Published in final edited form as:

ACS Nano. 2020 January 28; 14(1): 1084–1092. doi:10.1021/acsnano.9b08750.

Sequence Multiplicity within Spherical Nucleic Acids

Ziyin N. Huang,

Northwestern University, Evanston, Illinois

Lisa E. Cole,

Northwestern University, Evanston, Illinois

Cassandra E. Callmann,

Northwestern University, Evanston, Illinois

Shuya Wang,

Northwestern University, Evanston, Illinois

Chad A. Mirkin

Northwestern University, Evanston, Illinois

Abstract

The synthesis and evaluation of spherical nucleic acids (SNAs) incorporating two physically and chemically distinct classes of oligonucleotides (ODNs) at programmed ratios are described. These SNAs are single entity agents that enter the same target cell at defined stoichiometries, and as such allow one to control important cell signaling and regulatory processes. To study the effect of sequence multiplicity within such structures, we synthesized SNAs consisting of a mixture of class A CpG and class B CpG, immunostimulatory ODNs that activate two different toll-like receptor 9 signaling pathways, each in a sequence-specific fashion. These dual-CpG SNAs exhibit high cellular uptake and codelivery of the two ODNs, relative to mixtures of the linear ODN counterparts, and remain highly associated inside the cell over time. Furthermore, the dual-CpG SNAs augment dendritic cell maturation, compared to the same amounts of oligonucleotides delivered in linear or SNA form but not conjugated to one another. Consequently, these structures constitute a platform for designing oligonucleotide-based combination therapeutics with highly tailorable activities.

Graphical Abstract

Corresponding Author: chadnano@northwestern.edu.

Author Contributions

Z.N.H., L.E.C., C.E.C., S.W., and C.A.M. designed experiments; Z.N.H. performed experiments and analysis; L.E.C., C.E.C., and S.W. assisted with isolation of femur bone from mouse; Z.N.H. and L.E.C. performed primary bone marrow-derived DC generation; and Z.N.H., C.E.C., and C.A.M. wrote the manuscript with input from all authors.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.9b08750>.

Oligonucleotide synthesis and sequences used, additional discussion on CpG-A SNAs, supplementary figures of additional characterization (cryoTEM images, DLS, agarose gel), flow cytometry, and confocal microscopy data (PDF)

The authors declare no competing financial interest.

Keywords

spherical nucleic acids; DNA nanotechnology; biotechnology; microscopy; immunotherapy

Spherical nucleic acids (SNAs) are a class of nanostructures consisting of a nanoparticle core surrounded by a shell of highly oriented and densely packed oligonucleotides.^{1,2} Such structures are typically more stable than comparable linear strands in biological milieu and exhibit high cellular internalization *via* scavenger receptor-mediated endocytosis.³ SNAs made with liposome cores, termed liposomal SNAs (LSNAs), are biocompatible and suitable for a wide variety of biomedical applications.^{4–9} To date, with the exception of the use of secondary strands as diluents,¹⁰ material-directing units,^{11,12} or probe entities,^{13–15} SNAs have been prepared exclusively from a single oligonucleotide sequence. However, there are numerous applications where it may be beneficial to deliver multiple oligonucleotide sequences to the same target cell.¹⁶ Therefore, we sought to elucidate the additive effects of sequence multiplicity (i.e., multiple distinct oligonucleotide sequences on a single SNA construct) on the chemical and physical properties of SNAs in the context of a cell-regulatory process, immunostimulation.

Previous work has shown that SNAs with excellent immunostimulatory and immunomodulatory properties can be generated by employing synthetic oligodeoxynucleotides that contain unmethylated cytosine-guanosine sequences, known as a CpG motif (CpG ODN), as the oligonucleotide shell.^{5–8} Treatment with immunostimulatory SNAs has demonstrated superior antitumor efficacy in multiple mouse models of cancer.^{5,8} CpG ODNs, oligonucleotides that mimic the genome of most bacteria and DNA viruses, are toll-like receptor 9 (TLR9) ligands that induce downstream activation of the myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway, which leads to secretion of type I interferons (IFN) and cytokines.^{17,18} CpG ODNs are generally categorized into a few classes. Among them, class A and class B CpG ODNs (CpG-A and CpG-B, respectively) were the first two classes identified and are optimal in activating different immune pathways, while other CpG classes show intermediate responses.¹⁹ CpG-A has a partial phosphodiester (PO) backbone that consists of an internal CpG-containing palindrome and a partial phosphorothioate (PS) backbone that consists of poly(G) sequences on the terminals of the strands, while CpG-B has a fully PS backbone with hexamer CpG motifs. It has been reported in the literature that the combination of CpG-A and CpG-B

