Ternary Complex Consisting of DNA, Polycation, and a Natural Polysaccharide of Schizophyllan to Induce Cellular Uptake by Antigen Presenting Cells

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A natural polysaccharide called schizophyllan (SPG) can form a complex with polynucleotides, and the complex has been shown to deliver biofunctional short DNAs such as antisense DNAs and CpG-DNAs. Although it is a novel and efficient method, there is a drawback: attachment of homo-polynucleotide tails [for example, poly(dA) or poly(C)] to the end of DNA is necessary to stabilize the complex, because DNA heterosequences cannot bind to SPG. The aim of this paper is to present an alternative method in which SPG/DNA complexes can be made without using the tails. The basic strategy is as follows: since SPG can form hydrophobic domains in aqueous solutions, hydrophobic objects should be encapsulated by this domain. DNA alone is highly hydrophilic; however, once DNA/polycation complexes are made, they should be included by the SPG hydrophobic domain. The aim of this paper is to prove the formation of the polycation/DNA/SPG ternary complex. Gel electrophoresis showed that presence of SPG influenced the migration pattern of polycation+DNA mixtures. With increasing the SPG ratio, the zeta potential (ζ) of the polycation+DNA+SPG mixture decreased drastically to reach almost $\zeta = 0$ and the particle size distributions were altered due to the ternary complex formation. Confocal laser scanning microscopy revealed that the polycation/DNA/SPG ternary complexes showed high uptake efficiency when the complexes were exposed to macrophage-like cells (J774.A1). IL-12 secretion was enhanced when CpG-DNA was added as the ternary complex. These features can be ascribed to the fact that J774.A1 has a SPG recognition site called Dectin-1 on the cellular surface and the ternary complex can be ingested by this pathway.

Introduction

Polymeric nanoparticles have been demonstrated to be able to deliver drugs and thus enhance therapeutic efficacy.[1,2] Among others, the small gel particles that can encapsulate biologically active DNAs seem to be a major trend for future chemotherapy.[2] Akiyoshi et al.[3] are the first to demonstrate that cholesterol-bearing pullulan can form a nanosize gel and the gel particle spontaneously encapsulates proteins and other biofunctional compounds. They explained this encapsulation by the distinct hydrophobic domain (or pocket) that is provided by the combination of the hydrophilic polysaccharide and the hydrophobic cholesterol and thus can move dynamically to engulf hydrophobic materials from solutions. Utilizing natural polysaccharides is considered to be a good and new approach.[4,5] This is because natural polysaccharides can biodegrade into nontoxic components, provide solubility,[5] and can be used as a cell-specific ligand.[4]

Meanwhile, Sakurai and Shinkai[6−8] found that a natural polysaccharide called schizophyllan (SPG) forms a complex with polynucleotides, carbon nanotubes, and some hydrophobic polymers. SPG is composed of β-(1→3)-D-glucan main chain and one β-(1→6)-D-glycosyl side chain links to the main chain at every three glucose residues, as presented in Figure 1. In nature, SPG takes the form of a triple helix, and one triple helix can denature to three random coils by dissolving with dimethyl sulfoxide (DMSO) or alkaline solutions (pH > 12). They showed that one major driving force of the complexation ability of SPG is the combination of hydrophobic and hydrogen-bonding interactions. This feature suggests that renatured SPG can provide a hydrophobic domain that is similar to cholesterol-bearing pullulan.[3]

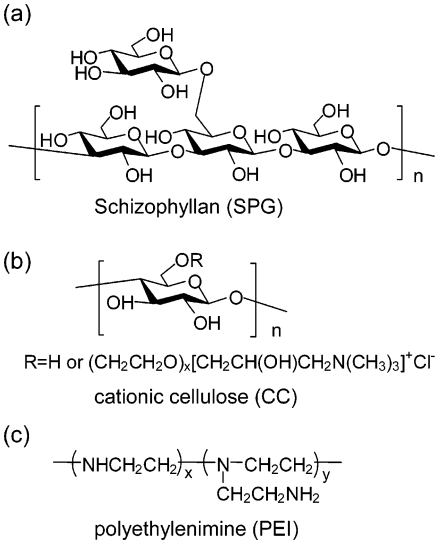

Figure 1. Chemical structures of SPG, CC, and PEI.

Recent work reported that β-1,3-glucans, especially for zymosan and other cell-wall glucans, are recognized by a receptor called Dectin-1[9−11] and are internalized by phagocytosis.[10] Most antigen presenting cells (APCs) have Dectin-1 on

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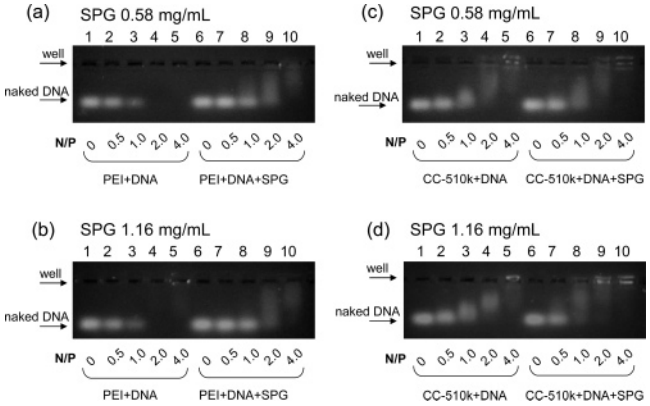

Figure 2. Changes of the gel electrophoresis patterns when N/P ratio was increased, comparing the polycation+DNA systems (lanes 1−5) with the ternary polycation+DNA+SPG mixtures (lanes 6−10).

the surface.[12] These findings indicate that SPG can play a role that can guide the encapsulated material to APCs as well as encapsulate drugs. APCs play a major role of immuno response, and recent studies revealed that oligodeoxynucleotides containing unmethylated CpG sequences (CpG-DNAs) are known as an immune adjuvant to effectively induce Th1 response when CpG-DNAs are engulfed by APCs.[13−16]

Mizu et al.[17] are the first to demonstrate that SPG can form a complex with a CpG-DNA sequence and deliver it to endosome to enhance cytokine secretion. They attached a $(dA)_{40}$ tail to the 3′ end of the CpG-DNA to stabilize the complex, because DNA heterosequences cannot bind to SPG. The key sequence of CpG is 18 bases, and thus the (dA) tail is twice as long as the key sequence. This is considered a drawback for the use of SPG for CpG-DNA delivery. The aim of this paper is to present a novel alternative method by using the hydrophobic pocket provided by renatured SPG.

