Formation of a DNA/*N*-Dodecylated Chitosan Complex and Salt-Induced Gene Delivery

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ABSTRACT: An *N*-dodecylated chitosan (CS-12) was synthesized from dodecyl bromide and chitosan and was assembled with DNA to form a polyelectrolyte complex (DNA/CS-12 PEC). UV was used to examine the thermal stability of DNA embedded in PEC. The results indicate that the incorporation of dodecylated chitosan can enhance the thermal stability of DNA. The analysis of AFM image shows that PEC develops a globule-like structure composed of 40–115 DNA molecules. Dissociation of PEC was investigated by the addition of low molecular weight electrolytes. The added small molecular salts dissociate the PEC, inducing DNA to release. The ability of Mg^{2+} to dissociate PEC is greater compared to that of Na^{+} and K^{+}. From AFM images, it can be visualized that DNA remains intact and undamaged due to the protection from DNase offered by alkylated chitosan. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 3391–3395, 2001

Key words: DNA; dodecylated chitosan; polyelectrolyte complex; atomic force microscopy

INTRODUCTION

In the past decade, DNA complexes with cationic liposome, poly(L-lysine), lipoglutamate, chlorophyll, dendrimers have been developed for transferring genes into eukaryotic cells.[1–5] In vitro studies have established that the formation of DNA-based complexes is a consequence of a self-assembly process triggered by electrostatic interactions between DNA host and polycationic guests. In the construction of gene delivery systems, DNA–lipid complexes are well investigated.[6–8] In his original work, Felgner proposed that cationic liposomes were attached to DNA strand like beads on a string. Rädler found that λ-phase DNA–lipid complexes formed multilamellar liquid crystalline aggregates with two-dimensional (2D) in-plane order of DNA sandwiched between the lipid membrane.[9]

It has been experimentally verified that a significant structural change in the liposomes and DNA occurs with the addition of cationic to DNA.[10,11] Recently, Sato et al.[12] prepared a DNA–polygalactosamine complex and investigated its interaction with cells. It was observed that the complex showed a high cell uptake and no cytotoxicity. Murata reported that *N,N,N*-trimethyl chitosan–DNA complex has a cellular recognition ability and shows potential as a gene delivery carrier.[13] To achieve efficient gene deliv-

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ery via encapsulation of DNA into a polycation, two basic requirements must be met: effective dissociation of DNA from its complex, and DNA protection from nuclease attack.

In the present study, an *N*-dodecylated chitosan was synthesized and incorporated with DNA to form a self-assembly complex. The structure of DNA–dodecylated chitosan complex was analyzed using AFM. The dissociation of the complex induced by small molecular salts was investigated, and DNA protection from nuclease provided by chitosan was visualized by AFM.

MATERIALS AND METHODS

Materials

Sodium salt of DNA from salmon testes (average M_w: 2000 kDa, ~ 2000 bp) was purchased from Sigma Chemical Co. (St. Louis, MO). Chitosan (average M_w: 700 kDa) was provided by Sigma. Dodecyl bromide (analytical grade) was supplied by Beijing Chemical Co. (China). DNase I was purchased from Sigma. All other reagents were analytical grade.

Synthesis of Dodecylated Chitosan (CS-12)

In this investigation, 2 g of chitosan was suspended in 40 mL of isopranol, followed by the addition of 5 N NaOH (20 mL). The mixture was vigorously stirred at 6°C for 30 min, after which 0.48 g of dodecyl bromide was added. After 4 h, the reaction mixture was poured out and neutralized with hydrochloric acid. The precipitate was filtered off, washed thoroughly with methanol, and dried in vacuum at 50°C to obtain dodecylated chitosan powder.

The increase in the characteristic bands of methylene band at 2853 cm^{-1}, 1451 cm^{-1}, and the decreased NH_2 band at 1589 cm^{-1} in the FTIR spectrum confirmed the formation of alkylated chitosan. The degree of substitution (DS) determined by potentiometric titration[14] was 22%.