ODNs has shown striking synergy, resulting in *in vivo* mouse survival after tumor challenge.
20

While this synergistic effect has been observed in *in vivo* tumor models, immune activation as a function of the ratio of CpG-A and CpG-B has not been elucidated. The SNA platform is ideal for carrying out such studies due to its modularity and programmability. Delivering multiple entities on the same particle to immune cells is beneficial over administration of simple mixtures of the individual components, because the nanoparticle scaffold allows for delivery of programmed ratios of each component to the same target cell.^{21,22} In this study, we incorporated two physically (e.g., structural features) and chemically (e.g., backbone chemistry) distinct oligonucleotide sequences, CpG-A (ODN 1585) and CpG-B (ODN 1826), into LSNAs (Scheme 1) to study the intracellular trafficking of the SNAs and their downstream activation of DCs.

RESULTS AND DISCUSSION

To generate SNAs, 3'-cholesterol-terminated oligonucleotides and 50 nm 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes were mixed in a predetermined ratio to achieve the desired CpG ODN loading on the SNA scaffold.^{4,8} First, single-component SNAs, comprised of either all CpG-A or all CpG-B DNA, were synthesized and characterized to understand the impact of sequence and secondary DNA structure on the DNA loading of SNAs. In particular, CpG-B SNAs (Figure S2) have been well-characterized and studied as immunotherapeutics with a maximal loading of 75 CpG-B ODN per 50 nm liposome.^{5,8,9} Conversely, CpG-A ODN has been proposed to have nanoparticle-like properties because it contains a poly(G) sequence that forms G-quadruplexes that can potentially stabilize the palindrome sequence.^{23,24} Thus, CpG-A is rarely incorporated into nanomaterials and has never been used as a component of SNAs. However, these higher-order structures of CpG-A are salt- and temperature-dependent;^{23,25} thus, we hypothesized that CpG-A aggregation could be circumvented by incorporating CpG-A into SNAs during the SNA assembly process (see Supporting Information). Therefore, we first studied the oligonucleotide content of CpG-A in SNAs and determined through agarose gel electrophoresis that up to 150 cholesterol-terminated CpG-As could be inserted into 50 nm DOPC liposomes (Figure 1a). This observation was confirmed by the size increase, as a function of oligonucleotide equivalents, as determined by dynamic light scattering (DLS, Figure 1b). Cryogenic transmission electron microscopy (CryoTEM) analysis revealed that unfunctionalized liposomes pack tightly together, presumably due to the absence of negatively charged surface ligands and therefore minimized electrostatic repulsion between particles (Figure 1c). However, when the liposomes were transformed into SNAs through their external modification with CpG-A, there was a marked increase in interparticle spacing (Figure 1d), a trend that was also observed with CpG-B SNAs (Figure S2). For example, CpG-A SNA interparticle spacing increased with increased oligonucleotide loading from 21.0 ± 3.5 nm (50 strands/SNA, Figure S3) to 27.7 ± 3.7 nm (150 strands/SNA, Figure 1d). These results not only show that higher oligonucleotide loading density leads to higher particle surface coverage, but also creates a more radially oriented ODN shell within the SNA structure. When the DNA/liposome ratio exceeded 150:1, an upward shift in mobility by gel electrophoresis was observed (Figure 1a), indicating a significant size increase in the

population, presumably due to aggregation of the SNAs caused by sequence self-complementarity (see discussion in Supporting Information). To avoid aggregation-specific effects on cellular uptake and downstream immune activation, SNAs incorporating 150 strands of CpG-A ODN per particle were used in further studies.