Materials and Methods

Materials. Mitsui Sugar Co., Ltd. (Japan) kindly supplied SPG. The weight-average molecular weight (M_w) and the number of repeating units were found to be 1.5×10^5 and 231, respectively.[18] The preparation of the rhodamine B-labeled SPG is described elsewhere.[19] Daicel chemical industries Ltd. (Japan) supplied cationic cellulose (CC). M_w of CC was determined with gel permeation chromatography (Shodex OHpak SB-804 and 806M columns) to be 5.1×10^5, and we denoted it by CC-510k. Sonication of CC-510k for 12 h with a SONIFIER 150 (Branson Ultrasonics Co., U.S.A.) lowered M_w to 4.7×10^4, and we denoted it by CC-47k. Branched polyethylenimine (PEI) with 25 KDa was obtained from Sigma-Aldrich. Calf-thymus DNA was obtained from Amersham Biosciences (U.S.A.). The average length of the DNA is 3000 bases. A DNA sequence with 5′- TCC ATG ACG TTC CTG ATG -3′ was used and denoted by CpG-DNA.[20] CpG-DNA and fluorescein isothiocyanate (FITC)-labeled CpG-DNA were synthesized by Hokkaido System Science (Hokkaido, Japan) and purified with high-pressure liquid chromatography. The fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco/BRL. Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. Ltd.

Sample Preparation. A water solution of the polycation sample (CC-510k, CC-47k, or PEI) was added to a DNA/Tris-HCl buffer solution (pH 7.5) to give a DNA/polycation complex. The final concentrations of Tris-HCl and NaCl were 20 and 50 mM, respectively. In the case of preparing DNA/polycation/SPG ternary complexes, there were three possible mixing orders, (1) DNA and SPG are first mixed and then the polycation is added, (2) DNA and the polycation are first mixed, and (3) SPG and polycation are first mixed. The first method was excluded because mixing polynucleotides with SPG at pH > 12 may lead hydrolysis of polynucleotides. For the second method, we found that DNA/polycation ion-complex; which were formed in the initial mixing, was precipitated and thus difficult to mix homogeneously with SPG in the subsequent mixing. The third method in which SPG coexisted with the polycation gave stable and reproducible complexes, comparing with the other methods (refer to the Supporting Information Figure S1). One possible explanation for excellence of the third method is that ionized polysaccharide hydroxyl groups may bind to the cationic groups of the polycation in the alkaline solution before mixing with DNA. In all the experiments, a large amount (980 μL) of the aqueous DNA solution (containing 20 μmol Tris-HCl and 47 μmol NaCl) was added to 10.5 μL of a SPG+polycation mixture in 0.28 M NaOH, followed by adding 0.5 M HCl to adjust pH =7.5. The final concentrations of Tris-HCl and NaCl were 20 and 50 mM, respectively. All the measurements were performed about 24 h after the sample preparation. The N/P ratio was determined as the molar ratio of nitrogen in the polycation to phosphorus in DNA, according to general manners.[21] [Note: DNA/polycation or DNA/polycation/SPG denotes the complexes made from DNA and polycation, or DNA, polycation, and SPG, respectively. In this paper "/" means the complex; PEI/DNA+polysaccharide means the mixture made of the PEI/DNA complex and polysaccharide. PEI/DNA+polysaccharide does not necessarily mean the ternary complex, it can be just a mixture of PEI/DNA and polysaccharide without any interaction.]

Gel Shift Assay and Size Determination. N/P ratio (changing from 0 to 4.0) dependence of the gel electrophoresis migration was carried out in a 2% agarose gel with a TBE buffer solution including GelStar at a voltage of 20 V for 90 min. The concentration of CpG-DNA was always kept at 8.3 μg/mL and that of SPG was 1.16 mg/mL or 0.58 mg/mL. The zeta potential (ζ) and the averaged hydrodynamic radius (R_h) were determined with a Malvern Zetasizer nano (Malvern, U.K.). In this measurement, calf thymus DNA was used.

Microscopy. Transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) images were acquired using a JEOL TEM-2010 (accelerating voltage 200 kV) and a TECNAI-20, FEI (accelerating voltage 200 kV), respectively. Energy dispersive X-ray spectroscopy (EDX) was used to detect P from DNA and to confirm the presence of DNA inside the particle. The sample was placed on 200-mesh carbon coated copper (Cu) grids. The composition for each sample was 9.0:1166 μg/mL for DNA+SPG and 9.0:46.6:1166 μg/mL for the DNA/CC-510k/SPG ternary complex, respectively. The TEM grid was dried under reduced pressure for 12 h before the observation. Confocal laser scanning microscopy (CLSM) was carried out to examine whether rhodamine B-labeled SPG and FITC-labeled CpG-DNA were co-localized when they were mixed in the presence of PEI. The composition for each sample was 9.0:9.4 μg/mL for DNA+PEI, 9.0:583 μg/mL for DNA+SPG, and 9.0:9.4:583 μg/mL for the DNA/PEI/SPG ternary complex, respectively. These samples were dialyzed and dropped onto a glass bottom dish. The observation of these samples was carried out after drying with a vacuum desiccator. The instrumentation and other experimental conditions are described below.

Cell Culture, Confocal Laser Scanning Microscopy, and IL-12 Secretion Assay. Murine macrophage-like cells, J774.A1, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The J774.A1 cells were maintained in DMEM supplemented with 10% FBS. All mediums contained a 1 wt % penicillin and streptomycin mixture. The cell incubation was always carried out at 37 °C in a fully humidified air containing 5 wt % of CO_2. J774.A1 cells were plated at a density of 1.5×10^4 cells/mL in a glass-bottom chamber (Lab-TekII Chembered Coverglass, Nalge Nunc, Rochester, NY). Following addition of each sample to the medium (150 μL), the cells were incubated for 6 h at 37 °C in a 5% CO_2 incubator. The cells were then washed twice with PBS (125 μL), fixed with 5% HCHO (100 μL) at 4 °C for 20 min. The cells were then washed twice with PBS (100 μL) and examined by confocal laser scanning microscopy (CLSM). Images of the samples were collected by CLSM on Nikon TE-2000 attached

