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Unmethylated CpG oligodeoxynucleotide induces inflammatory immune responses through cytokine production and has attracted increasing attention as an immunostimulator. However, there remains a challenging issue for the use of 'native CpG DNA'. In the present study, we prepared cationic nanometer-sized gels (nanogels) consisting of cycloamylose modified with cholesterol and diethylaminoethane to form hydrophobic cross-linking points and to add positively charge groups, respectively. The cationic nanogels and native CpG DNA formed nanometer-sized complexes. Complexes of native CpG DNA with cationic nanogels delivered native CpG DNA to macrophage-like cells and induced cytokine production. In addition, complexes of negative control oligonucleotides with cationic nanogels did not induce cytokine production, and the induction of cytokines using complexes of phosphorothioate-modified CpG with cationic nanogels was lower than that of native CpG DNA. These results suggest that the complex of native CpG DNA with cationic nanogels is a promising strategy for nucleic acid adjuvants.

Introduction

Unmethylated CpG DNA sequences that are preserved in bacterial genomic DNAs and many viral DNAs activate the vertebrate immune system.[1] Immune responses using bacterial extracts of attenuated mycobacteria Bacillus Calmette Guerin (BCG) have been studied since they were introduced as efficient agents for human cancer treatment more than a century ago. In the 1980s, Tokunaga et al. reported an immunostimulatory effect of BCG extracts for activating natural killer (NK) cells and tumor regression in mice was induced by the DNA fraction of the extracts. Purified BCG DNA enhances NK cell activity and the production of type 1 and type 2 interferons in vitro.[2] Further investigation by Krieg et al. confirmed the essential role of flanking bases around the CpG dinucleotides for immune stimulation, which conformed to the general consensus motif of XCGY, where X is any base except C, and Y is any base except G.[3] This concept was expanded to synthetic oligodeoxynucleotides containing unmethylated CpG DNA sequences, which also stimulate the innate immune system and are commercially available at present. Regarding the mechanism of immunostimulation, CpG DNA is first internalized by antigen-presenting cells, such as dendritic cells, macrophages, or B-cells, and transported through the endocytosis pathway. In endocytic vesicles, CpG DNA is recognized by Toll-like receptor 9, which induces the secretion of cytokines that widely activate both innate and adaptive immune systems.[4]

CpG DNA has been applied to adjuvants for vaccines against infectious pathogens, allergens and cancers in clinical trials. To retard degradation by nucleases within organisms, CpG DNA is generally modified with a phosphorothioate backbone (PS-CpG DNA), in which one of the non-bridging oxygen atoms at each of the phosphodiester linkages in CpG DNA is replaced with a sulfur molecule (**Fig. 1A**), thus, native CpG DNA is not used widely.[1] PS-CpG DNA can enhance nuclease-resistance and is commonly used for clinical research. However, recent studies showed that PS-CpG DNA caused unexpected side effects related to the PS-back bone, such as production of anti-PS-CpG DNA antibodies.[5] Therefore, it is desirable to develop a novel delivery system for native CpG DNA.

We reported that nanogels, nanometer-sized gels, consisting of self-assembled polysaccharides, have a high potential as novel carriers of bio-pharmaceuticals such as peptides, proteins, small interfering RNA and plasmid DNA. Nanogels have attracted growing interest for use as drug delivery systems.[6] We prepared physically cross-linked nanogels consisting of associated amphiphilic polysaccharides. For example, cholesterol-bearing pullulan (CHP) forms a stable nanogel in water by self-assembly through hydrophobic interactions of cholesterol groups.[7] The nanogel can trap proteins inside the polymer networks and assist protein refolding by molecular chaperone-like activity.[6] In addition, a novel cancer immunotherapy was developed using CHP nanogels complexed with cancer antigen.[8] We also prepared cationic CHP nanogels with amino groups that acted as a nanocarrier for intracellular protein delivery and for use in adjuvant-free intranasal vaccination.[9] Recently, we reported that cationic cycloamylose (CA) performed as an efficient carrier for nucleic acid delivery. CA is a cyclic α-1,4-linked polysaccharide including multiple hydrophobic cavity sites and a hydrophilic outer surface.[10] We prepared CA modified with spermine, a biogenic polyamine, which formed a complex with small interfering RNA[11] and plasmid DNA[12], which were effective at inducing gene silencing or higher gene expression than cationic linear structured amylose, respectively. We also

reported a novel gene delivery system using hexadecyl group-bearing cationic CA nanogels and a phospholipid-degrading enzyme.[13]

In this study, a novel cationic CA nanogels consisting of cholesterol (CH) and diethylaminoethane (DEAE) group-modified CA (CH-CA-DEAE) was synthesized (**Fig. 1B**), and used as a carrier for native CpG DNA (**Fig. 1C**). Recently, we reported cationic nanoballs consisting of enzymatically synthesized glycogen modified with cholesterol and DEAE groups (CH-ESG-DEAE), which effectively delivered proteins into cells.[14] The DEAE group continues to be widely used as a positively charged group in matrix gel beads of ion exchange column chromatography and locks the negatively charged materials such as nucleic acids, because the DEAE group contains a tertiary amine and has a stable positive charge at neutral and acidic pHs. Before amine groups with low pKa, such as polyethylenimine, were recognized as important groups for endosome escape, DEAE modified dextran was commonly used as a gene transfection carrier.[15] For CpG DNA delivery, endosome escape is not required and smaller changes in the positive charge of cationic carriers is preferred against the acidic conditions in endosomes. We confirmed the complex of native CpG DNA with CH-CA-DEAE nanogels by agarose gel electrophoresis, dynamic light scattering and zeta potential measurements. Subsequently, the cellular uptake of native CpG DNA using CH-CA-DEAE nanogels was investigated by confocal laser scanning microscopy. Finally, biological responses of the complex of native CpG DNA with CH-CA-DEAE nanogels were evaluated by cytokine secretion assay.