SNAs incorporating both classes of CpG ODN on the same nanoparticle scaffold were next synthesized by incorporating CpG-A and CpG-B into unfunctionalized DOPC liposomes at defined ratios (Table S2), based on the determined saturation limits of each single-component SNA. Agarose gel electrophoresis reveals that both CpG classes are incorporated into SNAs (Figure S8). To confirm the successful dual functionalization of SNAs with both CpG ODN, Forster resonance energy transfer (FRET), the energy transfer process from an excited fluorophore to an acceptor fluorophore (such as from fluorescein to Cy3) when the donor and acceptor are within the Förster radius, was employed.^{26,27} Dual-CpG SNAs functionalized with 50% fluorescein-labeled CpG-A and 50% Cy3-labeled CpG-B showed a significantly higher FRET efficiency as compared to a 50:50 mixture of two distinct sets of SNAs, each containing one fluorophore-labeled class of CpG ODN (Figure 2a). Such significantly higher FRET efficiency indicates that the two CpG ODN classes are in closer proximity when functionalized on the same SNA compared to when they are functionalized on separate SNAs, reflecting successful dual sequence functionalization on the same SNA.

To evaluate the uptake and immunogenicity of dual-CpG SNAs, mouse bone marrow-derived dendritic cells (DCs), a TLR9-presenting cell line widely utilized for *in vitro* immune stimulation studies using CpG ODNs, were used as a model system.²⁸ DCs incubated with SNAs showed significantly higher uptake as compared to cells treated with linear ODNs (Figure 2b,c) after 30 min. Dual-CpG SNAs showed similar uptake to SNAs functionalized with a single class of CpG ODN (Figures 2b,c, S8) on a per particle basis. As expected, the fluorescence of cells treated with dual-CpG SNAs incorporating fluorescein-labeled CpG-A and Cy5-labeled CpG-B at a 50:50 mol ratio was roughly half the intensity of cells treated with single-component SNAs, as each CpG component encompassed half of the total CpG ODN incorporated into the dual-CpG SNA scaffold. The Pearson correlation coefficient (ρ) is a measure of the linear correlation between two variables, where values greater than 0.8 indicate a very strong positive correlation. In cells incubated with 50:50 dual-CpG SNAs, the value of ρ when analyzing both CpG-A and CpG-B uptake indicates that there is a very strong positive correlation between the uptake of the two sequences ($\rho = 0.849 \pm 0.005$). In addition, the population of doubly positive cells (CpG-A+/CpG-B+) was roughly 5-fold higher in cells treated with dual-CpG SNAs as compared to cells treated with a 50:50 mixture of the two linear CpG ODNs not on SNAs (Figures 2e,f and S9). Together, these results indicate that dual-CpG SNAs lead to higher codelivery of CpG-A and CpG-B to cells. Interestingly, confocal microscopy revealed that even after extended periods (e.g., 48 h), the fluorescence from the two labeled CpG components of 50:50 dual-CpG SNAs still strongly overlapped as quantified by high Manders overlap coefficients, indicating that these materials remain in close proximity during cellular processing, while fluorescence from cells treated with a 50:50 mixture of both linear labeled CpG ODNs showed little overlap (Figures 2g,h and S10).