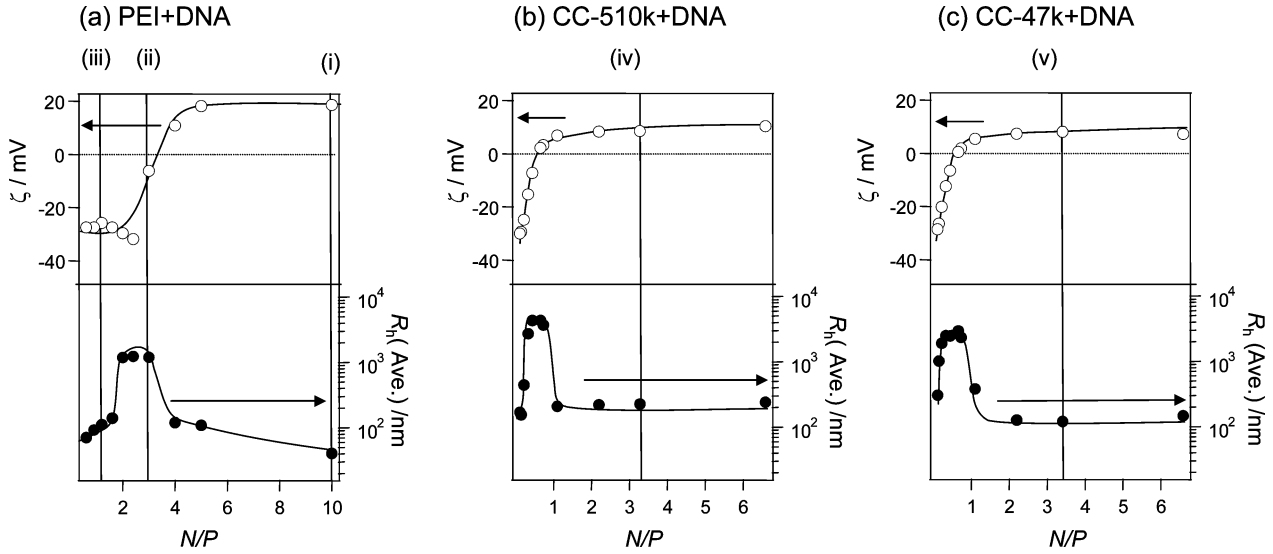

Figure 3. N/P dependence of the zeta potential (ζ) and the averaged hydrodynamic radius (R_h) for PEI+DNA (a), CC-510k+DNA (b), and CC-47k (c). At the compositions from i−v, we added polysaccharide to observe ζ and R_h as presented in Figures 4−6.

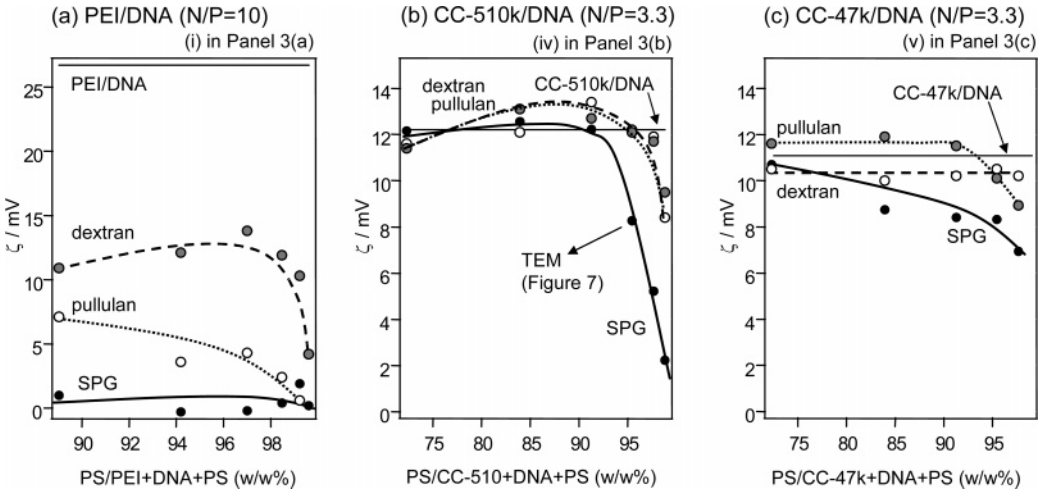

Figure 4. Polysaccharide composition (PS%) dependence of ζ for the ternary mixtures, comparing with the PEI/DNA (a), CC-510k/DNA (b), and CC-47k (c) systems.

with a confocal scan unit Radiance 2100 system (BioRad) using a 40× oil immersion objective. The excitation with filter set at 488 nm was suitable for observation of FITC-DNA emission. The excitation with filter set at 543 nm allowed detecting the fluorescence of the rhodamine B-labeled SPG. IL-12 secretion induced by exposing CpG-DNA was measured with a commercially available ELISA kit according to the instructions of the manufacturer (Endogene), after the cells were incubated at 37 °C for 48 h. The experimental details were described elsewhere.[17]

For CLSM, N/P was fixed at 3.3 for CC-510k system and at 10 for PEI system, and the concentrations for each sample were as follows: DNA, 1.0 μg/mL; DNA+SPG, 146.8 μg/mL (1:5.3 in molar ratio); CC-510k+DNA, 6.8 μg/mL (5.8 μg/ mL, 1.0 μg/mL); CC-510k+DNA+SPG, 6.8 μg/mL (5.8 μg/mL, 1.0 μg/mL, 145.8 μg/mL); PEI+DNA, 2.0 μg/mL (1.0 μg/mL, 1.0 μg/mL); and PEI+DNA+SPG, 147.8 μg/mL (1.0 μg/mL, 1.0 μg/mL, 145.8 μg/mL). Except for DNA alone and PEI+DNA+SPG, all samples had a positive ζ. We chose these compositions in order to compare between cation-induced and Dectin-1 mediated cellular uptakes. For IL-12 secretion assay, we used the samples with $\zeta = 0$ and SPG-complexed sample because we intended to elucidate cytokine secretion only induced by Dectin-1 mediated cellular entry. The composition for each sample was 72:31 μg/mL for DNA/PEI, 72:583 μg/mL for DNA+SPG, and 72:31:583 μg/mL for the DNA/PEI/SPG ternary complex, respectively. These samples were concentrated 10-fold by centrifugation in microcon and added to J774.A1 cell cultures. The final concentration of DNA was 72 μg/mL. Other experimental conditions are described elsewhere.[17]

