Complex formation between cationic β-1,3-glucan and hetero-sequence oligodeoxynucleotide and its delivery into macrophage-like cells to induce cytokine secretion†

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A cationic polysaccharide bearing a β-1,3-glucan main-chain structure (CUR-N^+) forms a complex with a hetero-sequence oligonucleotide, that is, a CpG ODN, and facilitates the transportation of the resultant complex into a murine macrophage-like cell J774.A1, which induces an efficient secretion of a cytokine (IL-12) as compared with that induced by conventional carriers such as poly(ethyleneimine) (PEI) and poly(L-lysine) (PLL).

Introduction

Research toward exploitation of gene and drug delivery systems using polymeric materials has rapidly grown over the past decade.[1] Cationic polymers are frequently employed for the delivery of antisense DNAs because of their ability to form complexes with the DNAs through electrostatic interactions.[2] Recently, increasing attention has been paid to unmethylated CpG dinucleotide-containing oligodeoxynucleotides (CpG ODNs) in immunotherapy.[3] The unmethylated CpG motif is frequently found in bacterial DNAs, which thereby stimulates cell-mediated immune responses in mammals. Recent studies have revealed that the CpG ODNs are excellent vaccine adjuvants against infectious disease, cancer and allergy.[3] To bring this therapy into practical usage, design of a good delivery system becomes indispensable. We recently succeeded in delivering a CpG ODN into a macrophage-like cell employing chemically modified schizophyllans (SPGs)‡ as carriers and demonstrated that cytokines are efficiently secreted.[4] One serious drawback exists in this system, however, that we must introduce a dA-tail to the CpG ODN, because the SPG derivatives can bind homo-sequence ODNs but not hetero-sequence ODNs.[5,6] As a result, we must inevitably use the 'expensive' dA-tail-connected CpG ODN.

We have endeavoured to design artificial β-1,3-glucan derivatives to extend and modify the ability of native β-1,3-glucan to bind hydrophobic guest molecules. Recently, we found that CUR-N^+, which is a cationic polysaccharide derived from curdlan (CUR) (Fig. 1) using 'click chemistry',[7] shows interesting binding properties toward a homo-sequence polynucleotide (poly(C)) and various kinds of hydrophobic guest molecules in aqueous solution.[8] Herein, we describe the unique binding phenomenon between CUR-N^+ and a hetero-sequence oligonucleotide, that is, the CpG ODN and the transportation of the resultant complex into a murine macrophage-like cell in detail. Most importantly, we have found that CUR-N^+ can bind to a hetero-sequence ODN *even though it bears no dA-tail*. Subsequently, the ability of the CUR-N^+–CpG ODN complex to induce cytokine (IL-12) secretion has been compared with those of PEI and PLL to assess the feasibility of this delivery system.

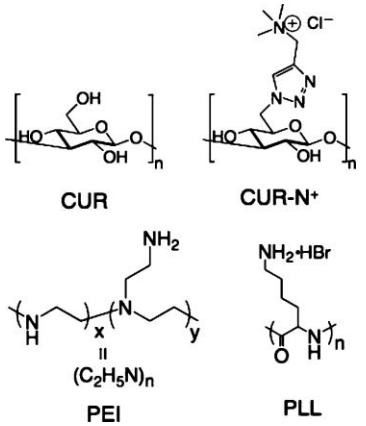

Fig. 1 Chemical structures of CUR, CUR-N^+, PEI (C_2H_5N is counted as the monomer unit in this study) and PLL.

Results and discussion

Synthesis and materials

As a CpG ODN, 5′-TCC AT*G A<u>CG</u> TT*C CTG ATG-3′ (18 mer, the key sequence[9] in italic) was used and CUR-N^+ (M_w = 32 000 (M_w/M_n = 1.6)) was synthesized according to the reported method.[8,10] The molecular weights (M_w) of PEI and PLL used in this study are 25 000 (branched) and 15 000–30 000, respectively.

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‡ Schizophyllan (SPG) is a natural polysaccharide produced by the fungus *Schizophyllum commune*, which consists of a β-1,3-glucan main chain bearing a β-1,6-glucoside side chain at every third glucose unit.