Although CpG ODNs activate TLR9 and lead to the secretion of cytokines and interferons, different classes of CpG ODNs induce different immune responses due in part to different intracellular trafficking of the oligonucleotides.^{29,30} After entering cells, CpG-A binds to TLR9 preferentially in the early endosome, which activates the interferon regulatory factor 7 (IRF7) pathway and leads to the secretion of type I IFN (e.g., IFN- α). In contrast, CpG-B activates TLR9 primarily in the late endosome, where the nuclear factor KB (NF- κ B) pathway is predominately activated and secretes proinflammatory cytokines. The detailed mechanisms of different activation pathways are still not completely understood, but it is proposed that both the sequence of the oligonucleotides and the pH of the intracellular organelles may contribute. Both factors likely impact the morphology of the CpG-TLR9 complex and consequently the extra-organelle portion of TLR9 responsible for downstream activation. Thus, we generated a series of dual-CpG SNAs with different ratios of CpG-A to CpG-B loading, ranging from 0% CpG-B (100% CpG-A) to 100% CpG-B (0% CpG-A) to study the effects of codelivery on downstream activation, with oligonucleotide loading confirmed by agarose gel electrophoresis (Figure S8). After incubating DCs with 1 μ M SNAs (by total CpG concentration) for 24 h, we found that high CpG-B loading promoted cytokine secretion (Figures 3a,b and S11), indicative of activation of the NF- κ B pathway, while high CpG-A loading led to high IFN- α production, indicative of activation of the IRF7 pathway (Figure 3c). Further, cells treated with single-component CpG-A SNAs or CpG-B SNAs stimulated higher cytokine and interferon production as compared to their linear counterparts. Interestingly, although cytokine secretion increased steadily with increasing CpG-B loading in SNAs, the linear ODN mixture reached a maximum level of cytokine secretion at 30% CpG-B with no enhanced cytokine production observed at higher ratios of CpG-B to CpG-A (Figure 3a,b). Similar behavior was observed for IFN- α production by linear ODN mixtures as a function of CpG-A ratios (Figure 3c).

We hypothesized that the differences in immune activation by SNAs as compared to linear ODNs was a result of the difference in intracellular trafficking of the two constructs. In the case of linear CpG ODNs, each component traveled independently to its preferred intracellular pathway for downstream activation, since the two components were not structurally associated. However, when CpG-A and CpG-B were coupled onto the same entity in dual-CpG SNAs, they remained highly associated over time (Figure 2g). Thus, CpG-A and CpG-B were trafficked to the same location, leading to only a portion of each CpG type delivered to its preferred intracellular organelle for TLR9 activation at any given time. To confirm this hypothesis, we studied the intracellular trafficking of different SNAs using confocal microscopy (Figures S12–S14), as well as the localization of CpG in intracellular organelles (Figures 3d,e, S15). We found that the trafficking of CpG-A and CpG-B ODNs was primarily dictated by the SNA scaffold with sequence identity having a minimal effect on intracellular trafficking when the ODNs were on an SNA. Conversely, the trafficking of mixtures of linear ODNs was dictated by each ODN sequence identity. CpG SNAs, regardless of sequence, were trafficked to early endosomes, late endosomes, and lysosomes at approximately the same rates and on the same time scales (Figure 3d). In general, SNAs colocalized with the early endosome after uptake, followed by a rapid decrease with a concomitant increase of colocalization with the late endosome. There was only an ~0.5 h difference between CpG-A and CpG-B SNAs that colocalized with the early

endosome, and all SNAs showed colocalization with the late endosome with a peak at ~2–3 h. Colocalization of SNAs in lysosomes steadily increased, reaching a plateau at 4 h which was maintained up to 24 h. On the contrary, mixtures of linear CpG-A and CpG-B ODNs were trafficked very differently (Figure 3e). After uptake, linear CpG-A ODN maintained high colocalization with the early endosome, where CpG-A/TLR9 binding is preferred, with a slight decrease in colocalization over time. In contrast, linear CpG-B ODN showed high colocalization with the late endosome at early time points, indicating very rapid traveling to late endosomes, where CpG-B/TLR9 binding is favored. These results may guide the design of SNAs for preferentially delivering various oligonucleotide sequences to specific intracellular organelles following this particular spatiotemporal path, which is otherwise impossible to control for the corresponding linear ODN counterparts.

We further hypothesized that maximum DC activation could be achieved by codelivering an intermediate ratio of CpG-A and CpG-B on the same SNA. Costimulation markers are antigen nonspecific signaling proteins that are necessary for antigen-presenting cells (e.g., DCs) to activate T cells.³¹ In addition to cytokine, chemokine, and interferon productions, TLR9 activation also leads to the upregulation of CD80 and CD86 (also known as B7-1 and B7-2, respectively), two proteins that can bind to CD28 on T cells for critical costimulation and T cell activation.^{32,33} After incubating DCs with dual-CpG SNAs for 24 h, SNAs at different CpG-A/B ratios demonstrated almost consistently superior performance to incubation as compared to linear CpG ODNs at the same ratios (Figures 4, S16). Expression of CD80 and CD86 peaked at 70 mol % CpG-A and 30 mol % CpG-B ratio on SNAs, and the percentage of the CD80⁺/CD86⁺ double-positive population was also the highest at this CpG ratio. These results suggest that dual-CpG SNAs with controlled oligonucleotide ratios can achieve maximal DC maturation, potentially because of the intracellular trafficking pathway taken by SNAs, allowing cells to take advantage of activation by both CpG classes. After SNAs enter cells, a relatively higher amount of CpG-A than CpG-B was needed to effectively bind TLR9 in the early endosome and induce significant downstream activation, because SNAs only remain in the early endosome for a brief time (Figure 3d). Conversely, a relatively lower amount of CpG-B was needed because the SNAs remain colocalized with the late endosome for extended periods, where CpG-B is processed.