Results and Discussion

Gel Shift Assay. Figure 2 shows how the gel electrophoresis pattern changed when SPG (0.58 or 1.16 mg/mL) was present in PEI+DNA mixtures (Figure 2a,b) or CC-510k+DNA mixtures (Figure 2c,d). The well positions and the naked-DNA bands are indicated by arrows. As shown in Figure 2a, with increasing N/P, the amount of free DNA decreased and finally disappeared at N/P = 4.0. Generally, PEI strongly binds to DNA to lead its conformation in highly compact forms. This compact PEI/DNA complex cannot be stained with GelStar or be interfered with by intercalaters.[22] This is the reason that there was no band at the large N/P ratios of lanes 4 and 5. When 0.58 mg/mL of SPG was present in the PEI+DNA mixture [0, 0.59, 1.18, 2.36, 4.72 μg/mL for PEI, lanes 6−10 in the Figure 2a], a broad DNA band was observed at an N/P ratio of 2.0 or 4.0 (lanes 9 and 10). This feature is quite in contrast with that of lanes 4 and 5. It seems that SPG interrupted the formation of the compact PEI/DNA complex. When the added amount of SPG was increased (Figure 2b), similar results were obtained, confirming the conclusion obtained in Figure2a.

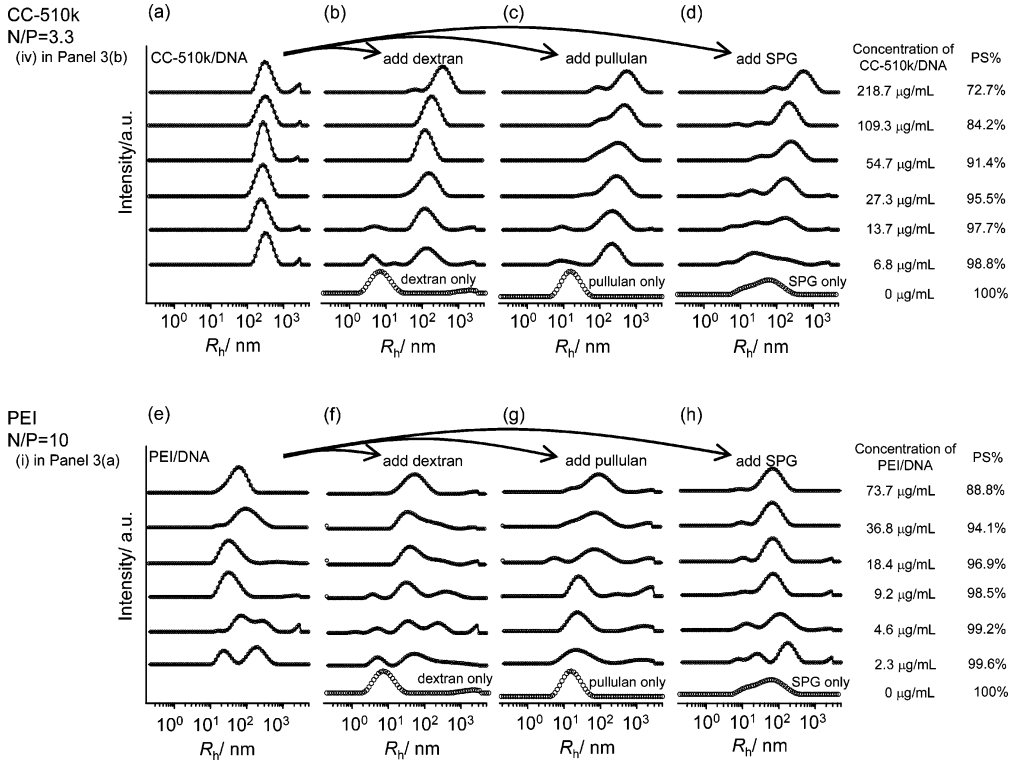

Figure 5. Changes of the particle size distribution upon mixing with each polysaccharide for CC-510k/DNA (N/P = 3.3) and PEI/DNA (N/P = 10). Each curve lining up sideways at the same height has the same polycation/DNA concentration as presented at the right side.

