content in the media. Thus, we can distinguish between changes in (i) pure water or water containing a small amount of buffer and (ii) aqueous solutions containing high salt concentration.

When particles were placed in either deionized water or pH 7.6 10 mM Tris HCl buffer, particles show water uptake from the medium and swelling could be observed. The swelling continues during the entire time interval studied. Only in the case of particles placed in the buffer was there a return to the original particle weight.

Quite different profiles were obtained in the presence of high salt content. A general trend is that particles showed an initial swelling and then dissolved. The time in the swollen state before dissolution depends on the DNA state and the ionic species.

Thus, CTAB−dsDNA particles placed in 150 mM NaBr monotonously dissolve with time. However, in NaCl, they showed an initial water uptake from the medium, full dissolution being achieved only after 400 h. In the case of particles prepared with denatured DNA, both water uptake and dissolution were observed. The time of stabilization became longer in the case of NaCl than in the case of NaBr solution.

The stability of the gel particles is given mainly by the electrostatic attraction between DNA and the oppositely charge surfactant. A weakening of this association is expected to result in a partial or complete dissolution. The electrostatic attraction is obviously expected to be weaker in the presence of electrolyte. Direct illustrations of this for systems of polyelectrolyte and oppositely charged surfactant are the increase in CAC as salt is added[42] and the decreased tendency for phase separation;[45] as salt is added, the two-phase region is reduced, eliminated, and even replaced by a region of segregative phase separation.

Differences in CTAB−DNA interactions as a function of the secondary structure of DNA were also emphasized during the swelling behavior experiments in the presence of high salt content. While CTAB−dsDNA particles placed in 150 mM NaBr monotonously dissolve with time, particles formed with denatured DNA showed an initial swelling and dissolved only after 600 h. The observed response would to be related with the capacity to form stronger DNA−surfactant complexes in the latter system, to which higher flexibility and amphiphilic character contribute.

The fact that there is a faster dissolution with bromide than with chloride points to a stronger association of the former ion to the DNA−surfactant complexes. The fact that we need to go beyond simple electrostatic theory to explain such systems was illustrated nicely in the works of Ilekti et al.[49,50] and Hansson et al.[51] on mixtures of sodium poly(acrylate) and CTAB. It was shown that there is a clear competition between the polyions and the bromide ions for associating with the cationic surfactant aggregates. At high dilution where the translational entropy per charge of the ions dominates, the binding of polyions is favored; as the system becomes more concentrated, there is an increasing association of bromide ions to the aggregates. Already some time ago, it was demonstrated by NMR that larger anions have a strong tendency to associate to nonpolar groups.[52–54] This association is due to dispersion forces and follows the polarizability of the ion. The so-called Hofmeister of lyotropic series listing the influence of ions and solubility is an illustration of this.[55–57]

Kinetics of DNA Release. Further studies have explored the effect of ionic strength on the release of DNA from the different particles. Figure 3 shows the observed accumulative DNA release. Generally, the release pattern resembles that observed in the swelling/dissolution profiles (see Figure 2). Thus, in the case of CTAB−dsDNA, particles placed in NaBr solution exhibited a fast burst effect (82% DNA released in the first 2 h) as a consequence of the dissolution profile observed. Particles placed in either water, pH 7.6 10 mM Tris HCl buffer, or NaCl showed no initial burst release; in the first 24 h, only 1.6, 2.0 and 3.3% of DNA was released, respectively. Total recovery of DNA was obtained when particles were placed in aqueous solutions containing high salt concentration. A similar ionic strength effect was observed in the case of particles formed with denatured DNA. However, in this case, slower kinetics were observed. In addition, for systems where total recovery was not achieved, a lower percentage of DNA release was observed; for instance after 1200 h, particles placed in pH 7.6 10 mM