Experimental

Materials

CA (Mn = 1.9×10^4 g/mol, Mw/Mn =1.08) was kindly provided by Ezaki Glico Co., Ltd. (Osaka, Japan). Diethylethylenediamine, 1,1'-carbonyl diimidazole (CDI) and branched polyethylenimine (PEI, Mw = 2.5×10^4 g/mol) were purchased from Sigma-Aldrich Co. (St. Louis, Mo). Cholesteryl N-(6-isocyanatohexyl) carbamate was purchased from NOF Co. (Tokyo, Japan). Native CpG DNA (5'-TCC ATG ACG TTC CTG ATG CT- 3'), 5'-TAMRA-labeled CpG DNA, negative control oligonucleotides (GpC DNA; 5'- TCC ATG AGC TTC CTG ATG CT- 3') and phosphorothioate-modified CpG DNA (PS-CpG DNA) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). Tris(hydroxymethyl)aminomethane (Tris)-acetate-ethylenediaminetetraacetic acid (EDTA) buffer, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, Minimum Essential Media (MEM) and Non-Essential Amino Acids Solution (NEAA) were purchased from Life Technologies Co., (Carlsbad, CA).

Synthesis of CA-DEAE and CH-CA-DEAE

DEAE was introduced to the hydroxyl groups of CA by conventional CDI activation.[12] Briefly, CA (0.893 g; 5.50 mmol) was dissolved in 10 mL of dry dimethylsulfoxide (DMSO), and CDI (624 mg, 3.85 mmol) was added to the mixture under dried nitrogen. After the solution was stirred for 5 h, diethylethylenediamine (5.52 mL, 38.5 mmol) was added to the mixture at room temperature. After stirring for 24 h, the reaction mixture was dialyzed with the Spectra/Pore 6 dialysis bag (molecular weight cutoff 2000; Spectrum Laboratories Inc., Rancho Dominguez, CA), using distilled water for 5 days at room temperature, and lyophilized to yield DEAE-bearing CA (CA-DEAE). Subsequently, CH-CA-DEAE was synthesized as follows: CA-DEAE in dry DMSO was reacted with cholesteryl N-(6-isocyanatohexyl) carbamate in dry pyridine at 70°C for 8 h. The reaction solution was dropped into excess ether/ethanol (95/5, v/v), and the precipitates were washed with ether/ethanol. The precipitates were dissolved in DMSO, dialyzed against distilled water, and lyophilized to yield CH-CA-DEAE. The degree of substitution of the cholesterol group was determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (500 MHz; solvent, DMSO-d_6/D_2O 9/1 (v/v)).

Complex formation of native CpG DNA with cationic carriers

Cationic carriers (2 mg/mL) were suspended in Dulbecco's phosphate-buffered saline (PBS, pH 7.4) and sonicated for 15 min with a sonicator probe. The suspensions were filtered through a Millipore filter (pore size: 0.22 μm). Native CpG DNA solution (110 μg/mL in PBS) was added to an equal amount of cationic carrier solution, in which the cationic carriers were adjusted at various C/P ratios (number of cationic groups in cationic carriers/number of phosphate groups in the native CpG DNA) in PBS. The complex solutions were kept for 15 min at room temperature. The final concentration of native CpG DNA was adjusted to 55 μg/mL in PBS.

Agarose gel electrophoresis

The complex solutions were loaded on 4.0% (w/v) agarose gels prepared from Tris-acetate-edta (TAE, 40 mM Tris-acetate, 1 mM EDTA) buffer. Electrophoresis was performed for 30 min at 100 V, and the DNA bands were visualized by staining gels with GelStar® Nucleic Acid Gel Stain (Lonza Group Ltd., Basel, Switzerland) in TAE buffer for 20 min at room temperature. Electrophoresis profiles were observed by BioDoc-It® Imaging System (UVP, Upland, CA). For nuclease-resistance assay, the samples were treated with DNase I (TAKARA Biotechnology Co., Ltd., Shiga, Japan) for 4 hours at 37 °C, followed by agarose gel electrophoresis. For release profile assay, native CpG DNA and PS-CpG DNA was dissociated from CH-CA-DEAE/CpG (C/P=8) using heparin (0-100 μg/mL) for 15 min at 37 °C, followed by agarose gel electrophoresis.

Particle sizes and zeta potentials

The particle sizes and zeta potentials of the complexes of native CpG DNA with cationic carriers were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The hydrodynamic diameter of the particles was determined by dynamic light scattering (DLS). The detector angles were 173° for DLS and 90° for zeta potentials at 25°C.

Cell culture

J774A.1 and RAW264.7 (RCB0535) cell lines were provided by Health Science Research Resources Bank (Tokyo, Japan) and RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (Tsukuba, Japan), respectively. J774A.1 cells were cultured in DMEM containing 10% FBS and 1% antibiotics. RAW264.7 cells were cultured in MEM containing 10% FBS, 1% antibiotics and 1% NEAA. Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cytotoxicity

The macrophage-like cell cells (J774A.1 or RAW264.7) were harvested in a 96-well plate at an initial density of 1.0×10^4 cells/well and cultured in 100 μL media for 15 h. Then, 10 μL of each sample was added to each well. The final concentration of DNA was 5 μg/mL and the concentration of cationic carriers was adjusted to various C/P ratios. After incubation for 24 h, 10 μL of WST-8

reagent (Dojindo, Kumamoto, Japan) was added to each well. After further incubation, the absorbance at 450 nm derived from WST-8 formazan was measured and expressed as a percentage relative to non-treated cells.