CONCLUSIONS

The SNA architecture allows for the delivery of diverse ODN classes at programmable ratios and with high uptake and codelivery to the same target cell. By generating a series of SNAs incorporating CpG-A and CpG-B on the same nanoparticle scaffold at different ratios, we determined that the SNA architecture provides high intracellular association of these two distinct ODN classes. Furthermore, codelivery of both CpG classes on the dual-CpG SNA scaffold leads to synergistic DC costimulatory molecule activation, where cytokine and IFN secretion is proportional to the fraction of each of their activating CpG classes (CpG-B and CpG-A, respectively). Although the two sequences used in this report are both TLR9 ligands, the high association and intracellular trafficking behaviors are governed by the SNA scaffold and are independent of sequence, as these sequences are trafficked to the same location despite being both physically and chemically diverse. Therefore, these findings

should be generalizable to the design and synthesis of SNAs containing any combination of multisequence SNAs for desired applications.

EXPERIMENTAL SECTION

Materials.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids as a solution in chloroform. TLR9 ligands CpG ODN 1585 (Class A), 1826 (Class B), and their corresponding GpC control sequences were synthesized on an ABI 3900 DNA oligonucleotide synthesizer with all reagents purchased from Glen Research (see Supporting Information).

Cell Lines.

Bone marrow-derived DCs were collected from C57BL/6 wildtype mice and processed following the literature protocol,³⁴ and the cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 100 U/mL penicillin-streptomycin (pen-strep, Gibco), and 20 ng/mL recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF, eBioScience). JAWSII, an immortal murine bone marrow-derived DC cell line, was purchased from ATCC (CRL-11904) and cultured in alpha minimum essential medium (Gibco) supplemented with 1 mM sodium pyruvate (Gibco), 5 ng/mL GM-CSF, 20% FBS, and 100 U/mL pen-strep.

Preparation of Liposomal SNAs.

In a typical synthesis, 4 mL of a chloroform solution of DOPC lipid (25 mg/mL) was transferred into a glass vial, and the lipid was dried under nitrogen gas for 30 min and stored *in vacuo* overnight to completely remove organic solvent and generate a thin lipid film. The film was then rehydrated in 10 mL of PBS. The resulting suspension was vortexed for 30 s and sonicated for 30 min. Following five freeze-thaw cycles, the suspension underwent serial extrusions in 200, 100, 80, and 50 nm track-etch membranes to obtain uniform-size liposomes. Liposomes were concentrated using 50K MWCO MicroKros tangential flow filtration (Spectrum Laboratories), and the final concentration was measured using the phosphatidylcholine assay kit (Sigma). To functionalize oligonucleotides onto the surface of liposomes, 100 μ M 3'-cholesterol-functionalized oligonucleotides were mixed with a predetermined amount of liposomes, calculated following Table S2. Samples were held overnight at 37 °C under shaking at 500 rpm.

Materials Characterization.