Preparation of a DNA–CS-12 Complex

At an equimolar ratio of the phosphate of DNA to the cation of CS-12, mixing of aqueous solutions of DNA and dodecylated chitosan produced a precipitate. Moreover, the precipitate was insoluble, even in strongly polar solvents such as dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), and formic acid. Therefore, a ratio of 1 : 2 of these components was selected. At this ratio, the mixing of DNA and hydrochloric acid solution of CS-12 generated only a turbid substance, which was lyophilized to produce a white powder.

UV Measurement

Fifty milligrams of DNA/CS-12 was dissolved in formic acid to form a dilute solution, which was heated from 25°C to 85°C. The absorbance of heat-treated solution with respect to each temperature was measured at 260 nm with a 756MC UV spectrophotometer. In comparison, an aqueous solution of DNA was checked with the same conditions. The concentration of DNA in dilute solution was 50 μg/ml.

Dissociation of DNA/CS-12 Complex

In this study, 20 mg of DNA/CS-12 complex samples was immersed in 25 mL of NaCl, KCl, and $MgCl_2$ solutions of various concentrations, respectively, and kept at 37°C for 48 h. The DNA released was determined at 260 nm with a 756 MC UV spectrophotometer with a 10-mm pathlength cuvette.

Fifty milligrams of DNA/CS-12 complex film and pure DNA was placed into deionized water, respectively, to which 10 μl of 200 μg/ml DNase solution was added. After 8 h of incubation at 37°C, the complex film was washed repeatedly with deionized water and placed into 0.1 mol/L $MgCl_2$ solution for an additional 48-h dissociation at 37°C. For AFM samples, 5-μL aliquots of pure DNA aqueous solution and a salt solution of complex were deposited onto a freshly cleaved mica disk, respectively. The deposited solutions were dried at room temperature. Imaging was conducted on an Atomic Force Microscopy (Nanoscope IIIa system, Digital Instruments, Santa Babara, CA). A Si_3N_4 probe on a triangular cantilever was used to obtain images in tapping mode.

RESULTS AND DISCUSSION

Thermal Stability and Aggregated State of DNA/CS-12 Complex

Figure 1 exhibits the absorbance of formic acid solution of the complex and aqueous solution of DNA as a function of temperature. The pure DNA shows a noticeable hyperchromic effect. Although DNA in the complex is relatively stable, a reason-

able explanation is that caused by the interaction with chitosan, DNA is condensed and sandwiched between CS-12, protecting it from thermal denaturation. Besides, the long alkyl side-chains in chitosan further act as a shield.

Many researchers now use fluorescence microscopy to observe that single DNA changes its structure in a discrete manner between coil and compact globules with the addition of cationic species.[15,16] However, the resolution limit of wavelength of fluorescence light fails to present a more accurate analysis of the structure of DNA-based complex.[17] In view of that, we use atomic force microscopy to analyze the morphology of DNA/CS-12 complex. Figure 2 displays the AFM image of DNA/CS-12 complex. One can clearly see that DNA is aggregated and the complex appears globular structure. The diameter varies form 0.09 μm to 0.23 μm, and the average height is $\sim$ 18 nm. To estimate approximately the number of DNA molecule encapsulated in one globule, a rough quantitative analysis is carried out as follows. The total volume of the globule is calculated in terms of the volume of spherical cap:

$$V_G = \frac{1}{3}\pi h^2(3R - h) \tag{1}$$

The total volume of DNA is

$$V_d = \pi r^2 L \tag{2}$$

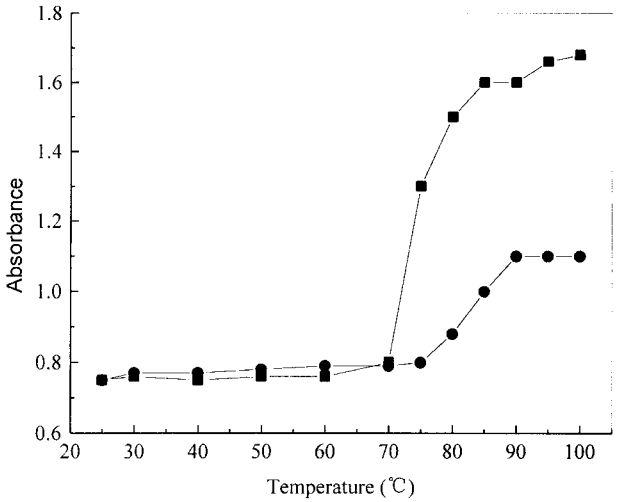


Figure 1 The variation in the absorbance values of pure DNA in aqueous solution (■) and DNA/CS-12 in formic acid solution (●) vs temperature.

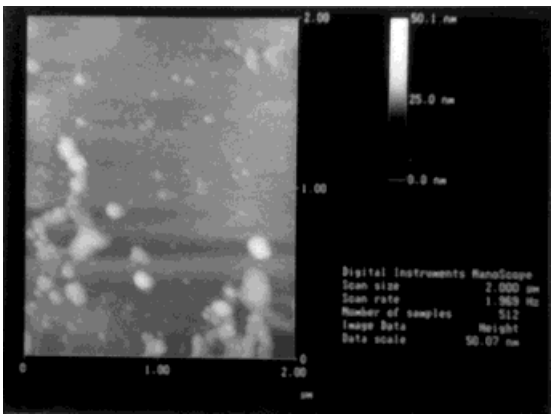


Figure 2 AFM image of DNA/CS-12 complex.

where

$$r = 10 \text{ Å}. L = n \times l$$

where n is the number of base pairs, and l, the distance between two neighboring base-pair, is equal to 3.4 Å.

The number of DNA molecule contained in one globule is

$$N = V_G/V_d \tag{3}$$

From the above formula, a single globule consists of 40–115 DNA molecules, which is shielded by chitosan, especially its long alkyl side-chains.

Delivery of DNA/CS-12 Complex

It has been established both theoretically and experimentally, that the dissociation of the polyelectrolyte complex could be achieved by changing the pH value, or ionic strength, or both.[18,19] The addition of a low-molecular-weight electrolyte is an effective way to destroy PEC. The charge screening of the components by added salts leads to a decrease in the number of interpolyelectrolyte salt bonds within PEC followed by dissociation of PEC to the initial polyions.[20] In our experiment, we check the dissociation of PEC in NaCl, KCl, and $MgCl_2$ water–salt solutions. It is necessary to point out that in the beginning, we found DNA can be dissociated from PEC into solution even in deionized water, which exceeds our expectation. We believe that not all DNA molecules are embedded in the complex and that some DNA molecules are located on the surface of complex. The DNA molecules on the surface are prone to

release in deionized water. Thus, we washed the complex samples with deionized water repeatedly till no DNA is detected, and then examine the dissociation of PEC induced by small molecular salts. Figure 3 shows the dependence of the amount of DNA delivered on the concentration of salt solution. As the concentrations of salts are 0.001*M,* no evident DNA release is observed; with a further increase of concentration, the dissociation of complex occurs and the amount of DNA delivered continuously increases. Moreover, the release amount in $MgCl_2$ is greater than in NaCl and KCl. This is related to the different affinity of ions to DNA. Mg^{2+} has a much higher affinity to DNA compared with Na^+ and K^+.[19] Therefore, it shows greater dissociation ability. Our result is basically consistent with the data obtained by Izumrudov et al.[19] However, we observed no significant difference between Na^+ and K^+ for the dissociation of DNA/CS-12 complex. It is speculated that this is originated from the little difference in the affinity of Na^+ and K^+ to DNA.