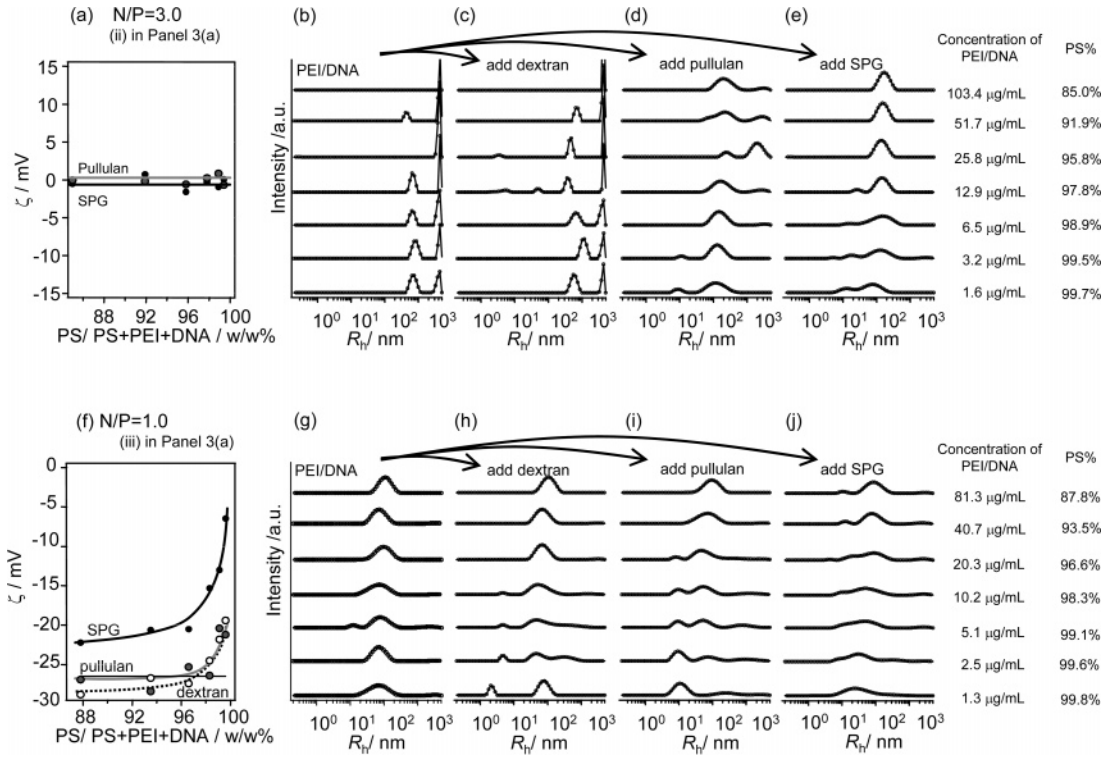

Figure 6. Polysaccharide composition (PS%) dependence of ζ for the ternary mixtures and changes of the particle size distribution upon mixing with each polysaccharide for the PEI/DNA systems at N/P = 3.0 and 1.0. Each curve lining up sideways at the same height has the same PEI/DNA concentration as presented at the right side.

When CC-510k was used instead of PEI [lanes 1−5 in Figure 2c,d], the complexation did not cause DNA to become invisible, indicating that the CC-510k/DNA complex is less compact compared with PEI/DNA. This feature can be ascribed to the structural difference between CC and PEI, i.e., CC has a positive nitrogen atom per 25 carbons in the repeating unit (4%), and on the other hand, PEI has a positive nitrogen atom per 2 carbons in the repeating unit (33%). The atoms other than the positive nitrogen should sterically interrupt the ion pair formation and the aggregation. Therefore, CC should form a less compact aggregate than PEI.

With increasing the N/P ratio in the CC-510k+DNA+SPG system, the migration band became smear and finally most DNA stayed at the well at N/P = 4.0. When lane 9 (N/P = 2.0) in

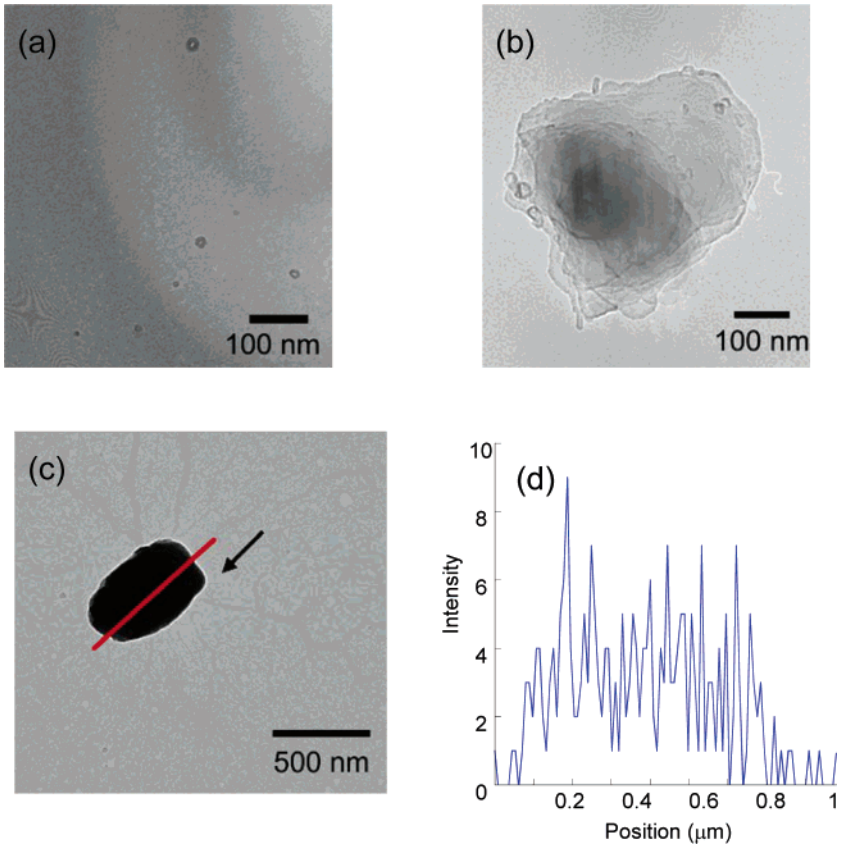

Figure 7. TEM images for a DNA and SPG mixture (a) and DNA/CC-510k/SPG ternary complex (b). The EDX line (c) scan was presented in panel d for P.

panels c and d of Figure 2 were compared, it seems that most DNA is present at the well for 1.16 mg/mL; on the other hand, there is a broad band observed for 0.58 mg/mL addition. This means that the presence of SPG influences the migration pattern again, suggesting that SPG can interfere with the CC-510k/DNA complex. This conclusion is confirmed by the comparison between the lanes 5 and 10, where more DNA is present at the well when SPG is present.

Zeta Potential and Particle Size. Figure 3 shows N/P dependence ζ and R_h when PEI or CC was mixed with DNA. R_h reached the maximum at N/P = 2.4 for the PEI+DNA mixture and at N/P = 0.7 for the CC system. The zeta potential increased continually from negative to positive values with increasing N/P. When ζ comes to zero, R_h gives the maximum for all the systems, indicating that ion-pair formations and the resulting electrical cancellation caused large aggregates.[23] The maximum R_h value of the CC system is almost twice as large as that of the PEI system.

Since all of the phosphodiesters of DNA are deprotonated in neutral solutions, the N/P ratio at $\zeta = 0$ should be related to the molar ratio of cationized amino groups in CC. In the case of CC, N/P = 0.7 gives $\zeta = 0$ corresponding to all of the amines being positively charged as presented in the chemical structure (see Figure 1). For PEI, when N/P = 2.4 and $\zeta = 0$, it is suggested that some of the main chain amines are not protonated.[23,24]

For the PEI+DNA system, the maximum value of R_h is 5 μm. With increasing the N/P ratio, R_h rapidly decreases and stays at R_h = 90−120 nm for N/P > 4, confirming the former studies.[25] This decrease can be explained by the fact that further addition of anions provides solubility and thus the size of the aggregate becomes smaller. When CC was added to DNA, the maximum value of R_h is about twice as large as that of PEI/DNA complexes, being consistent that CC/DNA complexes form less compact aggregate than PEI/DNA complexes.

