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Nanoparticles formation of Philippine carrageenan with pDNA/polyethylenimine and pDNA/chitosan as monitored by atomic force microscopy (AFM) for potential use in gene delivery

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Abstract

Philippine carrageenan, was interacted with pDNA/PEI and pDNA/chitosan nanoparticles to study its effect on DNA condensation for potential use in non-viral gene delivery. Complexation and DNA condensation was monitored by atomic force microscopy. A cloud like structure was observed in the image of carrageenan-PEI complex as compared with the complex of carrageenan-chitosan which is more compact showing a fibrous structure. When pDNA was complexed with -carrageenan a distinct morphology was observed. The structure showed the formation of linear strands of DNA into a web-like structure or a network without interlacing with the single stranded chains of carrageenan. Using an N/P ratio of 5, pDNA was not condensed using the different types of carrageenan. All of the images formed a cloud-like or dendritic structures. The complex of chitosan and carrageenan showed a poor medium for DNA condensation as observed in the micrograph. pDNA is more condensed with PEI-carrageenan complex than chitosan-carrageenan combination. The electrostatic interaction of carrageenan with PEI was able to condense the DNA at N/P ratio of 3.3 better than chitosan-carrageenan interaction with an N/P ratio of 5. Comparing the three types of Philippine carrageenan in the interaction of pDNA/PEI, iota type forms a good complex with PEI that can condense DNA, which is an important aspect in gene delivery.

Key words : Atomic force microscopy, DNA condensation, Polyethylenimine, Carrageenan, Chitosan

INTRODUCTION

Carrageenans constitute a group of sulphated galactans originating from red marine algae Rhodophycea and are well known to the food industry because of their suspending, thickening and gelling properties [1]. The disaccharides (1→3) -β-D-Gal-(1→4)-α-D-Galρ-(1→) are the basic repeating units in these linear polysaccharides which make carrageenan a good texture enhancers as well as stabilizers in the food and pharmaceutical/ cosmetic industries [1]. Recent studies show the importance of carrageenans in new drug delivery systems (DDS) since it provide more control over the rate of release [2,4,5,6]. Previous study showed the formation of ternary complex of pDNA-PEI- *iota* carrageenan as determined by gel retardation assay and monitored by atomic force microscopy (AFM) [7].

Through these promising results, the use of other types of Philippine carrageenan like *kappa* and *lambda* for *in vitro* gene delivery was explored in this study. AFM was used to characterize the morphology of pDNA-PEI and pDNA-Chitosan nanoparticles coated with carrageenan to form interpoly electrolyte complexes. AFM was also used to investigate the effect of carrageenan in DNA condensation.

Polyethylenimine (PEI) and chitosan has been exhaustively studied in various *in vitro* and *in vivo* gene transfer [8,9,10,11,12, 13]. Numerous studies were also conducted in the formation of polyplex of PEI conjugates and grafted copolymers for gene delivery [14,15,16,17,18] as well as galactosylated chitosan including grafted copolymers [19,20,21,22,23,24]. But the potential use of *kappa* and *lambda* type carrageenan to form interpolyelectrolyte complexes with PEI and chitosan in non-viral gene delivery system has not been studied yet.

MATERIALS AND METHODS

Chemicals

PEI (average MW=25KDA, branched with degree of polymerization of 580) was obtained from Aldrich Company (St Quentin Fallavier, France). κ-carrageenan (Bengel KK-100, Lot No. XO300-2), ι-carrageenan (Benvisco SI-100, Lot No.M1400-1) and λ-carrageenan(Benvisco SL-100, Lot No. S2703-2) was obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Chitosan hydroacetate (40 kDa) was obtained from Yaizu Suisankagaku Industry (Shizuoka, Japan). Degree of acetylation is about 87.5%.

Plasmid pGL3(SV40) was amplified in E. coli and purified by using Promega protocol.

All other chemicals used were of analytical grade. Dulbecco's PBS (-) was obtained from Nissui Pharmaceutical Co. Ltd (Japan).