Confocal laser scanning microscopy

To observe the intracellular localization of native CpG DNA delivered by cationic carriers, complexes were prepared using TAMRA-labeled CpG DNA. Cells were harvested in an IWAKI glass-bottom dish (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at an initial density of 2.0×10^4 cells/well and cultured in 1 mL media for 15 h. Then, 100 μL of each sample was added to each well. The final concentration of DNA was 5 μg/mL. After incubation for 4 h, the culture medium was removed, the cells were washed twice with PBS and fresh medium was added. The cells were visualized by confocal laser scanning microscopy (CLSM; LSM510META, Carl Zeiss, Germany) with excitation by He-Ne laser (543 nm).

Enzyme-Linked Immunosorbent Assay

Cells were harvested in a 96-well plate at an initial density of 5.0×10^4 cells/well and cultured in 100 μL media for 15 h. Then, 10 μL of each sample was added to each well. The final concentration of DNA was 5 μg/mL. After incubation for 24 h, the supernatants were collected and the concentration of IL-12 was determined using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Pierce Endogen Inc., Rockford, IL).

Results and discussion

Complex formation of native CpG DNA with cationic nanogels

In this study, cholesterol and cationic group modified cycloamylose (CH-CA-DEAE), was used as a carrier of native CpG DNA. The synthetic scheme of CA-DEAE and CH-CA-DEAE is shown in **Fig. 1B**. The DEAE groups were attached to CA by conventional CDI activation. The substitution degree of DEAE groups in CA-DEAE was 66 in 100 glucose units as measured by ^{1}H-NMR. The CA-DEAE was reacted with cholesteryl N-(6-isocyanatohexyl)carbamate by isocyanate chemistry. The substitution degree of cholesterol groups in CH-CA-DEAE was 3.2 in 100 glucose units. The particle sizes of CA-DEAE and CH-CA-DEAE were determined by DLS. The sizes of CA-DEAE and CH-CA-DEAE were 7.55 ± 0.01 nm and 25.8 ± 0.6 nm, respectively. This result suggested that the cholesterol group of CH-CA-DEAE formed physical cross-linking points by hydrophobic interactions and associated to form CH-CA-DEAE nanogels.

CA-DEAE or CH-CA-DEAE nanogels were added to native CpG DNA at various C/P ratios in PBS. The complexes of native CpG DNA with CA-DEAE and CH-CA-DEAE nanogels were evaluated by agarose gel electrophoresis (**Fig. 2**). The free DNA band disappeared when adding CA-DEAE or CH-CA-DEAE nanogels in lanes when C/P = 4 and 8. This finding suggested that CA-DEAE and CH-CA-DEAE nanogels formed ionic complexes at C/P ratios above 4.

Particle sizes of the complexes were determined by DLS (**Fig. 3A**). Agarose gel electrophoresis at C/P = 0.5, 1 and 2, demonstrated native CpG DNA did not form a complex with CA-DEAE and CH-CA-DEAE nanogels. The particle sizes of the complexes of this range of C/P ratios were larger than 500 nm in both cases of CA-DEAE and CH-CA-DEAE nanogels. However, the particle sizes of both complexes deceased when C/P = 4. Although the complex using CA-DEAE formed >1000 nm sized particles at C/P = 4, the size of the complex using CH-CA-DEAE nanogels was 198.1 ± 86.9 nm. A previous study using hexadecyl group-bearing cationic CA as gene carriers also showed that the physical cross-linking of cationic nanogels triggered smaller size complexes.[13] Cellular delivery of native CpG DNA preferred nanometer-scaled particles and the complex of native CpG DNA with CH-CA-DEAE nanogels (CH-CA-DEAE/CpG complex) can be used at C/P = 4 and 8. By zeta potential analysis, there was no significant difference between CA-DEAE and CH-CA-DEAE nanogels and each complex showed a positive charge at C/P > 2 (**Fig. 3B**).

Cell viability and cellular delivery of native CpG DNA to J744A.1 cells

Cytotoxicity of CH-CA-DEAE nanogels was measured by WST-8 assay. First, we used the J744A.1 macrophage-like cell as a model cell line, which produces inflammatory cytokines after successful intracellular CpG DNA stimulus. **Figure 4A** shows the cell viability of J744A.1 cells treated with the complex of native CpG DNA with PEI (PEI/CpG) and CH-CA-DEAE/CpG complexes at different C/P ratios. Although PEI is one of the most effective gene carriers[16], cytotoxicity of PEI to J744A.1 cells is high and the viability of J744A.1 cells was approximately zero at C/P > 2. However, J744A.1 cells were viable after incubation with CH-CA-DEAE/CpG complexes even at C/P = 4. This finding suggested that the cytotoxicity of CH-CA-DEAE/CpG complexes was low and therefore could be used as carrier of native CpG DNA to J744A.1 cells.

The efficiency of CH-CA-DEAE nanogels as a cellular delivery carrier of native CpG DNA was evaluated by CLSM using J744A.1 cells. For the CLSM study, native CpG DNA was labeled with red fluorescent dye (TAMRA). CLSM study was conducted using PEI/CpG and CH-CA-DEAE/CpG complexes at C/P=1 and 4, respectively, because cell viabilities were relatively high using these C/P ratios. Cytotoxicity assays demonstrated PEI/CpG and CH-CA-DEAE/CpG complexes formed at C/P = 1 and 4, respectively. Intracellular distribution of free DNA, PEI/CpG (C/P=1) and CH-CA-DEAE/CpG (C/P=4) complexes is shown in **Fig. 4B**. The highest fluorescent intensity inside cells was observed using the CH-CA-DEAE/CpG (C/P=4) complex, while the fluorescent intensities of free native CpG DNA and PEI/CpG complex were low. Furthermore, native CpG DNA delivered by CH-CA-DEAE nanogels at C/P = 4 was localized to intracellular vesicles such as endosomes, suggesting the CH-CA-DEAE/CpG complex can effectively deliver native CpG DNA to J744A.1 cells and activate inflammatory gene expression.