Complex formation between CUR-N$^+$ and ODN

We evaluated the complexation ability of CUR-N$^+$ toward the CpG ODN by spectroscopic measurements. Fig. 2A (a) shows the UV–vis and circular dichroism (CD) spectra of the CpG ODN in aqueous solution (no salt and buffer, pH = 6.0) at 25 °C. When we added CUR-N$^+$ to this solution, the UV–vis and CD spectra changed significantly depending on the amount of added CUR-N$^+$, indicating that complexation occurred. These spectral changes can be ascribed to the conformational changes of the CpG ODN induced by the complexation with CUR-N$^+$ because the CD intensity and absorbance of CUR-N$^+$ itself is negligible in this wavelength region. The CD intensity was increased with an increase in the amount of CUR-N$^+$ up to [CUR-N$^+$]/[CpG ODN] = 1 and then it was decreased and saturated at [CUR-N$^+$]/[CpG ODN] = 2 (Fig. 2B (a), [CUR-N$^+$] and [CpG ODN] denote the concentration in monomer unit and base unit, respectively). At [CUR-N$^+$]/[CpG ODN] = 1 (Fig. 2A (b)), the CD intensity was increased and the absorbance was decreased compared with those of CpG ODN alone. These facts indicate that the base stacking was intensified at this composition. Conversely, at [CUR-N$^+$]/[CpG ODN] = 2 (Fig. 2A (c)), the CD intensity was decreased and the absorbance was increased, which is indicative of weakened base stacking.§ These results suggest that CUR-N$^+$ can take two different types of complex with the CpG ODN depending on the feed ratio and the base alignment of the CpG ODN in each complex is different, the base stacking of the CpG ODN is intensified in a 1 : 1 complex and weakened in a 1 : 2 complex. Since the hydrophobic domains of *native* β-1,3-glucans such as SPG and CUR show almost no affinity with hetero-sequence RNAs and DNAs,[5,6] we presume that electrostatic interactions between CUR-N$^+$ and the CpG ODN contribute effectively to this complexation mode.

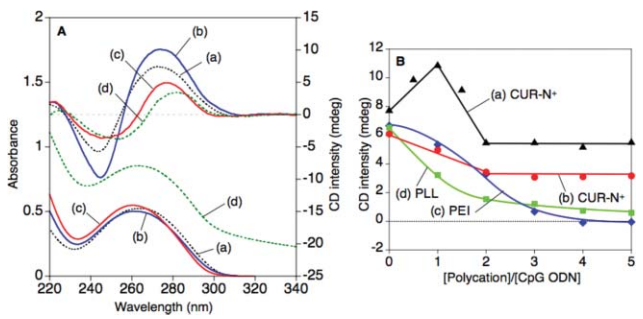


Fig. 2 (A) CD and UV–vis spectra of [CUR-N$^+$]/[CpG ODN] = 0 (a), 1 (b) and 2 (c) in aqueous solution (pH = 6.0–6.5) and (d) [CUR-N$^+$]/[CpG ODN] = 2 in PBS aqueous solution (pH = 7.4) at 25 °C with a 1 cm cell. (B) Plots of CD intensity at 281 nm against [CUR-N$^+$]/[CpG ODN] (a) in aqueous solution and (b) in PBS aqueous solution, (c) [PEI]/[CpG ODN] and (d) [PLL]/[CpG ODN] in PBS aqueous solution ([CpG ODN] = 6.3 × 10^{-5} M).

To evaluate the stability of the CUR-N$^+$–CpG ODN complex under the physiological conditions, we again measured UV–vis and CD spectra of the CUR-N$^+$–CpG ODN complex in phosphate-buffered saline (PBS) aqueous solution (pH 7.4) at 25 °C (Fig. 2A (d)). As shown in Fig. 2B (b), the CD intensity of the CpG ODN was decreased with an increase in the amount of CUR-N$^+$ and saturated at [CUR-N$^+$]/[CpG ODN] = 2, which was accompanied by an increase in the baseline of the UV–vis spectra. These results suggest that the CUR-N$^+$–CpG ODN complex consists of a 2 : 1 complex and results in aggregates in the presence of salt; the presence of salt would destabilize a 1 : 1 complex presumably due to the shielding of electrostatic interactions. By the addition of PEI or PLL, the CD intensity of the CpG ODN decreased continuously and finally reached almost zero (Fig. 2B (c) and (d)). This behaviour is typically observed for polyion complexes[1b,2] and can be rationalized by the formation of ill-defined aggregates, in which the base stacking collapses. Interestingly, no such complete collapse was observed for the CUR-N$^+$–CpG ODN complex. Fig. 3 depicts a proposed scheme for the complex formation between the polycations and the CpG ODN.