To assess CpG-A incorporation into SNAs, agarose gel electrophoresis was performed using 1% agarose (Sigma) with SYBR Safe DNA gel stain in 0.5× tris/borate/EDTA (TBE) (ThermoFisher Scientific) under 120 V for 1 h. To assess dual-CpG loading, fluorophore-labeled oligonucleotides (fluorescein-CpG-A and Cy5-CpG-B) were used, and agarose gel electrophoresis was performed using 0.5% agarose in 1× TBE without further staining. Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Zetasizer Nano with ~10 nM samples by particle. CryoTEM was performed with a Hitachi HT7700 TEM with a Gatan cryo-transfer holder, and imaging was performed under

a 120 kV accelerating voltage. CryoTEM samples were prepared by loading 4 μL of sample (100 μM CpG SNA stock solution or 1.33 μM liposome solution) onto 300-mesh copper TEM grids with lacey carbon films (Electron Microscopy Sciences) using a FEI Vitrobot Mark IV with its chamber equilibrated at 4 $^{\circ}\text{C}$ and 100% humidity. The samples were blotted for 5 s and plunged into liquid ethane before they were transferred and stored in liquid nitrogen. FRET measurements were performed using a Synergy plate reader (Biotek) with 10 nM samples by oligonucleotides. FRET efficiency (E) was calculated as

$$E = 1 - \frac{F_{\text{DA}}}{F_{\text{D}}}$$

where F_{DA} and F_{D} are the donor fluorescence intensities with and without an acceptor, respectively.

Confocal Microscopy and Image Analysis.

Cells (2×10^4 JAWSII) in 0.5 mL phenol red-free cell culture medium were plated on gelatin-coated 15 mm coverslips in 24-well plates overnight and incubated with 5 μM SNA by CpG oligonucleotide concentration. Fluorescein-labeled CpG-A ODN and/or Cy5-labeled CpG-B ODN at high concentration were used to ensure a detectable signal after a prolonged time. To track the same amount of SNAs trafficking over time, all cells were treated for 30 min. For the 30 min time points in the intracellular trafficking studies, cells were incubated with media containing either SNAs or linear CpG ODNs for 30 min, then washed, removed from media, fixed, and stained. For all other time points, cells were incubated in SNA or CpG-containing media for 30 min, washed twice, and incubated with fresh media for the additional amount of time prior to fixation and staining. At the end of the treatment, coverslips were incubated in 4% paraformaldehyde in PBS (diluted from a 32% aqueous solution from Electron Microscopy Sciences) for 15 min for fixation and 0.2% Triton-X in PBS (diluted from Triton-X from Sigma) for 10 min for permeation.

For the CpG association study (Figure 2), cells were incubated in Image-iT FX Signal Enhancer (ThermoFisher Scientific) for 30 min and then in Alexa Fluor 568 Phalloidin for 30 min. Phalloidin labels the actin of the cytoskeleton, which here serves as the confirmation of DC structure (the dendrite growth over time may also serve as confirmation of DC maturation, though it is difficult to quantify). For the intracellular trafficking study, coverslips were incubated in 2% bovine serum albumin (BSA) and 0.2% Triton-X in PBS for 1 h for blocking. Rabbit anti-EEA1 (Abclonal), rabbit anti-Rab9 (Abclonal), rabbit anti-LAMP1 (Abcam), and rabbit anti-LC3 (Sigma-Aldrich) primary antibodies diluted 1:1000 in 1% BSA, 0.1% Triton-X in PBS were used for early endosome, late endosome, lysosome, and autophagosome labeling, respectively. Alexa Fluor 568-labeled goat antirabbit IgG H&L secondary antibody (Abcam) diluted 1:1000 in 1% BSA, 0.1% Triton-X in PBS was used to fluorescently tag the intracellular organelle markers. All treatment and staining steps used 0.4 mL volumes, and coverslips were washed twice using 1 mL of PBS or thrice using PBS-Triton-X (for antibody washes) after each step. After staining, coverslips were mounted onto glass slides using ProLong Glass with NucBlue nuclear stain (ThermoFisher Scientific) and

cured overnight. Confocal microscopy was performed using Leica TCS SP8 LSCM with hybrid detectors (HyD) and HyVolution.³⁵