Cytokine secretion of J744A.1 cells using native CpG or GpC DNA

In both cytotoxicity and cellular delivery studies, CH-CA-DEAE nanogel was a promising carrier for native CpG DNA delivery to J774A.1 cells. To evaluate the biological reaction of cells, J774A.1 cells were incubated with native CpG DNA in different formulations: free native CpG DNA, PEI/CpG (C/P=1), CA-DEAE/CpG (C/P=1, 2, 4 and 8) and CH-CA-DEAE/CpG (C/P=1, 2, 4 and 8) complexes. After administration for 24 h, IL-12 concentrations in culture medium were measured by ELISA. **Figure 5A** shows IL-12 secretion from J744A.1 cells. The highest IL-12 secretion was observed using the CH-CA-DEAE/CpG (C/P=4) complex. In addition, we prepared complexes of GpC DNA (negative control oligonucleotides) with PEI, CA-DEAE and CH-CA-DEAE nanogels, and evaluated IL-12 secretion after administration of these samples to J744A.1 cells. GpC DNA did not induce IL-12 secretion in J744A.1 cells by any formulation (**Fig. 5B**). These results indicated that cytokine production by CA-

DEAE/CpG complexes or CH-CA-DEAE/CpG complexes in **Fig. 5A** did not derive from stimulation of cationic polymers such as CA-DEAE or CH-CA-DEAE but rather from the specific reaction of native CpG DNA.

Generally, the efficiency of gene expression depends on delivery efficiency and cytotoxicity of carriers. Proteoglycans of the extracellular matrix have a negative charge derived from sulfated glycosaminoglycans such as chondroitin sulfate. Therefore, a stronger positive charge is preferred for gene carriers for delivery of genes to cells. However, a higher concentration of amine-derived cation of polymers also shows high toxicity to cells. Therefore, the most efficient C/P ratio of gene expression is the maximum C/P ratio that can minimize cytotoxicity.[16] In our system, the uptakes of CpG by CH-CA-DEAE nanogel increased in the systems with the higher C/P ratio (C/P=1, 2, 4) and the higher IL-12 productions were observed. In the case of C/P =8, however, the decrease of the IL-12 production was observed due to relatively high cytotoxicity. Both free native CpG DNA and PEI/CpG complexes showed little IL-12 production because these approaches do not deliver native CpG DNA intracellularly to J744A.1 cells (**Fig. 4B**). A comparison between CA-DEAE/CpG and CH-CA-DEAE/CpG at the same C/P value demonstrated CH-CA-DEAE/CpG stimulated effective inflammatory gene expression, because the cholesterol group contributed to nucleolysis resistance. Rattanakiat et al. prepared cholesterol-modified CpG DNA, which formed nanoparticles by self-assembly, and evaluated immunostimulatory activity. They reported that the cholesterol modification increased the stability of oligodeoxynucleotides in serum.[17]

We investigated cytokine secretion and cytotoxicity assays using nanogel of cholesterol-bearing pullulan conjugated with DEAE (CHP-DEAE, substitution degrees of cholesterol and DEAE were 1.2 and 73 in 100 glucose units, respectively) instead of cycloamylose-based system. In the case of CHP-DEAE nanogels, the highest IL-12 secretion was 4.9 ± 0.5 μg/mL at C/P=4 (CH-CA-DEAE nanogel: 9.4 ± 1.4 μg/mL) whereas cytotoxicity of CHP-DEAE at C/P=4 was 41.5 ± 6.4 % (CH-CA-DEAE nanogel: 82.6 ± 5.2 %) (**Fig. S1** of electric supplementary information (ESI)). Both IL-12 secretion and cell viability of CHP-DEAE nanogels were lower than these of CH-CA-DEAE nanogels. Cycloamylose was preferred to pullulan as a base polysaccharide of the nanogels for CpG delivery.

Cell viability and cellular delivery of native CpG DNA to RAW264.7 cells

Cytotoxicity and delivery efficiency of CH-CA-DEAE nanogels were also evaluated using RAW264.7 cells, another model macrophage-like cell line. Cytotoxicity of PEI/CpG and CH-CA-DEAE/CpG complexes at different C/P ratios were measured by WST-8 assay (**Fig. 6A**). Similar to J744A.1 cells, the cell viability of CH-CA-DEAE/CpG complexes was higher at C/P <4 than C/P=8, and that of the PEI/CpG complex was lower than the CH-CA-DEAE/CpG complex. Nevertheless, cell viability of RAW264.7 cells was relatively higher than J744A.1 cells when using the PEI/CpG complex, suggesting RAW264.7 cells have a higher resistance to cationic polymers than J744A.1 cells.

Cellular delivery of native CpG DNA to RAW264.7 cells was also evaluated using TAMRA-labeled native CpG DNA by CLSM. As for the J744.A1 cell study in **Fig. 4B**, PEI/CpG and CH-CA-DEAE/CpG complexes were prepared at C/P = 1 and 4, respectively. Intracellular distribution in RAW264.7 cells is shown in **Fig. 6B**. Similar to J744.A1 cells, red fluorescence was detected in RAW264.7 cells using CH-CA-DEAE/CpG (C/P=4) complexes, while the fluorescent intensities of native CpG DNA without carrier was low. In the case of the PEI/CpG complex, weak fluorescence was detected both inside and outside RAW264.7 cells.