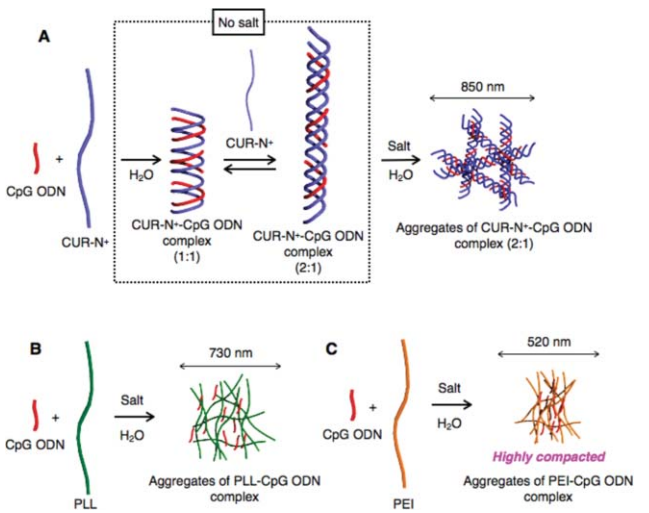


Fig. 3 Schematic illustration for the formation of (A) CUR-N$^+$–CpG ODN complexes, (B) PLL–CpG ODN complex and (C) PEI–CpG ODN complex. The hydrodynamic radii of each aggregate were determined with DLS (Fig. S11 in ESI†).

These aggregates of polycation–CpG ODN complexes in PBS aqueous solution were characterized by dynamic laser light scattering (DLS) and ζ potential measurements (Fig. S11 in ESI†). The aggregates of the complex formed between CUR-N$^+$, PEI and PLL and the CpG ODN at [polycation]/[CpG ODN] = 2 have sizes of 850 nm, 730 nm and 520 nm and ζ potentials of +13.3 mV, −12.4 mV and +16.1 mV, respectively. The anionic charge of the aggregates of the PEI–CpG ODN complex at [PEI]/[CpG ODN] = 2 can be attributed to the unprotonated amino groups of PEI (pK_a = *ca.* 5.5) under these conditions. The size of the aggregates of the PEI–CpG ODN complex at [PEI]/[CpG ODN] = 4 is 560 nm with a ζ potential of +6.8 mV.

Gel electrophoresis (Fig. 4) demonstrated that the CpG ODN complexed with the polycations lost mobility, indicating that the complexes are stable in the physiological solution. In addition, the CpG ODN in the presence of 4 equivalents of PEI was hardly stained with GelStar®, implying that the structure of the PEI–CpG ODN complex is so compacted that the staining molecule cannot access the nucleobases of the CpG ODN.

§ The similar perturbation of the CD band of single-stranded DNAs is reported for single-stranded DNA binding proteins, see ref. 11.

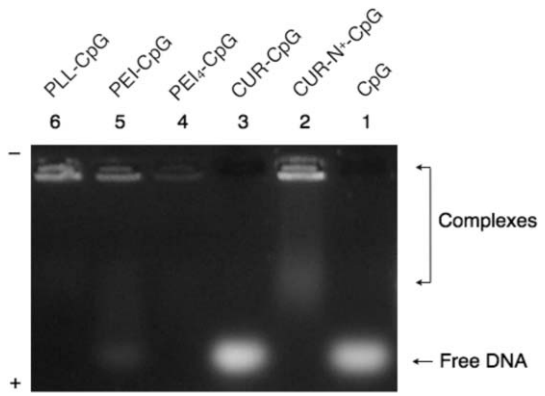


Fig. 4 Formation of the polymer–CpG ODN complexes confirmed by 2% agarose gel electrophoresis stained with GelStar®. (Lane 1, CpG ODN, lane 2, CUR-N^+–CpG ODN ([CUR-N^+]/[CpG ODN] = 2); lane 3, CUR–CpG ODN ([CUR]/[CpG ODN] = 2); lane 4, PEI_4–CpG ODN ([PEI]/[CpG ODN] = 4); lane 5, PEI–CpG ODN ([PEI]/[CpG ODN] = 2); lane 6, PLL–CpG ODN ([PLL]/[CpG ODN] = 2), [CpG ODN] = 35 μg mL^{-1}).

Several lines of evidence described above indicate that CUR-N^+ shows unique binding properties toward the hetero-sequence ODN (CpG ODN), which is stable in the physiological condition, as compared with those of conventional polycations such as poly(ethyleneimine) (PEI) and poly(L-lysine) (PLL).

Cellular uptake of complexes by a murine macrophage-like cell

Cellular uptake of the CpG ODN complexed with the polycations by a murine macrophage-like cell J774.A1 was monitored with a fluorescence activated cell sorting (FACS) analyzer, where FITC-labeled CpG ODN (FCpG ODN) was used as a marker. The molar ratio ([polycation]/[CpG ODN]) was fixed to 2 for the polycation–CpG ODN complexes and also 4 for the PEI–CpG ODN complex to evaluate the influence of ζ potential. Since the fluorescence intensity of FCpG ODN depends on microenvironments (Fig. S12–13 in the ESI†) and/or the presence of quenchers, the quantitative comparison may become inappropriate. Nevertheless, the efficient uptake of the complexed CpG ODN compared with that of the naked CpG ODN is evident from the marked shift of peaks (Fig. 5A). The major uptake mechanism is owing to electrostatic interactions between negatively-charged cell membrane and positively-charged complexes and subsequently occurring phagocytosis.[12]