Data processing and colocalization calculations were performed using the Leica Application Suite X (for HyVolution) and an in-house Matlab script. A total of 25 cells were included for the analysis of each sample. Images of individual detection channels were exported as grayscale maps and denoised using deep neural network. Z-stack layers of whole cells were arranged into a three-dimensional array as pseudo-3D reconstruction of the cell image for each channel. Colocalization analysis provides a quantitative means to evaluate the qualitative confocal image data. Manders overlap and colocalization coefficients^{36,37} were used in this study to analyze the colocalization of color pairs from the confocal images. For analyzing the association of the two CpG ODNs, the Manders overlap coefficient, a parameter describing the degree of overlap between two components in an image, was calculated. The Manders overlap coefficient, a derivative of the Pearson's correlation coefficient (r), is defined as

$$r = \frac{\sum_i a_i b_i}{\sqrt{\sum_i (a_i)^2 \cdot \sum_i (b_i)^2}}$$

where a_i and b_i are the gray scale values of the individual voxels i of the color components a and b , which in this case, are the fluorescein and Cy5 signals, respectively. For analysis of colocalization of CpG ODN in the organelles, the Manders colocalization coefficient (M_{ab}) is used which is defined by

$$M_{ab} = \frac{\sum_i a_i b_i > 0}{\sum_i a_i}$$

so that M_{ab} measures the fluorescence fraction of a colocalized with b , which in this case is the ODN signal (fluorescein and/or Cy5) colocalized in the organelle (Alexa Fluor 568). This coefficient is useful since it does not consider the pixels of the second signal (organelles) that do not overlap with the first signal (ODN). Both Manders overlap and colocalization coefficients are signal intensity independent, which make them advantageous in this study to compare markers that may have different signal intensities caused by different uptake amount (SNA vs linear oligonucleotides), CpG signal decrease over time, and difference in organelle antibody labeling.

Flow Cytometry.

All antibodies and staining buffers were purchased from BD Biosciences, and all antibodies were mixed and diluted to 1:200 in staining buffer. For the uptake study, 3×10^5 cells were treated with fluorescein-labeled CpG-A and/or Cy5-labeled CpG-B oligonucleotides with a total oligonucleotide concentration of 1 μ M in 1.2 mL microtubes. After a 30 min incubation at 37 °C, cells were washed three times with 1 mL of PBS and stained with 100 μ L of antibody solution containing rat antimouse CD11b and hamster antimouse CD11c. Pearson

correlation calculations (ρ) were performed using fluorescein (G) and Cy5 (R) signals of each cell into the formula

$$\rho(G, R) = \frac{1}{N-1} \sum_{i=1}^N \left(\frac{G_i - \mu_G}{\sigma_G} \right) \left(\frac{R_i - \mu_R}{\sigma_R} \right)$$

where μ_G and σ_G are the mean and standard deviation of G , respectively, and μ_R and σ_R are the mean and standard deviation of R , respectively. For the dendritic cell activation study, 3×10^5 cells were incubated with 200 μL of 1 μM CpG (linear or SNA) containing media for 24 h in 1.2 mL microtubes. The cells were then washed three times with 1 mL of PBS and stained with 100 μL of antibody solution containing rat antimouse CD11b, hamster antimouse CD11c, hamster antimouse CD80, and rat antimouse CD86 for 15 min at 4 °C. The cells were then fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. Flow cytometry was completed with a BD LSRFortessa flow cytometer system. A total of 30,000 events were collected for each sample, and DCs gated with CD11c+ population. Color compensation was performed using the AbC total antibody compensation bead kit (ThermoFisher Scientific). Data analysis was performed using FlowJo and Matlab.

Cytokine and IFN Measurements.