Cytokine secretion of RAW264.7 cells using native CpG or PS-CpG DNA

In this study, we used native CpG DNA to stimulate inflammatory gene expression. PS-CpG DNA, phosphorothioate-modified CpG DNA are frequently used in studies that utilize CpG DNA for immune stimulation, because native CpG DNA are eliminated by nuclease-mediated degradation and non-specific binding with plasma proteins.[18] We compared IL-12 secretion levels from RAW264.7 cells administrated with native CpG DNA and PS-CpG DNA using free DNA, PEI/CpG (C/P=0.25, 0.5, 1 and 2), CA-DEAE/CpG (C/P=1, 2, 4 and 8) and CH-CA-DEAE/CpG (C/P=1, 2, 4 and 8) complexes.

Figure 7A shows that free PS-CpG DNA stimulated IL-12 (138 ± 43 pg/mL), and all cationic carriers (PEI, CA-DEAE and CH-CA-DEAE nanogels) induced higher IL-12 secretion from RAW264.7 cells. Cytotoxicity of the PEI/CpG complex was relatively high at large C/P ratios, so maximum IL-12 secretion was induced using PEI/CpG complex at C/P = 0.5 (**Fig. 6A**). For the other carriers, maximum IL-12 secretion was induced at a relatively high C/P ratio (C/P = 4 for CA-DEAE/CpG complex and C/P = 8 for CH-CA-DEAE/CpG complex) because of the lower cytotoxicity of these carriers compared with PEI.

Free native CpG DNA stimulated a low level of IL-12 (4.7 ± 3.3 pg/mL), suggesting that native CpG DNA was degraded by nucleases and could not activate inflammatory gene expression in RAW264.7 cells (**Fig. 7B**). Incubation with PEI/CpG and CA-DEAE/CpG complexes did not increase IL-12 secretion. However, CH-CA-DEAE nanogels induced IL-12 secretion and a maximum level was produced at C/P = 8 (2161 ± 344 pg/mL). The experiments of agarose gel electrophoresis of the complexes mixed with DNase I as model nuclease[19] was carried out. From **Fig. S2** of ESI, the degraded DNA bands were not observed in CH-CA-DEAE/CpG systems (C/P=2, 4 and 8). CH-CA-DEAE nanogels protected native CpG DNA against nuclease-degradation. However, the degraded DNA bands were observed in CA-DEAE systems (C/P=1 and 2). The results corresponds to the efficiency of IL-12 secretion in **Fig. 7B**.

The following experiments were carried out to investigate the reason why CH-CA-DEAE nanogels enhanced IL-12 secretion by native CpG DNA more than PS-CpG DNA. The particle sizes of the complexes of CH-CA-DEAE nanogels with PS-CpG DNA were examined at different C/P ratios. The behavior of the change of the size of the complex of PS-CpG DNA as a function of C/P ratios was similar to that of native CpG DNA (**Fig. S3** of ESI). The average sizes of the complexes (C/P=4) of native CpG DNA and PS-CpG DNA were 198 and 107 nm, respectively. The cellular uptake of the complex with PS-CpG DNA (C/P=4) was not so different form the complex with native CpG DNA (C/P=4) by CLMS (**Fig. S4** of ESI). Finally, we investigated the release profile of native CpG DNA and PS-CpG DNA from the complexes of CH-CA-DEAE nanogels (**Fig. 8**)[20]. The release of DNA from the complexes after the addition of heparin was estimated by agarose gel electrophoresis. In the case of native CpG DNA, clear band appeared at the same position as free DNA by addition of more than 50 μg/mL heparin. On the other hand, the release of PS-CpG DNA from the complex was not observed even in the presence of 75 μg/mL heparin. Native CpG DNA was released from the CH-CA-DEAE/CpG complex more effectively than PS-CpG DNA. These results suggest that the release property of

CpG DNA after uptake to the cell may effect the cytokine production.

Conclusions

CH-CA-DEAE self-assembled to form nanogels by hydrophobic interactions of cholesterol groups. CH-CA-DEAE nanogels formed nanometer-scaled complexes with native CpG DNA by electrostatic interactions. A comparison of free DNA, PEI/CpG, CA-DEAE/CpG and CH-CA-DEAE/CpG complexes demonstrated the most effective cellular uptake and cytokine secretion in macrophage-like cells was induced by the CH-CA-DEAE/CpG complex. Furthermore, negative control oligonucleotides did not induce cytokine expression, suggesting cytokine secretion induced by CH-CA-DEAE/CpG complexes did not derive from stimulation by cationic polymers. Of note, the production of cytokines by native CpG DNA using CH-CA-DEAE nanogels was higher than by phosphorothioate-modified CpG DNA. In conclusion, CH-CA-DEAE nanogel is a promising carrier for the delivery of nucleic acid adjuvants such as native CpG DNA.