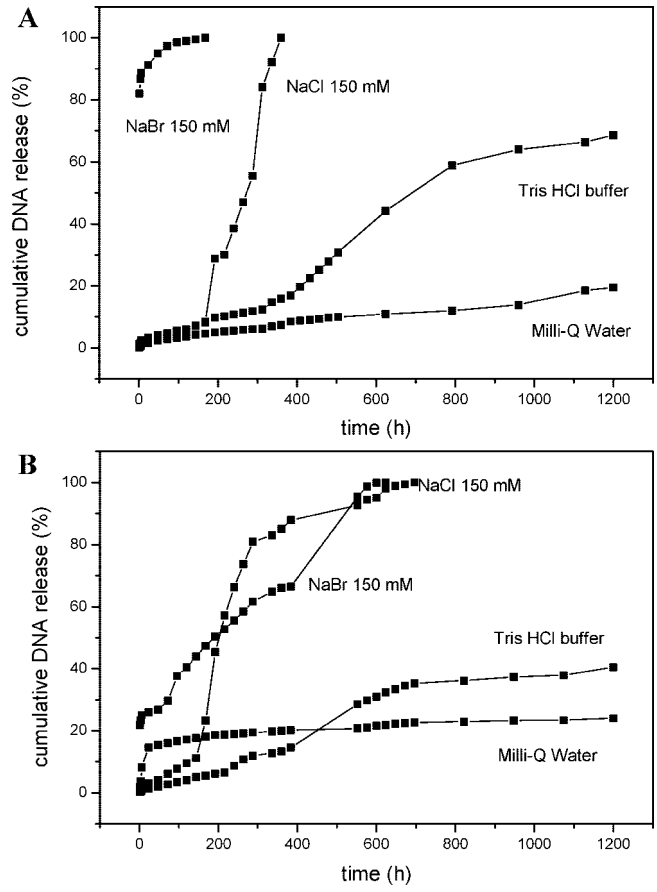

Figure 3. Release of DNA from CTAB−dsDNA (A) and CTAB−ssDNA particles (B) in different solutions.

Tris HCl buffer showed 69 and 40% of DNA released from the particles containing dsDNA and ssDNA, respectively.

There is thus a striking difference between the release of DNA from CTAB−dsDNA and CTAB−ssDNA particles, which suggests that the interaction is much stronger in the case of ssDNA. The results described here are also consistent with the DNA entrapment studies and the DNA distribution derived from them. As mentioned above, these results are in agreement with studies of the precipitation of DNA from solution on the addition of DTAB.[30,36,37] This study demonstrated that for ssDNA solutions a lower amount of surfactant is necessary to induce phase separation than for dsDNA solutions.

Particle Morphology. Scanning electron microscopy imaging was carried out in order to establish possible differences in the morphologies between the different particles prepared. Figure 4 shows the micrographs of freeze-dried particles from the systems CTAB−dsDNA and CTAB−ssDNA. The lyophilization procedure constitutes a standard protocol for the preparation of samples prior to their examination by means of SEM. Although this procedure would cause some deformation in shape and arrangement, the nature of the DNA (dsDNA or ssDNA) would be the most important factor in the diverse morphology of the obtained DNA gel particles.

Clear similarities in the outer surface morphology between the two formulations were thus found. However, interestingly, the surface of the inner structure revealed a different texture. Much larger pores and channel-like structures were found in the inner surface of particles formed with native DNA. These results suggest that these particles would be more porous, which could explain the higher degree of swelling. The structure of the particles formed with denatured single-stranded DNA is