We examined the cytotoxicity by use of a WST-8 assay (Fig. S14 in ESI†) and found that polymer–CpG ODN complexes showed almost no cytotoxicity under the conditions that cytokine secretion was examined (*vide infra*), except for the PEI–CpG ODN complex at [PEI]/[CpG ODN] = 4. Then, we measured the amount of IL-12 secreted from the J774.A1 cells by an ELISA kit, comparing the naked CpG ODN, the polycation–CpG ODN complexes and the polycations themselves (Fig. 5B and S15 (for entire data) in ESI†). As shown in Fig. 5B, when the naked CpG ODN was added, almost no IL-12 was secreted presumably due to degradation of the CpG ODN by deoxyribonuclease as well as its low permeability to the cell membrane. In sharp contrast, the CpG ODN complexed with CUR-N^+ and PLL showed a significant amount of IL-12 secretion. The complexed CpG ODN

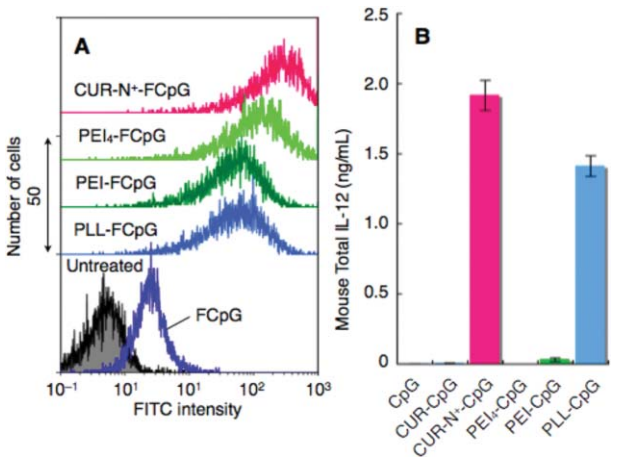


Fig. 5 (A) FACS analyses of untreated and naked FCpG ODN and macrophage-like cells (J744.A1) treated with polycation–FCpG ODN complexes. (B) Effect of polycation complexation on CpG ODN-mediated cytokine (IL-12) secretion (see Experimental for details).

would be protected from the degradation[8,13] and be transported efficiently into the cell (Fig. 5A). Among the polycations, CUR-N^+ achieved the highest performance (1.9 ng mL^{-1}, 62- and 1.4-fold of PEI ([PEI]/[CpG ODN] = 2) and PLL, respectively). PEI hardly induced the secretion of IL-12 even though the CpG ODN complexed with PEI was efficiently ingested as evidenced by the FACS analyses. Most probably, this low efficiency can be explained by two reasons (1) the CpG ODN molecules complexed with PEI were so compacted that they could not be released in the endosomal compartments such as late endosome and lysosome, where the CpG ODN recognition receptor of Toll-like receptor 9 (TLR-9) is present[14] and (2) the proton sponge effect[15a] of the PEI–CpG ODN complex caused the endosomal escape of the CpG ODN before the complex reached the endosomal compartments. On the other hand, the CpG ODN complex with CUR-N^+ and PLL should be transferred and remain in the endosomal compartments without the proton sponge effect[15b] and be easily recognized by TLR-9.

Confocal fluorescence microscopic images of the J774.A1 cells treated with the CUR-N^+–CpG ODN complex were obtained by triple-labeling experiments using a nucleus-selective dye (DAPI), FITC-labeled CUR-N^+ (FCUR-N^+) and rhodamine(ROX)-labeled CpG ODN (RCpG ODN). As shown in Fig. 6, red dots are distributed heterogeneously within cytosol in a spotty fashion, which indicates the localization of the RCpG ODN in vesicles, presumably in the endosome or lysosome. In addition, the yellow color in the merged image evidences noticeable colocalization of FCUR-N^+ and the RCpG ODN. These results indicate that the CUR-N^+–CpG ODN complex is transported predominantly into the endosomal compartments, which is essential for efficient secretion of IL-12.