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Notes and references

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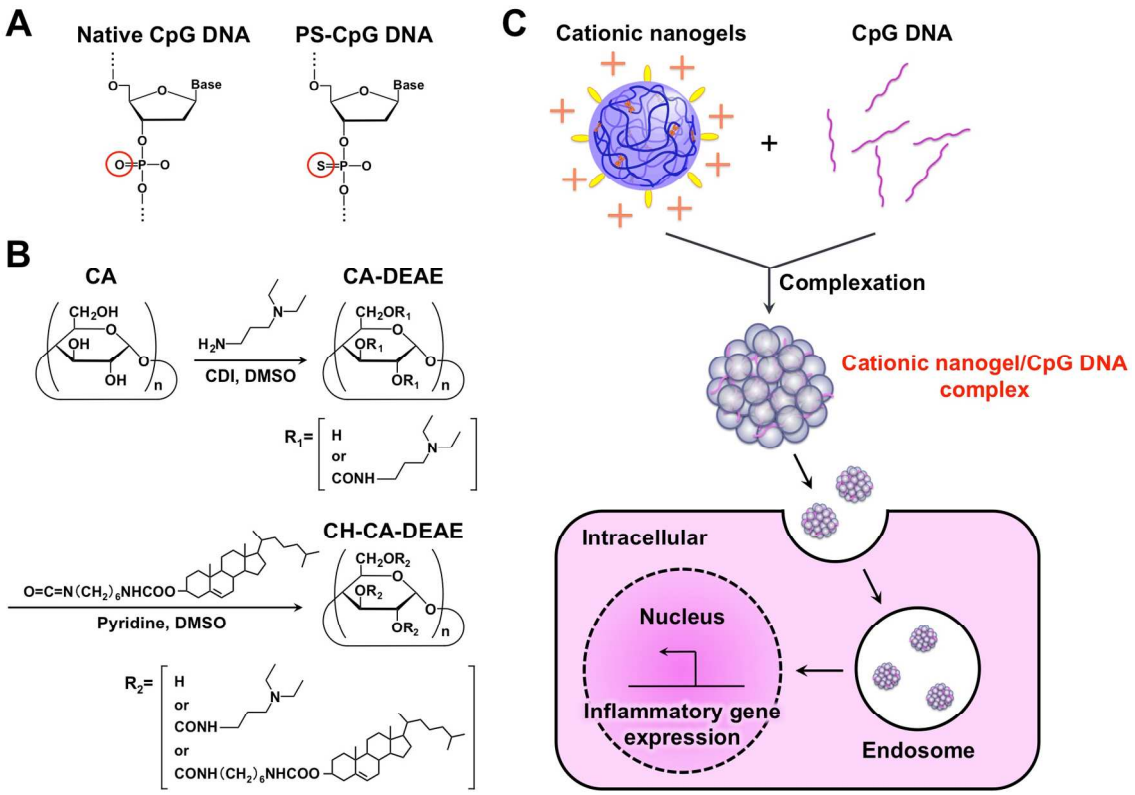

Fig. 1 Schematic illustration of (**A**) chemical structure of native CpG DNA (target oligonucleotides of this study) and PS-CpG (oligonucleotides used commonly consisting of phosphorothioate backbone), (**B**) synthesis of CA-DEAE and CH-CA-DEAE, and (**C**) cellular delivery and inflammatory activation of CpG DNA using cationic nanogels.

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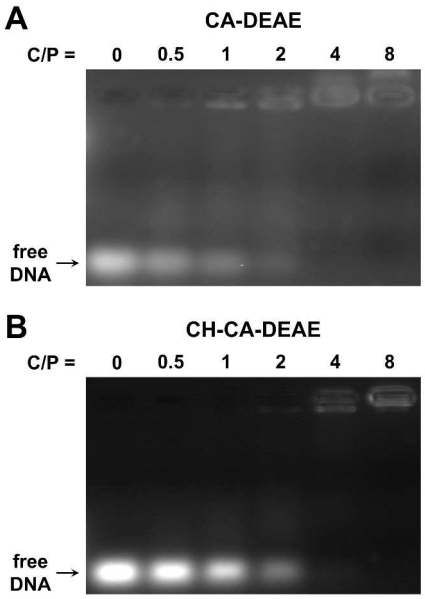

Fig. 2 Complex formation of native CpG DNA with (**A**) CA-DEAE or (**B**) CH-CA-DEAE nanogels confirmed through agarose gel electrophoresis.

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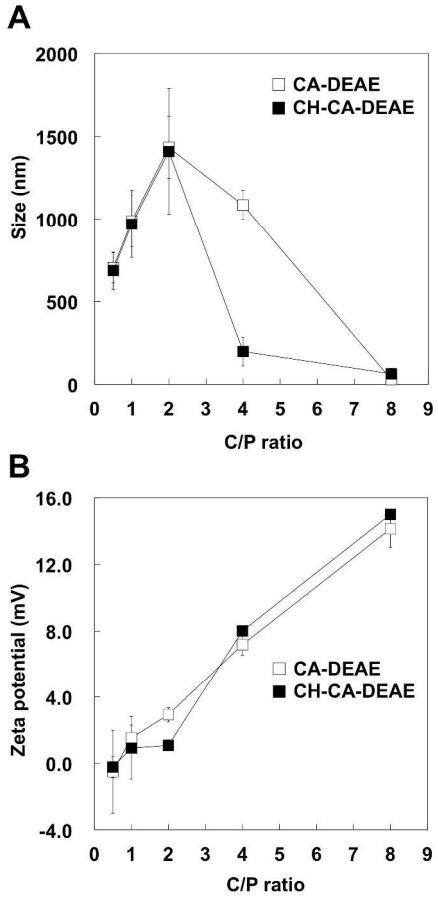

Fig. 3 (**A**) Particle sizes and (**B**) zeta potentials of complexes of native CpG DNA with CA-DEAE and CH-CA-DEAE nanogels (n=3).