Sample preparation

κ, ι and λ carrageenan were dissolved in PBS (-) solution at pH 6.5. Then mixed using a vortex machine, followed by ultrasonication and shaken at 200 rpm for 8 hours. Final concentration of each sample is: κ-carrageenan (4.67 mg/mL), ι-carrageenan (5.63 mg/mL) and λ-carrageenan (6.81 mg/mL). Chitosan (2.805 mg/mL) was prepared by dissolving in Phosphate Buffer Solution, PBS (-) solution then adjusted the pH to 6.5 and shaken at 200 rpm, 37 °C for 12 hours. PEI was dissolved in PBS (-) solution following the procedure of preparing the chitosan with a final concentration of 0.90 mg/mL. Complex formation was verified by electrophoresis on a 1% agarose gel with Tris-acetate (TAE) running buffer, 100V for 30 minutes. DNA was visualized with ethidium bromide (0.2 μg/mL).

Preparation of complex for AFM analysis

pDNA-Chitosan-Carrageenan

One (1) µL of pDNA (10 µg/µL) and 1 µL of chitosan solution (2.805 mg/mL) was diluted each with 49 µL of PBS (-) solution at pH 6.5 with a charge ratio of 1.8 : 1 (charges of nitrogen atoms in chitosan vs. of phosphate in DNA which means a N/P ratio of 5) and allowed to stand separately for 15 minutes at room temperature. Then mixed the two solutions and allowed again to stand for 15 minutes at room temperature. The carrageenan solution was slowly added to the mixture at different volumes (0 µL → 7.5 µL), mixed and repeatedly allowed to stand for 15 minutes at room temperature. Ten (10) µL of the mixture was applied in a drop deposition on to the surface of a silica glass as previously described [23,24] and allowed to dry at room temperature. Following adsorption for 2 minutes at room temperature, excess fluid was removed by absorption with filter paper. Then 50 L of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging.

pDNA-PEI-Carrageenan

All solutions for DNA, PEI and carrageenan were prepared in PBS (pH=7.3). One (1) µL of pDNA (10 µg/ µL) and 1 µL of PEI (0.9 µg/ µL) was diluted each with 49 µL of PBS(-) solution with a charge ratio of 5.3 : 1 (charges of nitrogen atoms in PEI vs. of phosphate in DNA which means a N/P ratio of 3.3) and allowed to stand separately for 15 minutes at room temperature. Then mixed the two solutions and allowed again to stand for 15 minutes at room temperature. The carrageenan solution was slowly added to the mixture at different volumes (0 µL → 30 µL), mixed and repeatedly allowed to stand for 15 minutes at room temperature. Ten (10) µL of the mixture was applied in a drop deposition on to the surface of a silica glass as previously described [23,24] and allowed to dry at room temperature. Following adsorption for 2 minutes at room temperature, excess fluid was removed by absorption with filter paper. Then 50L of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging.

Atomic force microscopy (AFM) measurement

Imaging was done through a commercial SPA-300 system of Seiko Instruments, Inc. Japan). A Si_3N_4 tip on the cantilever with a length of 100m and a depth of 400 nm (SN-AF01-A, Olympus Optical Co.) was used with tapping mode. Imaging was recorded in non-contact mode under ambient condition. Solutions of pDNA + PEI and pDNA + chitosan including carrageenan on the mica disk were observed as control.

RESULTS

The formation of the ternary complexes was determined by gel retardation assay. Release of DNA was not observed in the agarose gel chromatogram (Figure 1), As observed in the electrophoretic mobility of the free DNA (pGL3SV40), this was retarded with the formation of the complex at various charge ratios, confirming the formation of ternary complex of pDNA-PEI-carrageenan and pDNA-chitosan-carrageenan.

Prior to AFM imaging of interpolyelectrolyte complexes of pDNA-PEI-Carrageenan and pDNA-Chitosan-Carrageenan, each of the compounds were characterized first. Structures of , , type carrageenan are shown in Figure 2. The micrographs of the different types of carrageenan showed different morphologies. -carrageenan (Figure 2A) forms an aggregated network in conformity with the results of McIntire and Brant, 1999 [25] who uses water to dilute the carrageenan samples and prepared by aerosol spray deposition. No difference was noted in this study which makes use of PBS solution adjusted to pH 6.5. to dilute the carrageenan. The presence of single stranded chains were also