Conclusions

In conclusion, we have revealed that CUR-N^+ forms complexes with a CpG ODN and facilitates the transportation of the complexes into a macrophage-like cell and subsequent secretion of IL-12. It is particularly worthy to emphasize that cytokine secretion employing CUR-N^+ as the carrier was the largest

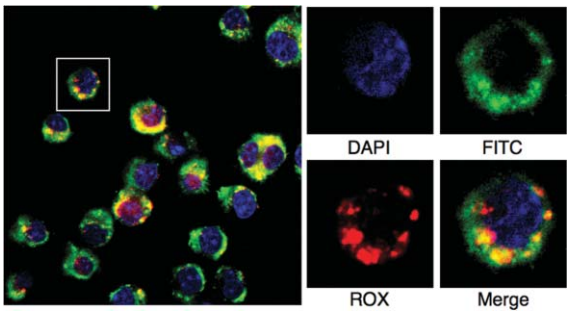


Fig. 6 Internalization and localization of the FCUR-N$^+$–RCpG ODN complex in the J774.A1 cells (see Experimental for details). The blue, green and red colors correspond to DAPI (nucleus), FITC (CUR-N$^+$) and ROX (CpG), respectively. The yellow color is the outcome of the merge between FITC and ROX.

among the polycations used in this study, indicating that CUR-N$^+$ could be one of the most suitable carriers for CpG ODN. Since hydrophobic molecules, which are insoluble in water and/or cannot be transported across the cell membrane by themselves, can be included in the hydrophobic region of CUR-N$^+$ and be transported with the CpG ODN, we will be able to engineer this delivery system into more sophisticated one.[3,16]

Experimental

General

^{1}H NMR spectra were obtained on a Brucker DRX600 or AV300M spectrometer. 2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) was used as reference for D_2O as solvent. The IR spectra were measured using a Perkin-Elmer Spectrum One FT-IR spectrometer. The absorption and circular dichroism (CD) spectra were measured in a 1 cm quartz cell unless otherwise noted using a Jasco V-570 spectrometer and a Jasco J-710 spectropolarimeter, respectively. The fluorescence spectra were measured using a Perkin-Elmer LS-55 luminescence spectrometer. Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF MS) spectra were recorded on a Voyager-DE PRO (PerSeptive Biosystems) by use of α-cyano-4-hydroxy-cinnnamic acid (CHCA) and 3-hydroxypicolinic acid (HPA) as the matrix. Dynamic light scattering (DLS) and ζ potential were measured using a Malvern Zeta Sizer Nano ZS incorporating non-invasive back scatter optics (NIBS, 173°) with a laser source of He–Ne 633 nm. Size exclusion chromatography (SEC) was performed with a Jasco PU-1580 Plus liquid chromatograph system equipped with a UV–visible detector (UV-1570 Plus), an RI detector (RI-2031 Plus) and a column oven (CO-2060 Plus). A SEC column (Shodex OHpak SB-806M HQ) was connected and H_2O containing 100 mM of $NaNO_3$ was used as the eluent at a flow rate of 0.5 mL min^{-1} at 40 °C.

Materials

Curdlan (MW = 1 000 000) and poly(L-lysine) hydrobromide (MW = 15 000–30 000) were obtained from Wako Chemicals. Poly(ethylenimine) (MW = 25 000) was purchased from Aldrich. As CpG ODN, we adopted the sequence of 5′-TCC ATG ACG TTC CTG ATG-3′ (MW = 5465.62). For CLSM and FACS, we used 3′-rhodamine- and 3′-fluorescein-labeled CpG ODN (RCpG ODN (MW = 6191.21) and FCpG ODN (MW = 6058.62), respectively). All ODN samples were synthesized at Hokkaido System Science (Hokkaido, Japan) and purified with high-pressure liquid chromatography. The lengths of the synthesized ODNs were confirmed with denature PAGE and MALDI-TOF MS spectral technique. RNase- and DNase-free distilled sterile water and ×10 PBS buffer (pH 7.4) were obtained from Nippon Gene (Japan) and used for all measurements. All other starting materials and solvents were purchased from chemical companies and used as received.

Sample preparation for measurements

Sample preparation for UV–vis and CD spectroscopic measurements of the CUR-N$^+$–CpG ODN complex in aqueous solution. An CpG ODN aqueous solution (3.13 mM (base unit), 50 μL) was diluted with sterile water (2500 μL). This solution was titrated with Cur-N$^+$ aqueous solution (15.6 mM (monomer unit) at an interval of 10 μL). The resulting solution was then subjected to UV–vis and CD spectroscopic measurements.