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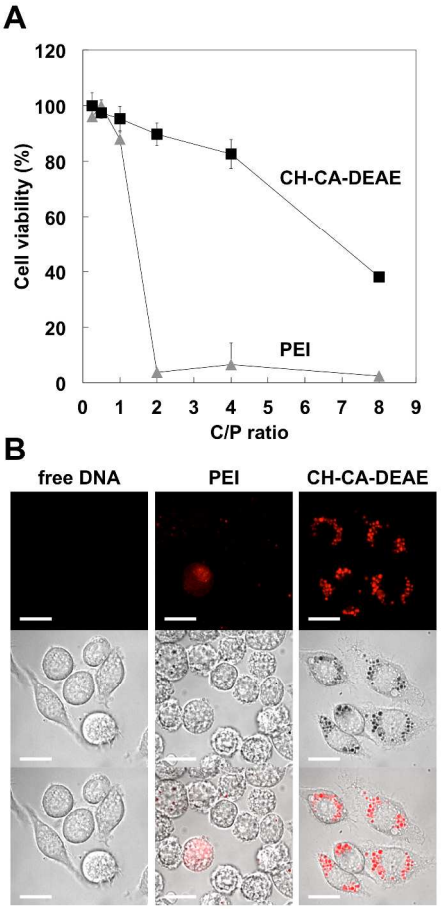

Fig. 4 (**A**) Cytotoxicity and (**B**) intracellular distribution of J744A.1 cells using CH-CA-DEAE nanogels. Cytotoxicity of PEI/CpG and CH-CA-DEAE/CpG complexes were measured by WST-8 assay. J744A.1 cells were incubated with samples for 24 h (n=3). Intracellular distribution of free DNA, PEI/CpG (C/P=1) and CH-CA-DEAE/CpG (C/P=4) complexes were evaluated by confocal laser scanning microscopy using TAMRA-labeled (red dye) native CpG DNA after incubation with J744A.1 cells for 4 h. Scale bars: 20 μm.

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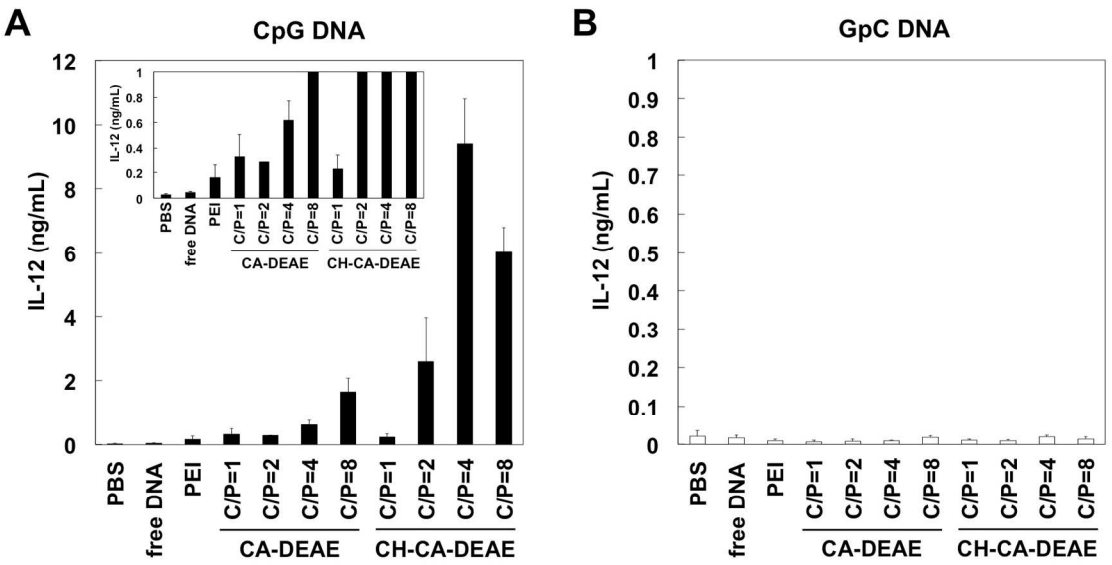

Fig. 5 IL-12 secretion of J744A.1 cells induced by (**A**) native CpG DNA and (**B**) GpC DNA (negative control oligonucleotides) complexed with PEI, CA-DEAE or CH-CA-DEAE nanogels. J744A.1 cells were incubated with PBS, free DNA, PEI/CpG (C/P=1), CA-DEAE/CpG (C/P=1, 2, 4 and 8) or CH-CA-DEAE/CpG (C/P=1, 2, 4 and 8) complexes for 24 h, and the IL-12 concentration in cultural medium was determined by ELISA (n=3).

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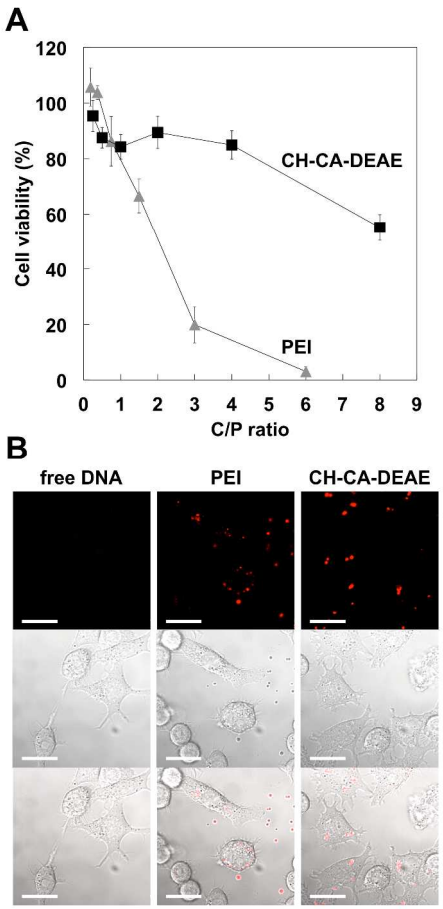

Fig. 6 (**A**) Cytotoxicity and (**B**) intracellular distribution of RAW264.7 cells using CH-CA-DEAE nanogels. Cytotoxicity of PEI/CpG and CH-CA-DEAE/CpG complexes were measured by WST-8 assay. RAW264.7 cells were incubated with samples for 24 h (n=3). Intracellular distribution of free DNA, PEI/CpG (C/P=1) and CH-CA-DEAE/CpG (C/P=4) complexes was evaluated by confocal laser scanning microscopy using TAMRA-labeled (red dye) native CpG DNA after incubation with RAW264.7 cells for 4 h. Scale bars: 20 μm.