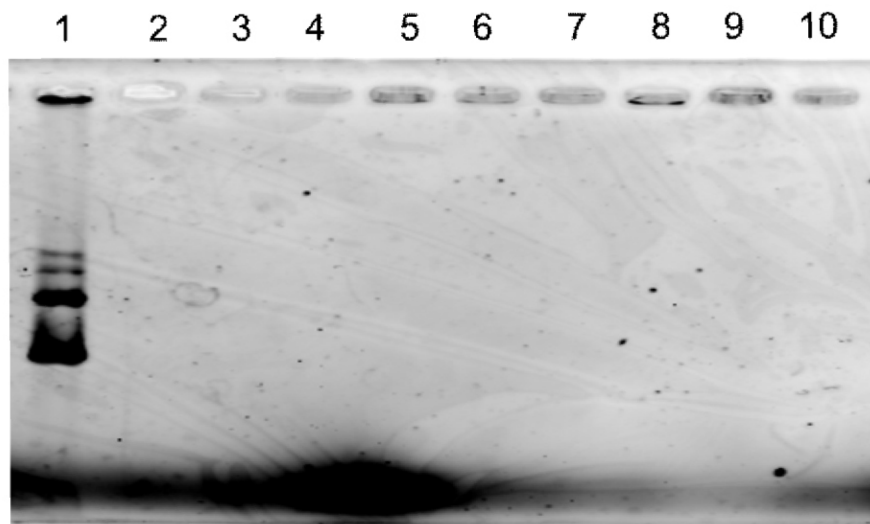

Figure 1. Complex formation of pDNA with carrageenan, PEI and chitosan by gel retardation assay. Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide. Lane1: pDNA (0.1 µg); Lane 2: pDNA+PEI (N/P = 5); Lane 3: pDNA + chitosan (N/P = 5); Lane 4: pDNA + carrageenan (ratio = 1:4); Lane 5: pDNA-chitosan-κ-carrageenan complex (N/P = 5, 1:1:0.5); Lane 6: pDNA-chitosan-ι-carrageenan complex (N/P = 5, 1:1:0.5); Lane 7: pDNA-chitosan-λ-carrageenan complex (N/P = 5, 1:1:0.5); Lane 8: pDNA-PEI-κ-carrageenan complex (N/P = 3.3, 1:1:4); Lane 9: pDNA-PEI-ι-carrageenan complex (N/P = 3.3, 1:1:4); Lane 10: pDNA-PEIλ-carrageenan complex (N/P = 3.3, 1:1:4).

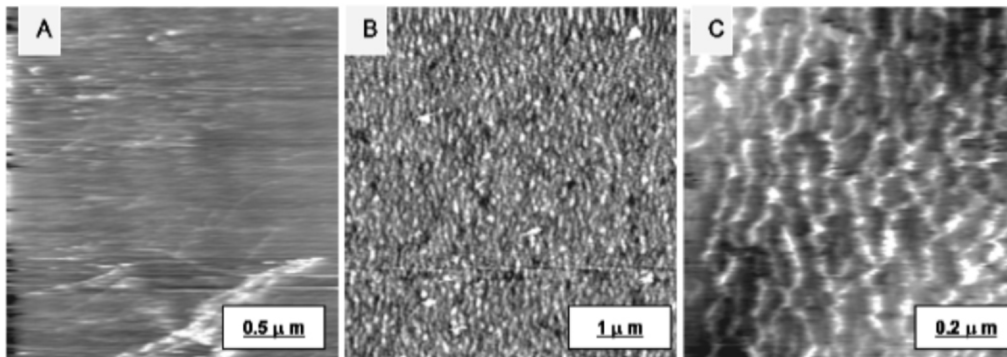

Figure 2. AFM micrographs of carrageenan: A. κ-carrageenan; B. ι-carrageenan; C. λ-carrageenan

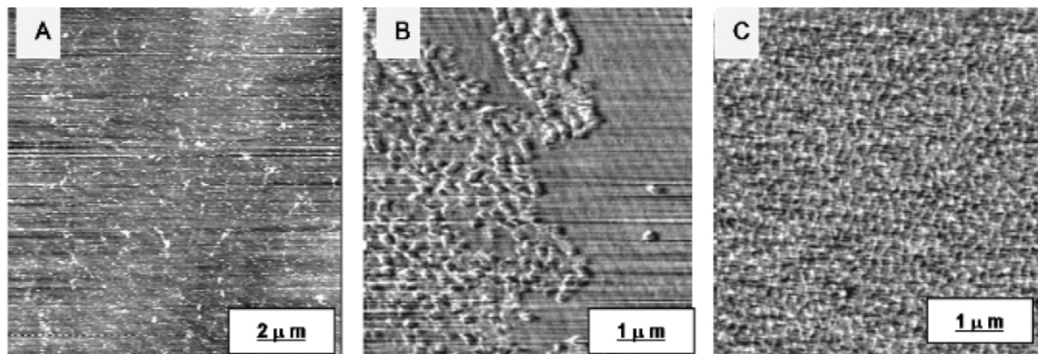

Figure 3. AFM micrographs of plasmid and polymers: A. pDNA (plasmid pGL3(SV40); B. chitosan (2.805 mg/mL); C. PEI (0.90 mg/mL)