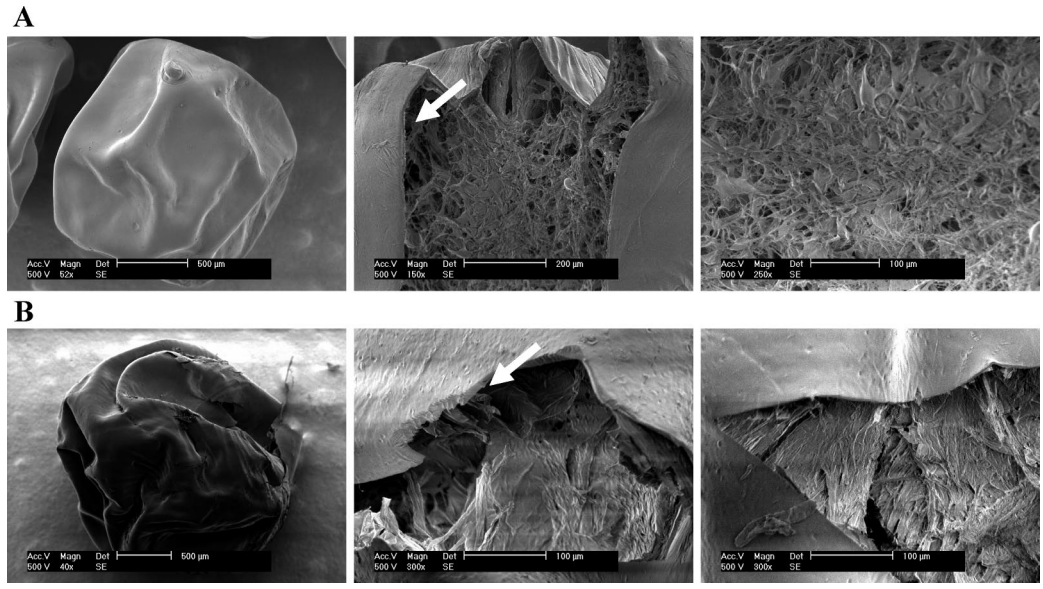

Figure 4. Scanning electron micrographs of individual CTAB−dsDNA (A) and CTAB−ssDNA (B) particles: outer surface (left), cross sections showing both the outer and inner sufaces (center), and inner surface (right). The arrows in the central panels emphasize the shell structure in the obtained particles (the central panels have been adapted from ref 25).