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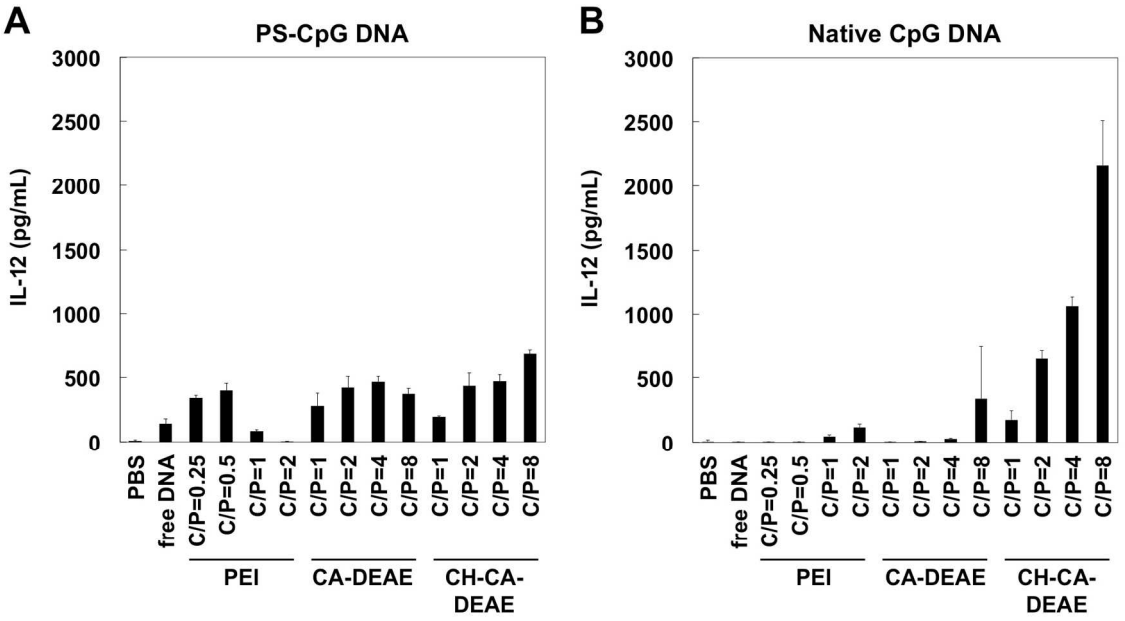

Fig. 7 IL-12 secretion of RAW264.7 cells induced by (**A**) PS-CpG DNA and (**B**) native CpG DNA complexed with PEI, CA-DEAE and CH-CA-DEAE nanogels. RAW264.7 cells were incubated with PBS, free DNA, PEI/CpG (C/P=0.25, 0.5, 1 and 2), CA-DEAE/CpG (C/P=1, 2, 4 and 8) or CH-CA-DEAE/CpG (C/P=1, 2, 4 and 8) complexes for 24 h, and the IL-12 concentration of supernatants was determined by ELISA (n=3).

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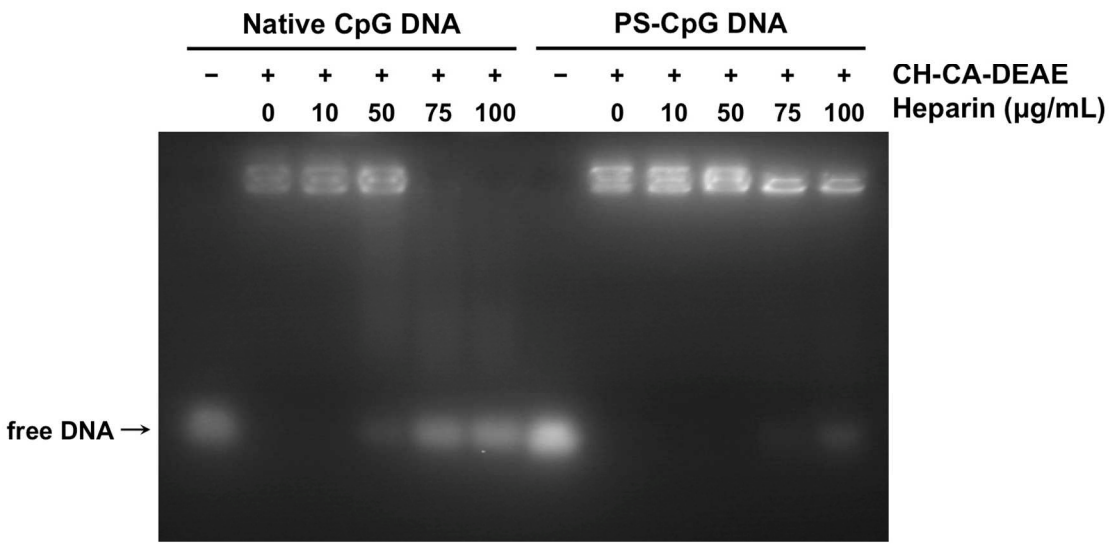

Fig. 8 Complex formation of native CpG DNA with CH-CA-DEAE nanogels after incubation with heparin confirmed through agarose gel electrophoresis.