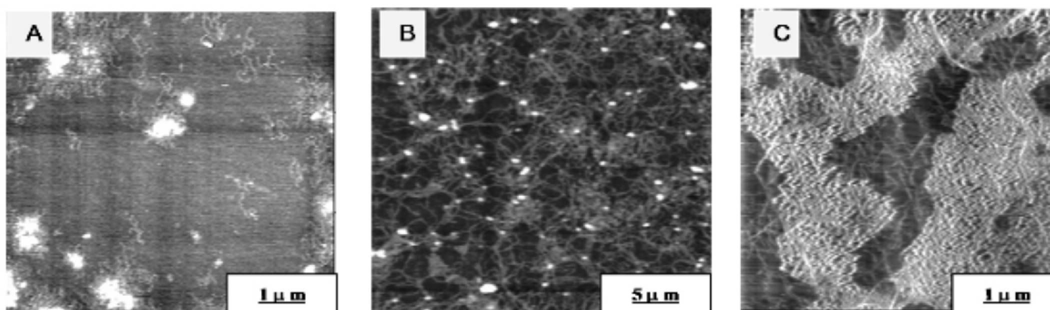

Figure 4. AFM images of plasmid coated with polymers. A. pDNA + chitosan (N/P = 5); B. pDNA + PEI (N/P = 5); C. pDNA + carrageenan (ratio = 1:4)

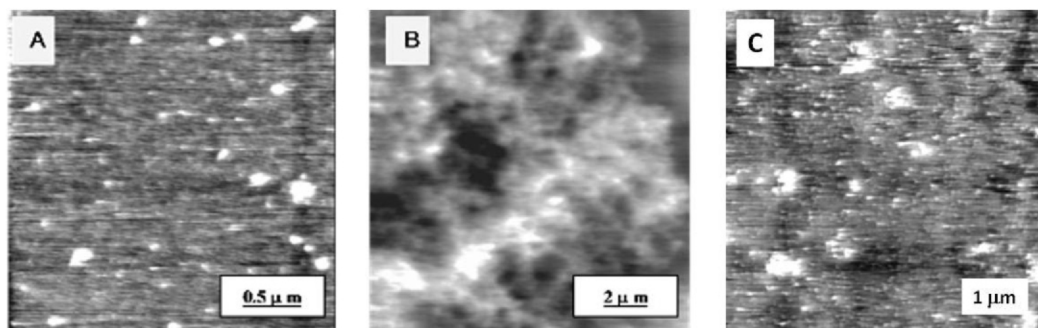

Figure 5. AFM micrographs of carrageenan mixed with PEI and chitosan. A. κ-carrageenan + Chitosan (1:1); B. κ-carrageenan + PEI (1:1); C. λ-carrageenan + PEI (1 :1)

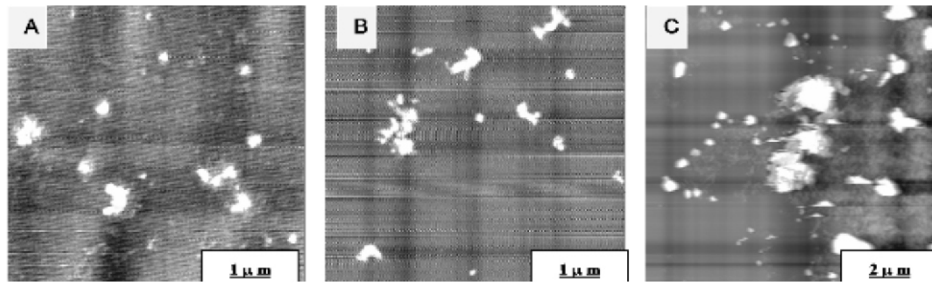

Figure 6. AFM micrographs of pDNA-chitosan-carrageenan complex (N/P = 5, 1:1:0.5)
A. κ-carrageenan; B. ι-carrageenan; C. λ-carrageenan