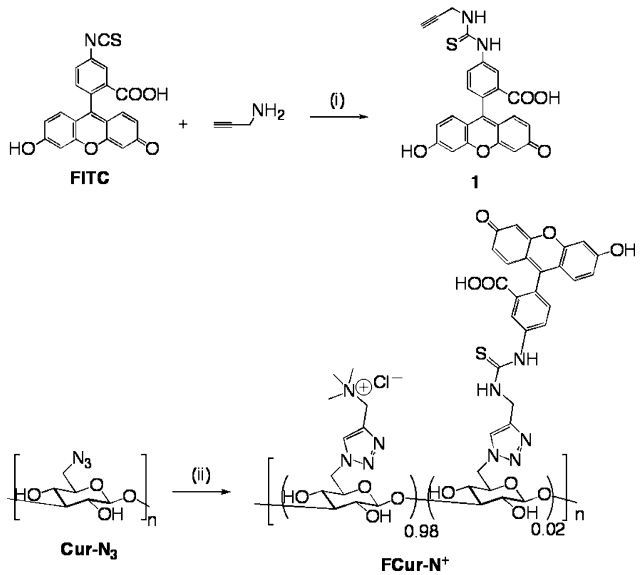
Sample preparation for UV–vis and CD spectroscopic measurements of CUR-N$^+$–CpG ODN, PEI–CpG ODN and PLL–CpG ODN complexes in PBS aqueous solution. A CpG ODN aqueous solution (3.13 mM (base unit), 50 μL) was diluted with PBS aqueous buffer (pH 7.4, 2500 μL). This solution was titrated with Cur-N$^+$ or PEI or PLL aqueous solution (15.6 mM (monomer unit) at an interval of 10 μL). The resulting solution was then subjected to UV–vis and CD spectroscopic measurements.

DLS and ζ potential measurements. A CpG ODN aqueous solution (3.13 mM (base unit), 20 μL) was diluted with PBS aqueous solution (1000 μL). To this solution was added an appropriate amount of polycation aqueous solution (15.6 mM (monomer unit), 8 μl or 16 μl). The resulting solution was then subjected to DLS and ζ potential measurements.

Synthesis

Synthesis of 1. To a solution of 5-fluorescein isothiocyanate, isomer I (FITC) (100 mg, 0.26 mmol) in EtOH–THF (3 : 2 (v/v), 15 mL) was added propargylamine (17.2 μL, 0.26 mmol) and the solution was cooled to 0 °C. To the solution was added triethylamine (36.2 μL, 0.26 mmol) and the resulting orange reaction mixture was warmed to room temperature and stirred for 3 h at room temperature under an argon atmosphere. The reaction mixture was concentrated *in vacuo* and the residue was purified by column chromatography (SiO_2, $CHCl_3$–MeOH = 5 : 1, v/v) to afford compound **1** as a yellowish-orange powder. Yield: 65 mg (75%). ^{1}H NMR (300 MHz, 0.5 mM, DMSO-d_6, 25 °C): δ = 9.94 (s, 1H), 8.38 (s, 1H), 7.73 (s, 1H), 7.12 (d, J = 8.4 Hz, 1H, ArH), 6.93–6.48 (m, 6H, ArH), 5.30 (s, 1H, ArH), 5.25 (s, 1H, ArH), 4.88 (s, 2H, CH_2), 1.87 (s, 1H, C≡CH); MALDI-TOF MS (CHCA): calcd for [M($C_{21}H_{31}NO_5$) + H]$^+$ 445.09, found. 445.11.

Synthesis of FCUR-N$^+$. To a solution of CUR-N_3 (94 mg, 0.5 mmol (monomer unit)) in DMSO (5 mL) (stirring for 2 hours at room temperature to dissolve CUR-N_3 in DMSO completely) was added water (0.5 mL), propylamine (0.5 mL), $CuBr_2$ (5.6 mg, 5 mol%), ascorbic acid (22.0 mg, 25 mol%) and compound **1** (4.4 mg, 0.02 eq. (monomer unit)). After the solution was stirred at



Scheme 1 (i) TEA, THF–EtOH, rt, 3 h, (ii) **1**, $CuBr_2$, ascorbic acid, propylamine, 90% DMSO aq., 60 °C, 24 h, then 1-propyltrimethylammonium chloride, rt, 3 days.

60 °C for 24 h, 1-propynyltrimethylammonium chloride (334 mg, 5 eq. (monomer unit)) was added and the resultant solution was further stirred at room temperature for 3 days. The solution was dialyzed by distilled water with SpectraPor membrane (MWCO: 8000, wet with 0.1% sodium azide) for 2 days, purified by gel filtration on Sephadex G-100 (Pharmacia) and lyophilized using a LABCONCO freeze dryer 4.5 to afford FCUR-N^+ as an orange powder. Yield: 102 mg. ^{1}H NMR (600 MHz, 1.0 mg mL^{-1}, D_2O, 25 °C): δ = 8.43 (s, 1H, triazole-H), 5.13 (br, 1H, H^1), 4.70 (br, 2H (overlapped with water), NCH_2), 3.85 (br, 2H, H^6 and H^3), 3.66 (br, 2H, H^6 and H^5), 3.38 (br, 2H, H^2 and H^4), 3.18 (br, 9H, NCH_3); FTIR (powder, cm^{-1}): 3373, 2923, 1576, 1475, 1073, 897, 551; UV–vis: λ_{max} = 495 nm, fluorescence: λ_{max} = 518 nm, SEC (0.1 M $NaNO_3$ aq., pullulan standards) $M_w = 3.6 \times 10^4$ and $M_w/M_n = 1.8$.