Formation of Ternary Complexes Observed with DLS. Figure 4 shows the polysaccharide composition (PS%) dependence of ζ for the ternary mixtures, where we used SPG, pullulan, or dextran as the polysaccharide. Here N/P was fixed at 3.3 for the CC systems and at 10 for the PEI system. The reason that we used the different N/P is that we wanted to compare the three complexes at almost the same ζ values. For convenience the plots were separated into three panels: a, b, and c for the PEI, CC-510k, and CC-47k ternary mixtures, respectively. In these experiments, we always kept the polysaccharide concentration at 583 μg/mL. PS% was defined by the weight percent of the added polysaccharide in the total amount. Dextran was used for comparison because it is highly hydrophilic and thus hydrophobic pockets are hardly formed in aqueous solutions. The solid line in each panel indicates the value of ζ for the corresponding DNA/polycation complex alone.

In panel b in Figure 4 with increasing the SPG PS%, ζ decreased drastically to reach almost $\zeta = 0$ at PS% > 98%. This drastic drop was not observed when dextran or pullulan was added. This decrease of ζ for SPG can be explained by assuming that the hydrophobic pocket of SPG should engulf the partially hydrophobic and positively charged complex and thus ζ became almost zero. When we carried out the same experiments for the CC-47k+DNA system in Figure 4c, there was no drastic decrease in ζ, the three polysaccharides showed similar behaviors, and ζ values stayed at the almost same values as those of the binary mixture of CC-47k+DNA. These facts suggest that the formation of the ternary complex requires a certain molecular weight. The similar molecular weight

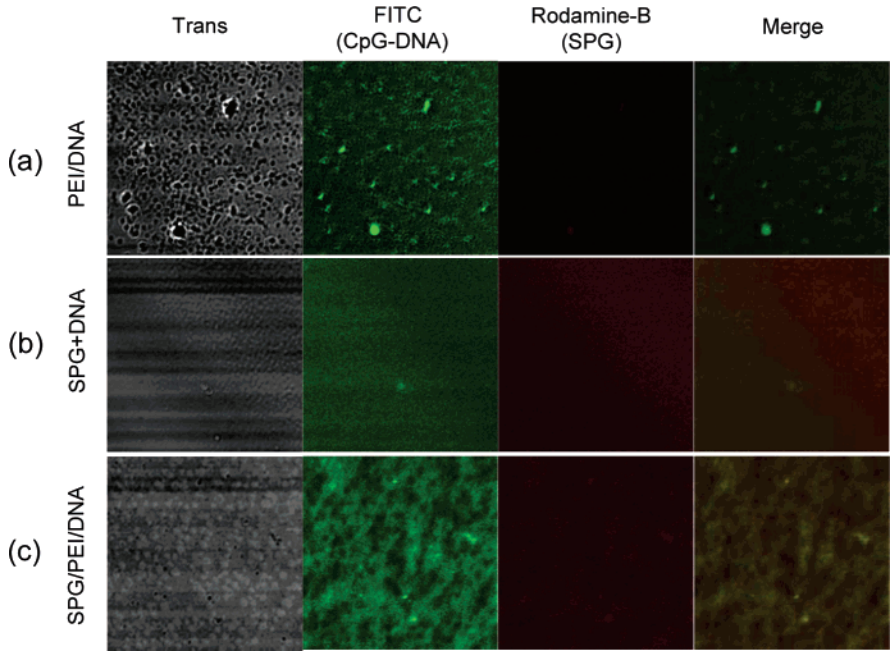

Figure 8. Confocal laser scanning microscopy images for PEI/DNA (a), SPG+DNA (b), and SPG/PEI/DNA (c).

effect was observed when we used a hydrolytically cleaved SPG (M_w = 33 000) instead of the present SPG in the Figure 4a experiment. (See Figure S1 in the Supporting Information.)

Figure 4a shows the PS% dependence of ζ when PEI was used as the polycation. The three polysaccharides showed lower ζ values than that of PEI+DNA complex, suggesting that the PEI+DNA complex can interact with not only SPG but also dextran and pullulan. It is interesting that the SPG system showed the lowest value and it was almost 0 mV for PS% > 80%, and pullulan comes in second. The results in Figure 4 lead the following conclusions: (1) PEI/DNA complexes tend to bind to the polysaccharides better than that of CC/DNA. (2) In the three polysaccharides, SPG is easiest to form a ternary complex. We do not have any explanation why the addition of dextran decreased ζ at this moment.

Panels a−d in Figure 5 compare the particle size distribution when one of the polysaccharides was present in the CC-510k/DNA complex, corresponding to N/P = 3.3, i.e., (iv) in Figure 3b. The CC-510k/DNA concentrations are indicated at the right side, and Figure 5a shows the distribution of only CC-510k/DNA. Panels b, c, and d show how the distributions were changed when we added dexran, pullulan, or SPG, respectively. The compositions of the added polysaccharide are indicated for each curve. It should be noted that each curve lining up sideways has the same CC-510k+DNA concentration as presented at the right side.

When the amount of pullulan (c) or dextran (b) was increased, the peak at R_h = 1000−500 nm (large particles) never disappeared even at PS% = 99%. In the range of PS% > 95%, there was a peak for the smaller particles around R_h = 10 nm, probably corresponding to the non-interacting dextran and pullulan. When the amount of SPG was increased (d), the larger particle peak was almost disappeared at PS% = 97.7%, confirming that SPG is easiest to form the ternary complexes. Figure 5e−h shows plots similar to those of Figure 5a−d, except for using PEI and N/P = 10. The presence of pullulan induced the most drastic changes in the distribution than the others, suggesting that DNA/PEI complexes were ingested by pullulan. Pullulan is a water soluble polysaccharide composed of maltotriose (α-1,4 linkage) units linked by α-1,6 glucosyl bonds. Unlike SPG, pullulan does not form triple helices and thus the encapsulation mechanism is not the same as SPG. Nakatani et al.[26] showed that amylose and pullulan enhanced fluorescence of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) in aqueous solution, while dextran scarcely enhanced. They ascribed this fluorescence enhancement to presence of hydrophobic domains. It is well-known that amylose has a hydrophobic domain and includes hydrophobic substance. We assume that the pullulan formed the hydrophobic space similar to amylose.

We do not have a reasonable explanation why pullulan showed more dramatic changes than that of SPG for the case of PEI. As presented in Figure 5e, the concentration dependence of the particle distribution for PEI/DNA shows rather complicated behavior, and presumably this complexity causes ambiguity in interpreting the data of Figure 5f−h.