AFM Visualization of DNA Protection

Figure 4 displays the AFM images of pure DNA and DNA released from complex with the addition of DNase. Pure DNA in the absence of dodecylated chitosan is hydrolyzed by DNase and has been broken into fragments, while DNA dissociated from the complex is well protected. One can clearly see that DNA remains intact and undamaged. Quong and Neufeld[21] reported that chitosan-coated DNA is not sufficiently protected, as it was exposed to nuclease. In our case, we observe that dodecylated chitosan offers a good protection for DNA. It is proposed that the incorporation of long hydrophobic alkyl chains lowers the permeability of DNase, shielding DNA from attack.

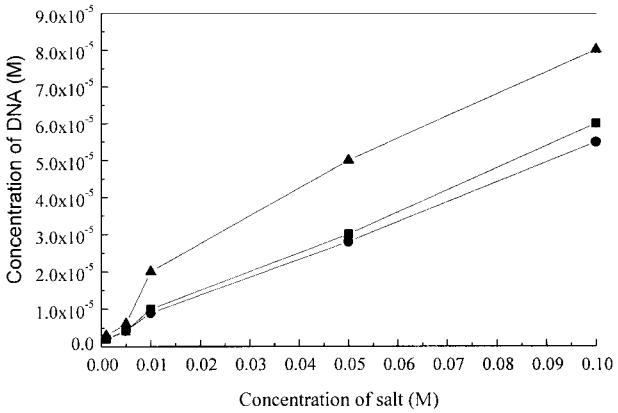
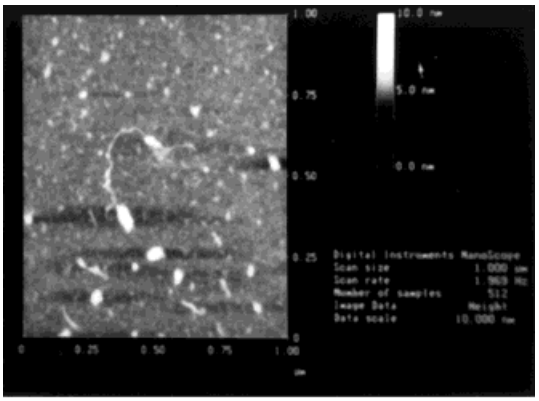
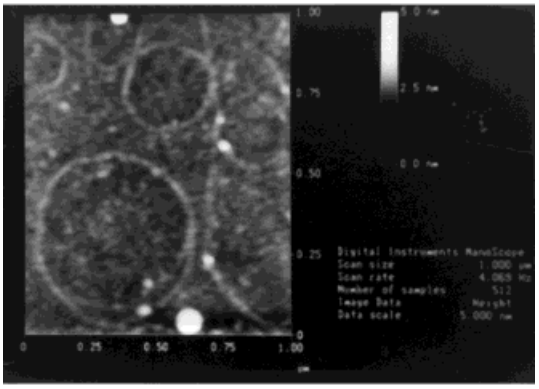


Figure 3 Dependence of the amount of delivered DNA on the concentration of NaCl (■), KCl (●), and $MgCl_2$ (▲) solutions.



(a)



(b)

Figure 4 AFM images of pure DNA (A) and DNA released from complex (B) with the addition of DNase.

CONCLUSIONS

An *N*-dodecylated chitosan (CS-12) was synthesized and assembled with DNA to form a polyelectrolyte complex (PEC). The thermal stability of the complex is enhanced due to the encapsulation of DNA in chitosan. The electrostatic interaction between DNA and CS-12 results in a globule-like structure containing 40–115 DNA molecules. The complex can be dissociated by the addition of small molecular salt solution. The ability of Mg^{2+} to break the PEC is greater than that of Na^+ and K^+. Importantly, it is visualized by AFM that the

DNA can be well protected by the dodecylated chitosan from nuclease.

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