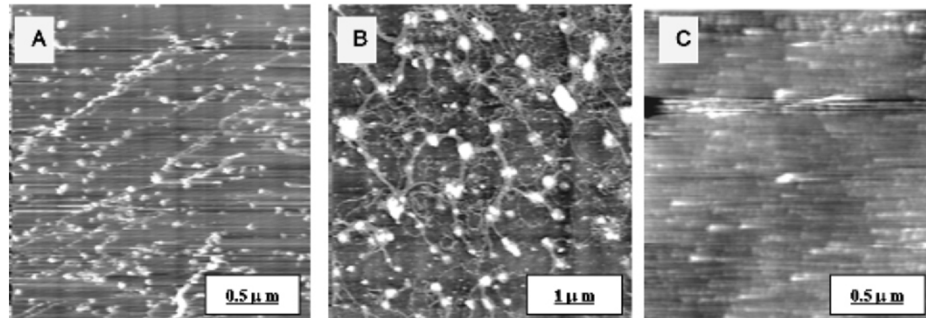

Figure 7. AFM micrographs of pDNA-PEI-carrageenan complex (N/P = 3.3, 1:1:4)
A. κ-carrageenan; B. ι-carrageenan; C. λ-carrageenan

found in the micrograph. To compare the results with -carrageenan [Figure 2B], it displayed a peculiar structure which is quite different from the structure previously reported by McIntire and Brant, 1999 [25]. Since the molecular structure of -carrageenan has "kinks" that increase its flexibility and reduce the space occupied by the molecule as described by Kirby et al, 1996 [26], this might explain the irregularities of the structure. In -carrageenan (Figure 2B) the structure exhibited a network or typical chains for the polymeric molecule. It has the same morphology as described by Oliva et al, 2003 [27] in their studies.

The topographies of pDNA, chitosan and polyethylenimine are shown in Figure 3. pDNA (Figure 3A) showed a typical topography of DNA molecules in coil and globule forms similar to the results of AFM studies conducted by Sergeyev et al, 1997 [28] for DNA condensation. DNA in an aqueous solution is a highly negative charged polymer with a long persistent length [29]. A dendritic topography was observed in the AFM image of chitosan (Figure 3B). According to Xiaoqing et al, 2004 [30], AFM images of chitosan forms a prominent and delicate dendritic structures which are fully-simulated and grow in cluster composed of some nano particles with varying values. The findings of Xiaoquing *et al* [30] were similarly observed in this study. In PEI (Figure 3C), dendritic topography was also observed. Menchaca *et al*, 2004 [31] reported in their study that PEI film does not produce nano ring structure which also conforms with our observation.

Structures of pDNA-chitosan (N/P=5), DNA-PEI (N/P=2.3) and pDNA-carrageenan are shown in Figure 4. A variety of condensed molecules of pDNA-chitosan (Figure 4A) and pDNA-PEI (Figure 4B) were observed. The presence of individual toroids, circular DNA and a network of toroids were revealed by AFM in both samples. Linear strands and loop-like structures were also observed which are intermediates in the condensation of DNA. PEI-DNA tends to form aggregates overtime [13]. The micrograph of pDNA-mixed with carrageenan (Figure 4C) showed a distinct morphology. The linear strands of DNA formed into a web-like structure or a network without interlacing with the single stranded chains of κ-carrageenan. This might be due to the negative charge of both samples forming a surface membrane.

The polymer complex of -carrageenan with chitosan and PEI exhibited different morphologies (Figure 5). Based on the micrographs, κ-carrageenan binds with PEI and chitosan due to electrostatic interactions between sulfhydryl groups of carrageenan and amine groups of chitosan and PEI. PEI with carrageenan (Figure 5B) showed a cloud like structure than with chitosan (Figure 5A) which is more compact. The cloud like structure of PEI-carrageenan complex might be due to the formation of a surface membrane whom Muralidhar *et.al* (2001) [32] describe as the reaction of polyneutralization since carrageenan is a polyanion. The compactness of carrageenan-chitosan complex might be due to the double-helical form of κ-carrageenan interacting with chitosan forming a fibrous structure which is insoluble in aqueous acidic solution.