Figure 6a shows the PS% dependence of ζ at N/P = 3.0 for the PEI system, and Figure 6b−e shows how the particle size distributions were changed by adding each polysaccharide. At this composition, the charge of PEI and DNA were neutralized with each other. The PEI/DNA and PEI/DNA+dextran systems showed presence of very large particles more than 5 μm. Although the plots were obtained, the reliability of the data is poor because the sizes of large particles are beyond the measuring limit of our instrument. It is interesting that when pullulan or SPG was present in the system, the micrometer-scale large particles disappeared and the particle size became smaller with increasing PS%. These features are consistent with the previous conclusion that SPG and pullulan easily form the ternary complex. Figure 6f shows the PS% dependence of ζ at N/P = 1.0 for the PEI system, where ζ is negative because of DNA-rich composition. Figure 6g−j shows how the particle size distributions were changed by adding each polysaccharide; again, SPG and pullulan induced the drastic changes in the distribution. Figure 6 shows that the ternary complex can be formed for the entire range of ζ and the order of tendency is SPG > pullulan ≫ dextran. This order equals the order for water solubility of the polysaccharides, in other words, the order of the ability to form hydrophobic pockets.

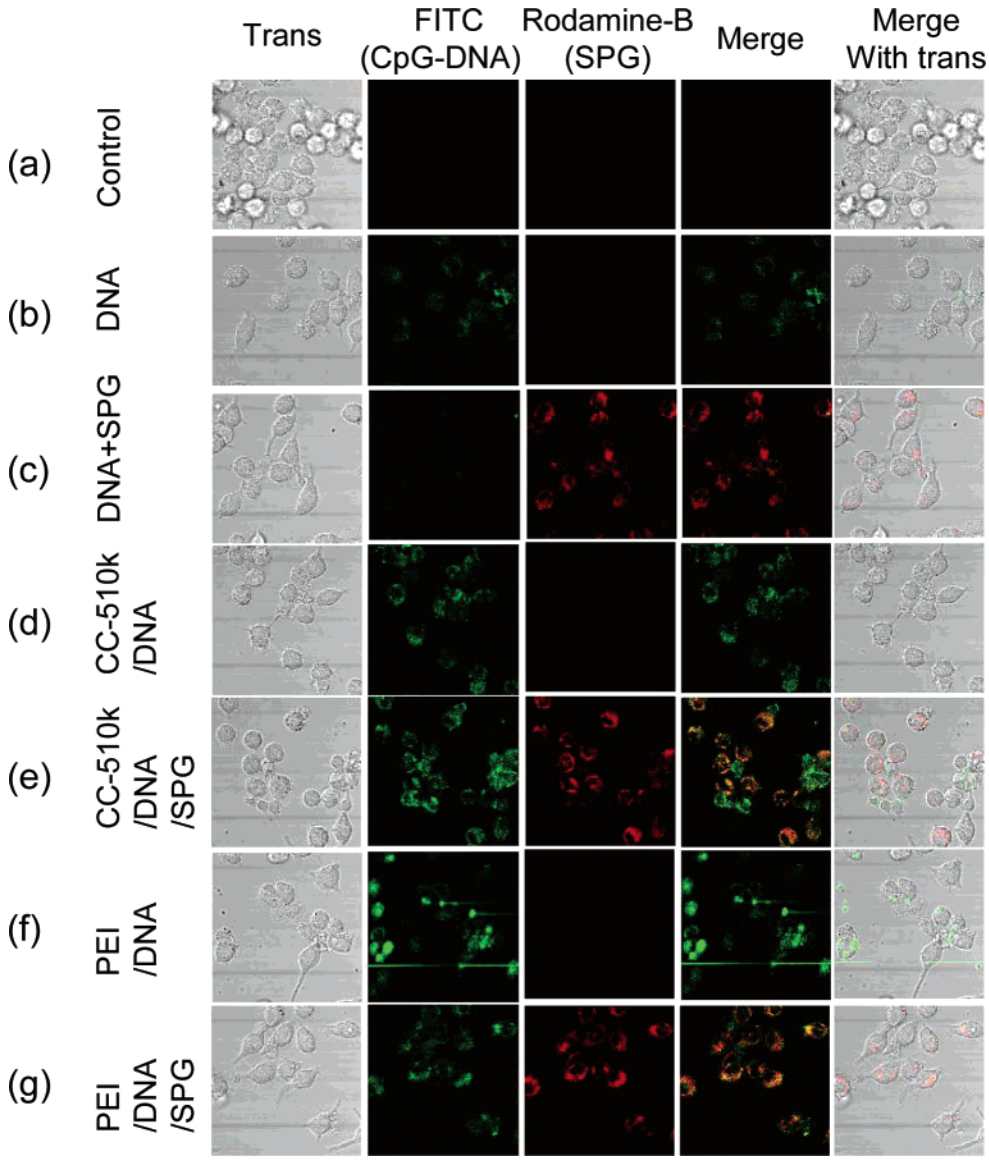

Figure 9. Confocal laser scanning microscopy images for J774.A1 to examine the cellular ingestion ability of the polycation/DNA/SPG ternary complexes.

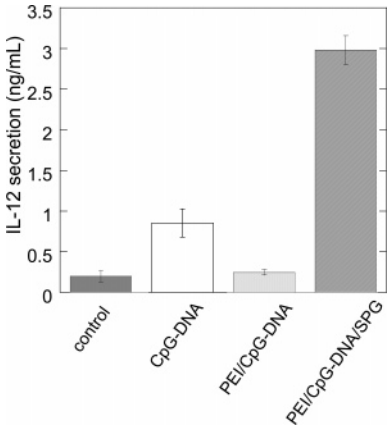

Figure 10. Comparison of IL-12 secretion after administrated naked CpG-DNA, PEI/CpG-DNA, and PEI/CpG-DNA/SPG.