Biological methods

Complex preparation for transfection experiment. 0.95 mg of a CpG ODN were dissolved in sterile H_2O (800 μL, 3.13 mM (base unit)). An appropriate amount of polymer solution (15.6 mM (monomer unit)) was added to the CpG ODN solution. The molar ratio ([polymer]/[CpG ODN]) was controlled to be 2 (and 4 for PEI). After the solution was kept at room temperature for 30 min to lead the complexation, we measured the gel electrophoresis migration pattern (2% agarose gel) to verify the complexation between the CpG ODN and the polymer. The bands were visualized with GelStar® (Amersham).

Cell culture and the cytokine secretion measurement. 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 medium contains a 1 wt% penicillin–streptomycin mixture. The cell incubation was always carried out at 37 °C in fully humidified air containing 5 wt% of CO_2. J774.A1 cells were plated in 96-well dishes using 1×10^6 cells per mL (1 well per 100 μL) and were allowed to attach to the plate for 5 h. An appropriate amount of the CpG ODN (35 μg mL^{-1}) and the polymer–CpG ODN complex (CpG ODN: 35 μg mL^{-1}, [polymer]/[CpG ODN] = 2 (and 4 for PEI)) were added to J774.A1 cell cultures. Subsequently, cells were incubated at 37 °C for 24 h and the supernatants were collected for ELISA assays. The secretion of IL-12 was determined using a commercially available ELISA kit according to the instructions of the manufacture (Endogen). The experiments were performed twice in triplicate.

Fluorescence activated cell sorting (FACS) analysis. J774.A1 cells were plated in a 48-well dish (1.5×10^6 cells per mL; 200 μL per well) and were allowed to attach to the well at 37 °C for 14 h. Then, the FCpG ODN and the polycation–FCpG ODN complex (FCpG ODN: 39 μg mL^{-1}, [polycation]/[FCpG ODN] = 2 (and 4 for PEI), see above for the complex preparation) were added to the J774.A1 cell cultures. The cells were incubated at 37 °C for 6 h, washed twice with PBS and harvested. The number of the cells having FITC was counted with an EPICS XL (Beckman Coulter).

Confocal microscopic observations. J774.A1 cells were plated at a density of 1.5×10^6 cells per mL in a glass-bottom chamber (Lab-Tek II Chembered Coverglass, Nalge Nunc, Rochester, NY). Following addition of the FCUR-N^+–RCpG ODN complex (RCpG ODN: 40 μg mL^{-1}, [FCUR-N^+]/[RCpG ODN] = 2.0) to the medium, the cells were incubated for 6 h at 37 °C in a 5% CO_2 incubator. The cells were then washed twice with PBS (200 μL), fixed with 5% HCHO (200 μL) in the presence of DAPI (for nuclear staining, 1 μL (1.0 mg mL^{-1})) at 4 °C for 20 min. The cells were then washed twice with PBS (200 μL), treated with one drop of antifade solution (SlowFade, Molecular Probes, Eugene, OR) and the images of the samples were collected using a fluorescence microscope (Eclips TE2000-U, Nikon, Tokyo, Japan) with a confocal scan unit (Radiance2100, Bio-rad, Tokyo, Japan) attached.

Cytotoxicity for carriers determined by a WST-8 assay. J774.A1 cells were plated in 96-well dishes using 1×10^6 cells per mL (1 well per 100 μL) and were allowed to attach to the plate for 5 h. An appropriate amount of the CpG ODN (35 μg mL^{-1}) and the polymer–CpG ODN complex (CpG ODN: 35 μg mL^{-1}, [polymer]/[CpG ODN] = 2 (and 4 for PEI)) were added to the J774.A1 cell cultures. Subsequently, the cells were incubated for 24 h before measurement of the cell growth. The cell number was evaluated by use of a Cell Counting Kit-8® (Dojindo, Japan), called a WST-8 assay. The WST-8 assay uses a tetrazolium salt, which produces a water-soluble formazane dye upon reduction mediated by dehydrogenase in living cells. After incubation for 24 h, 10 μL of the Cell Counting Kit-8 working solution (containing WST-8 and 1-methoxy-5-methylphenazinium methosulfate) were added to each well and the cells were incubated for 4 h at 37 °C. Plates were read on a microplate reader Multiskan JX (Thermo Labsystems) using a wavelength of 450 nm in comparison with 650 nm. Each control or treated cells was tested in triplicate wells, and the mean and standard deviation of the values were plotted.