Figure 6 shows the AFM images of pDNA complexed with chitosan and carrageenan. Using an N/P ratio of 5, pDNA was not condensed using the different types of carrageenan. All of the images formed a cloud-like or dendritic structures. The complex of chitosan and carrageenan showed a poor medium for DNA condensation as observed in the micrograph. Comparing it with the image of pDNA-chitosan (Figure 4A), toroids and circular DNA as well as network of toroids were not observed in Figure 6. Most likely, carrageenan competes with DNA in interacting with chitosan aside from forming an insoluble precipitate when mixed with carrageenan.

The micrographs of pDNA-PEI-carrageenan complex are

shown in Figure 7. As shown in the images, DNA is more condensed with PEI-carrageenan complex than chitosan-carrageenan combination. With an N/P ratio of 3.3, toroids and circular DNA with a network of toroids were observed in iota carrageenan (Figure 7B) than in kappa type (Figure 7A). No DNA condensation was observed in lambda type carrageenan (Figure 7C). As observed in the micrograph, aggregates were formed instead. This maybe due to the different structure of lambda carrageenan compared to kappa and iota type which carries three sulphate groups per repeating unit.

DISCUSSION

The atomic force microscope (AFM) is ideally suited for characterizing nanoparticles. It offers the capability of 3D visualization and both qualitative and quantitative information on many physical properties including size, morphology, surface texture and roughness. Statistical information, including size, surface area, and volume distributions, can be determined as well. A wide range of particle sizes can be characterized in the same scan, from 1 nanometer to 8 micrometers. In addition, the AFM can characterize nanoparticles in multiple mediums including ambient air, controlled environments, and even liquid dispersions [33]. The objective of this study is to monitor the complexation and DNA condensation through atomic force microscopy. The structure of carrageenan, polyethylenimine and chitosan were also investigated before complexation with DNA. The atomic force microscope (AFM), with its high resolution and continuous images of cells and biomacromolecules, has recently become a powerful tool for the study of biomolecular shapes, conformations, behavior and the relationship between function and molecular conformation[34]. Through the use of AFM, the macromolecular structures of various carrageenan were determined. It was shown that the structure is dependent on the concentration[25, 35]. κ-carrageenan forms both single and two stranded structures at a low concentration[35] which was evident in the result obtained while ι-carrageenan displayed a peculiar structure with a more flexible network. It was explained by Kirby et al, 1996 [26] that the molecular structure of ι-carrageenan has "kinks" that increase its flexibility and reduce the space occupied by the molecule. In λ-carrageenan a honeycombed structure is formed only at high concentrations[35]. Chitosan is also dependent on the concentration to form dendritic feature[30]. PEI on the other hand does not produce nano ring structure[31]. Atomic Force Microscopy (AFM) is used to image DNA with high accuracy and under physiological conditions[36,37]. The samples must be immobilized onto a flat surface in order to be imaged by AFM. In this study freshly cleaved mica was used for this purpose. The negatively charged backbone of the DNA can be utilized for immobilization onto charged substrates by means of electrostatic interactions. The AFM image of pDNA (uncomplexed) is of coil form of which the regions of the duplex were observed to fold back on themselves without binding or interacting to each individual molecules.

The method used to prepare the Philippine carrageenan with pDNA/polyethylenimine and pDNA/chitosan complexes provided results that appear to reflect differences in the accessibility of individual complexed DNA molecules, at the time of their formation, to other complexes and to mica. The complexes of PEI/carrageenan with pDNA shown here are well-condensed complexes as compared to chitosan/carrageenan with pDNA since condensation is present even after rinsing and drying with compressed air. Several conclusions can be drawn from this study. First, good DNA condensation was observed in PEI/carrageenan than chitosan/carrageenan. Second, good DNA condensation was obtained only with pDNA-PEI-ι-carrageenan complex than in pDNA-PEI-κ-carrageenan with the formation of toroids and circular DNA. DNA condensation is important not only for gene delivery, but also for other natural biological processes such as viral replication and cell division.

CONCLUSION

Between the complex of chitosan-carrageenan and PEI-carrageenan the latter condensed the DNA better than the former. Among the three types of carrageenan, iota type forms a good complex with PEI which can condense DNA for gene delivery. DNA forms a web-like structure or a network when mixed with carrageenan. The study also showed that through AFM imaging, structures of interpolyelectrolyte complexes of chitosan-carrageenan and PEI-carrageenan with DNA can be evaluated and investigated for gene delivery use.

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