TEM and CLSM To Support Formation of the Ternary Complex. Figure 7a,b shows typical TEM images of a DNA and SPG mixture (a) and a DNA/CC-510k/SPG ternary complex (b), respectively. For comparison, we carried out TEM observation for a DNA/CC-510k complex; however, we always observed particles that were too large to obtain clear TEM images (Panel d in Figure S3 of the Supporting Information). The DNA+SPG mixture always gave very small particles less than 10 nm; on the other hand, when we added CC-510k to this solution, the large particle appeared as presented in Figure 7b. One may think that this particle consists of only DNA and CC-510k; however, we never observed this size of particles for DNA/CC-510k complexes. These observations are consistent with Figure 5d. Therefore, we assume that this particle is the ternary complex. For EDX measurements, we selected a large particle such as that in Figure 7c, because a size of several hundred nanometers was necessary to measure a EDX line scan. In Figure 7c, the solid line presents the cross-section where the EDX line scan was obtained. Figure 7d presents the profiles for P atoms, showing that P atoms were concentrated in the particle. The presence of P atoms is attributed to DNA, confirming the inclusion of DNA into the ternary complex.

Figure 8 compares CLMS images for PEI/DNA, SPG+DNA, and SPG/DNA/PEI, where FITC-labeled CpG-DNA and rhodamine B-labeled SPG were used. These samples were dialyzed in order to avoid crystallization of salts. In order to obtain a particle large enough to give CLMS images (normally

at least 200 nm), we prepared the samples at considerably high concentrations with vacuum drying. PEI/DNA showed green color because only PEI/DNA complexed particles were present. In the images for SPG+DNA, both components are water soluble and thus no particles were observed. When the three components were present, the DNA/PEI formed a different shape from that of PEI/DNA and SPG present at the same position as PEI/ DNA, as indicated in the yellow dots in the merged images. This CLMS experiment supports the formation of the ternary complex as well as TEM does.

Cellular Uptake of CpG-DNA through the Ternary Complex. As mentioned in the Introduction, to deliver CpG-DNAs to intracellular endosome and/or lysosome, there are two major issues to overcome: instability of CpG-DNA in biological fluids and low uptake efficiency into cells. Encapsulation with SPG is considered to dissolve these two issues because the bound CpG-DNAs should be protected form enzymatic degradation[27] and SPG can be a leading material to macrophages. We carried out a preliminary assay to examine the cellular ingestion ability and its biological response by use of CLMS (Figure 9) and IL-12 secretion assay (Figure 10). As mentioned in Materials and Methods, N/P was fixed at 3.3 for CC-510k system and at 10 for PEI system for CLSM. Except for DNA alone and PEI+DNA+SPG, all samples had a positive ζ.

In Figure 9, FITC-labeled CpG-DNA and rhodamine B-labeled SPG were used, and thus the green and red colors indicate the localized place of DNA and SPG, respectively. Therefore, when CpG-DNA and SPG coexist, these parts become yellow after superimposition. We hardly observed uptake of naked CpG-DNA (b). When the cells were exposed to CpG-DNA+SPG mixture (c), uptake of SPG was observed. However, when CC-510k/DNA complex is added, the uptake of DNA into cells was detected because the CC-510k/DNA complex should have a positive charge favorable for ingestion. The CC-510k/CpG-DNA/SPG ternary (e) complex showed rather high uptake efficiency. This enhancement should be ascribed to Dectin-1 promoting endocytosis as described in our paper.[28] There are some cells colored in green as well as yellow in Figure 8e, indicating uptake of CpG-DNA/CC-510k uncomplexed with SPG. The PEI/CpG-DNA complex showed large aggregates, and the strong green dots are owing to the aggregate adsorbed on the dish glass-bottom. On the other hand, the PEI/CpG-DNA/SPG ternary complex did not show such aggregates. Most cells are yellow, indicating that most CpG-DNAs were engulfed into the cell as the ternary complex. The ζ values of the PEI/CpG-DNA/SPG ternary is zero (see Figure 4a), and thus the cellular ingestion should be ascribed to Dectin-1. It is considerably interesting that the amount of the ingestion of DNA is almost the same as that of CC-510k+DNA in which the interactions between polycations and positively charged cellular surface should play a major role in cellular entry.

Figure 10 compares IL-12 secretion level between naked CpG-DNA, PEI/CpG-DNA, and PEI/CpG-DNA/SPG complexes. It should be noted that N/P was fixed at 3.0 in this experiment, and thus ζ values for both PEI/CpG-DNA and PEI/CpG-DNA/SPG were zero (see Figure 6). The PEI/CpG-DNA gave the lowest secretion, almost the same as that of the control. This is because compacted PEI/CpG-DNA should hardly release the bound CpG-DNA, and the neutralized charge had no ability to enter the cells. Furthermore, the smaller size of the PEI/CpG-DNA/SPG complex is probably better for ingestion than the larger PEI/CpG-DNA complex (see Figure 6a). It is interesting that the order of the IL-12 secretion is the reverse of the ingestion level measured with microscopy (Figure 8). The PEI/CpG-DNA/SPG gave the highest secretion, which should be ascribed to enhanced uptake due to Dectin-1 and the less compact state of CpG-DNA in the complex (see Figure 2). Here, we should emphasize that the CpG-DNA used in this work has no poly(dA) tail. The present vitro assay proves that the polycation/CpG-DNA/SPG complex has great advantage to deliver functional oligo-DNAs to APCs.

For the CC-510k system, we could carry out the same assay; however, we should add a large amount of SPG to get $\zeta = 0$ (see Figure 4). This composition is a disadvantage for CpG-DNA delivery. We carried out the same experiments for positively charged PEI/CpG-DNA or CC-510k/CpG-DNA and their ternary complexes and found that there was no appreciable difference between the polycation/DNA complexes and the ternary ones. This is presumably because the Dectin-1 mediated entry was overwhelmed by the polycations induced one. Since it is well-known that polycations induced cellular entry is not applicable to in vivo owing to its nonspecificity, we believe that the present PEI/CpG-DNA/SPG is well positioned to be applied to in vivo.

Conclusions

This paper demonstrated that SPG can interact with CC/DNA or PEI/DNA based on agarose gel electrophoresis, DLS, and TEM. CLSM revealed that the CC/CpG-DNA/SPG and PEI/CpG-DNA/SPG ternary complexes showed high uptake efficiency. These results indicate that the hydrophobic pocket of SPG engulfs the partially hydrophobic and positively or negatively charged complex composed of polycation and DNA. Finally, the vitro assay showed that the PEI/CpG-DNA/SPG complex induced the most IL secretion, proving our idea works well. The present method should open a new approach to design effective gene carriers.

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Supporting Information Available. Additional figures and information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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