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Notes and references

1 (*a*) L. Gros, H. Ringsdorf and H. Schupp, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 305; (*b*) A. V. Kabanov, E. V. Batrakova and D. W. Miller, *Adv. Drug Delivery Rev.*, 2003, **55**, 151; (*c*) H. Otsuka, Y. Nagasaki and K. Kataoka, *Adv. Drug Delivery Rev.*, 2003, **55**, 403; (*d*) R. Duncan, *Nat. Rev. Drug Discovery*, 2003, **2**, 347; (*e*) R. Haag and F. Kratz, *Angew. Chem., Int. Ed.*, 2006, **45**, 1198.

2 (*a*) D. Luo and W. M. Saltzman, *Nat. Biotechnol.*, 2000, **18**, 33; (*b*) G. Zuber, E. Dauty, M. Nothisen, B. Belguise and J. P. Behr, *Adv. Drug Delivery Rev.*, 2001, **52**, 245; (*c*) D. Putnam, *Nat. Mater.*, 2006, **5**, 439.

3 (*a*) A. M. Krieg, *Nat. Med.*, 2003, **9**, 831; (*b*) G. K. Mutwiri, A. K. Nichani, S. Babiuk and L. A. Babiuk, *J. Controlled Release*, 2004, **97**, 1; (*c*) Y. Kuramoto, M. Nishikawa, K. Hyoudou, F. Yamashita and M. Hashida, *J. Controlled Release*, 2006, **115**, 226 references cited therein.

4 (*a*) M. Mizu, K. Koumoto, T. Anada, T. Matsumoto, M. Numata, S. Shinkai, T. Nagasaki and K. Sakurai, *J. Am. Chem. Soc.*, 2004, **126**, 8372; (*b*) N. Shimada, C. Coban, Y. Takeda, M. Mizu, J. Minari, T. Anada, Y. Torii, S. Shinkai, S. Akira, K. J. Ishii and K. Sakurai, *Bioconjugate Chem.*, DOI: 10.1021/bc0700178.

5 (*a*) K. Sakurai and S. Shinkai, *J. Am. Chem. Soc.*, 2000, **122**, 4520; (*b*) K. Sakurai, M. Mizu and S. Shinkai, *Biomacromolecules*, 2001, **2**, 641.

6 K. Sakurai, K. Uezu, M. Numata, T. Hasegawa, C. Li, K. Kaneko and S. Shinkai, *Chem. Commun.*, 2005, 4383.

7 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596.

8 M. Ikeda, T. Hasegawa, M. Numata, K. Sugikawa, K. Sakurai, M. Fujiki and S. Shinkai, *J. Am. Chem. Soc.*, 2007, **129**, 3979.

9 Y. Aramaki, S. Yotsumoto, H. Watanabe and S. Tsuchiya, *Biol. Pharm. Bull.*, 2002, **25**, 351.

10 (*a*) T. Hasegawa, M. Umeda, M. Numata, C. Li, A.-H. Bae, T. Fujisawa, S. Haraguchi, K. Sakurai and S. Shinkai, *Chem. Lett.*, 2006, **35**, 82; (*b*) T. Hasegawa, M. Umeda, M. Numata, C. Li, A.-H. Bae, T. Fujisawa, S. Haraguchi, K. Sakurai and S. Shinkai, *Carbohydr. Res.*, 2006, **341**, 35.

11 T.-C. Mou, M. Shen, S. Abdalla, D. Delamora, E. Bochkareve, A. Bochkarev and D. M. Gray, *Chirality*, 2006, **18**, 370.

12 S. D. Conner and S. L. Schmid, *Nature*, 2003, **422**, 37.

13 (*a*) M. Mizu, K. Koumoto, T. Anada, K. Sakurai and S. Shinkai, *Biomaterials*, 2004, **25**, 3109; (*b*) M. Mizu, K. Koumoto, T. Anada, R. Karinaga, T. Kimura, T. Nagasaki, S. Shinkai and K. Sakurai, *Bull. Chem. Soc. Jpn.*, 2004, **77**, 1101.

14 H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira, *Nature*, 2000, **408**, 740.

15 (*a*) O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J.-P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297; (*b*) N. D. Sonawane, F. C. Szoka, Jr. and A. S. Verkman, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **278**, 44826.

16 N. Shimada, K. J. Ishii, Y. Takeda, C. Coban, Y. Torii, S. Shinkai, S. Akira and K. Sakurai, *Bioconjugate Chem.*, 2006, **17**, 1136.