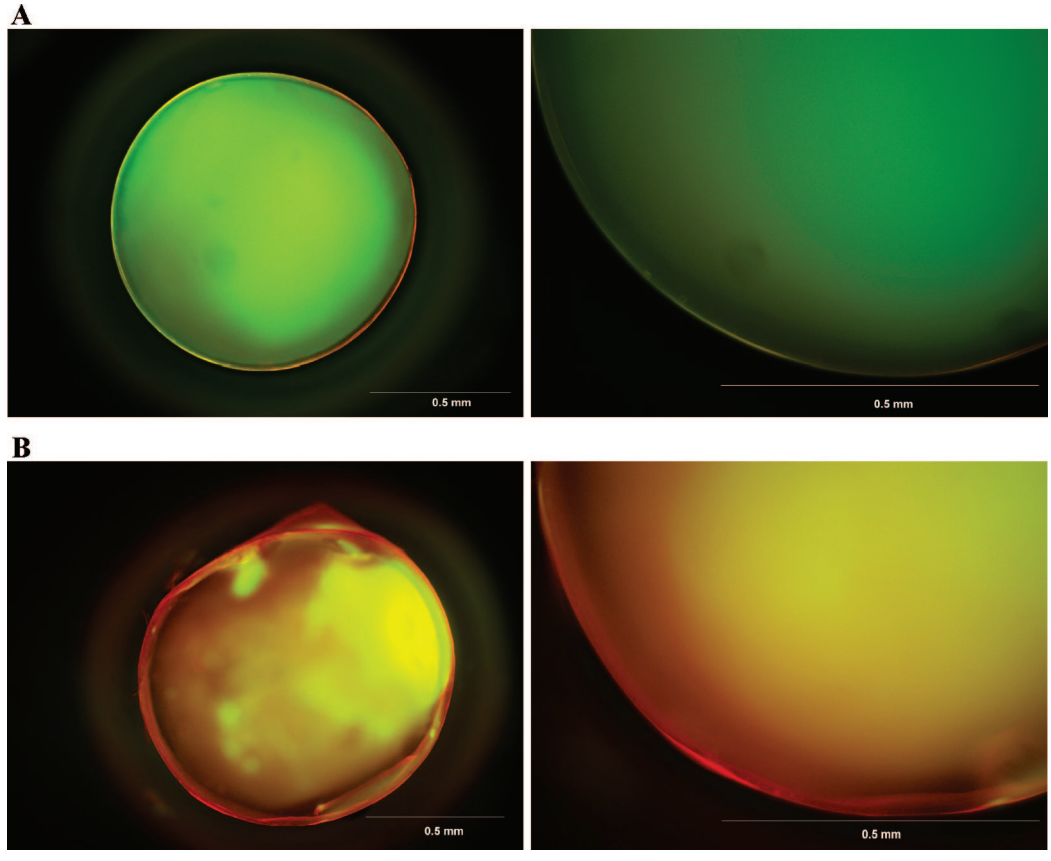

Figure 5. Fluorescence micrographs of CTAB−dsDNA (A) and CTAB−ssDNA (B) particles: individual particles (left) and the same particles at high magnification (right).

rough and less open than that of particles formed with native DNA. These observations seem then to be consistent with the respective swelling response (see Figure 2).

Considering our systems, the obtained structures seem to confirm the higher degree of interaction between ssDNA and CTAB, increasing the shell section of the obtained particles. Consequently, the core–shell structure seems to be crucial in the controlled encapsulation and release of the polyelectrolyte.

Studies of complexes formed by interfacial diffusion between cationic chitosan and negatively charged surfactant[58] demonstrated that the existence of an ordered structure formed at the interface. The capsule shells obtained may be considered as physical networks in which surfactant micelles form polycationic−multianionic electrostatic complexes as cross-link points. Preliminary results of small-angle X-ray spectroscopy (SAXS) measurements on the hydrated skin of

CTAB−dsDNA particles at 25 °C have shown three diffraction orders of a hexagonal repeat period ($q_{max} = 1.29$ nm^{-1} (results not shown)). These results would confirm the existence of an ordered nanostructure of the DNA−CTAB complexes, which would be involved in the stabilization of the obtained particles.

Secondary Structure of DNA in the Particles. Information about the secondary structure of the DNA molecules in the gels was obtained by fluorescence microscopy using acridine orange as the staining medium. Acridine orange has been used to label nucleic acids in solution and intact cells.[59–62] Acridine orange intercalates into double-stranded DNA as a monomer, whereas it binds to single-stranded DNA as an aggregate. Upon excitation at 470−490 nm, the monomeric acridine orange bound to double-stranded DNA fluoresces green, with an emission maximum at 530 nm. The aggregated acridine orange on single-stranded DNA fluoresces red, with an emission at about 640 nm.[60,61]

On the basis of the observation of green or red fluorescence, acridine orange has been used to differentiate native, double-stranded DNA from denatured, single-stranded DNA in the CTAB−DNA particles. Figure 5 shows fluorescence microghaps of individual particles.

These results are consistent with the secondary structure of the DNA used for the particle preparation. The fluorescence microscopy studies thus suggest that the formation of the particles is carried out with the conservation of the secondary structure of DNA.

4. Concluding Remarks

DNA gel particles were formed by interfacial diffusion on mixing solutions of DNA (either single-stranded (ssDNA) or double-stranded (dsDNA)) with solutions of the cationic surfactant, CTAB. The obtained particles were characterized with respect to the degree of DNA entrapment, surface morphology, and secondary structure of DNA in the particles. The swelling/deswelling behavior and DNA release were investigated in response to salt additions. Analysis of the data indicates clear differences in interaction between the surfactant and the state of DNA, single- or double-stranded.

Preliminary results of SAXS measurements have supported the existence of an ordered structure formed on the hydrated skin of the obtained particles. The formation of a physical network in which surfactant micelles form polyanionic−multicationic electrostatic complexes as cross-link points seems to play an important role in the stabilization of the DNA particles. Fluorescence microscopy studies suggest that the formation of the particles was carried out with the conservation of the initial secondary structure of DNA. Current studies are focused on characterizating the structure of these DNA gel particles and on modifying the experimental conditions for further application in gene transfection (if DNA plasmid could also be used as the complexation agent). Interestingly, these particles represent a conceptual step in the design and development of new nonviral vectors for the delivery of therapeutic DNA.

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