Cytokine and IFN release were measured using Luminex 200. Assays were performed using mouse magnetic Luminex assay kits purchased from R&D Systems for cytokines and ThermoFisher Scientific for IFN- α . Cells (2×10^5) were treated with 200 μL of media containing 100 nM CpG (linear or SNA) in a round-bottom 96-well plate for 24 h; after centrifugation for 5 min at 1000 rpm, the cell supernatants were collected and transferred to the Luminex assay plate without further dilution. Sample processing and measurements were performed following the manufacturer's specific protocols. Data analysis was performed using Milliplex Analyst software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under awards U54CA199091 and R01CA208783. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The project was also supported by the Prostate Cancer Foundation and the Movember Foundation under award 17CHAL08, and the IDP Sherman Fairchild Foundation through the Robert H. Lurie Comprehensive Cancer Center. This work made use of the IMSERC at Northwestern University and EPIC facility of Northwestern University's NUANCE Center, which has received support from the Soft and Hybrid Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205); the MRSEC program (NSF DMR-1121262) at the Materials Research Center; the International Institute for Nanotechnology (IIN); the Keck Foundation; and the State of Illinois through the IIN. Confocal microscopy was partially performed at the Biological Imaging Facility at Northwestern University, graciously supported by the Chemistry for Life Processes Institute, the NU Office for Research, and the Rice Foundation. Computation was supported in part through the computational resources provided for the Quest high performance computing facility at Northwestern University which is jointly supported by the Office of the Provost, the Office for Research, and Northwestern University Information Technology. Flow cytometry was supported by the Northwestern University Flow Cytometry Core Facility supported by Cancer Center Support Grant (NCI CA060553). Z.N.H. acknowledges support by the Northwestern University Graduate School Cluster in Biotechnology, Systems, and Synthetic Biology, which is affiliated with the Biotechnology Training Program

funded by NIGMS Grant T32 GM008449. L.E.C. acknowledges support from Northwestern University's Cancer Nanotechnology Training Program award T32CA186897. C.E.C. acknowledges support from the Eden and Steven Romick Post-Doctoral Fellowship through the American Committee for the Weizmann Institute of Science. S.W. was supported by a fellowship associated with the Chemistry of Life Processes Predoctoral Training Program T32GM105538 at Northwestern University.

REFERENCES

- (1). Mirkin CA; Letsinger RL; Mucic RC; Storhoff JJ A DNA-Based Method for Rationally Assembling Nanoparticles into Macroscopic Materials. *Nature* 1996, 382, 607–609. [PubMed: 8757129]
- (2). Cutler JJ; Auyeung E; Mirkin CA Spherical Nucleic Acids. *J. Am. Chem. Soc* 2012, 134, 1376–1391. [PubMed: 22229439]
- (3). Choi CH; Hao L; Narayan SP; Auyeung E; Mirkin CA Mechanism for the Endocytosis of Spherical Nucleic Acid Nanoparticle Conjugates. *Proc. Natl. Acad. Sci. U. S. A* 2013, 110, 7625–7630. [PubMed: 23613589]
- (4). Banga RJ; Chernyak N; Narayan SP; Nguyen ST; Mirkin CA Liposomal Spherical Nucleic Acids. *J. Am. Chem. Soc* 2014, 136, 9866–9869. [PubMed: 24983505]
- (5). Radovic-Moreno AF; Chernyak N; Mader CC; Nallagatla S; Kang RS; Hao L; Walker DA; Halo TL; Merkel TJ; Rische CH; Anantatmula S; Burkhart M; Mirkin CA; Gryaznov SM Immunomodulatory Spherical Nucleic Acids. *Proc. Natl. Acad. Sci. U. S. A* 2015, 112, 3892–3897. [PubMed: 25775582]
- (6). Guan C; Chernyak N; Dominguez D; Cole L; Zhang B; Mirkin CA RNA-Based Immunostimulatory Liposomal Spherical Nucleic Acids as Potent TLR7/8 Modulators. *Small* 2018, 14, No. 1803284.
- (7). Ferrer JR; Wertheim JA; Mirkin CA Dual Toll-Like Receptor Targeting Liposomal Spherical Nucleic Acids. *Bioconjugate Chem.* 2019, 30, 944–951.
- (8). Wang S; Qin L; Yamankurt G; Skakuj K; Huang Z; Chen PC; Dominguez D; Lee A; Zhang B; Mirkin CA Rational Vaccinology with Spherical Nucleic Acids. *Proc. Natl. Acad. Sci. U. S. A* 2019, 116, 10473–10481. [PubMed: 31068463]
- (9). Yamankurt G; Berns EJ; Xue A; Lee A; Bagheri N; Mrksich M; Mirkin CA Exploration of the Nanomedicine-Design Space with High-Throughput Screening and Machine Learning. *Nat. Biomed. Eng* 2019, 3, 318–327. [PubMed: 30952978]
- (10). Jin R; Wu G; Li Z; Mirkin CA; Schatz GC What Controls the Melting Properties of DNA-Linked Gold Nanoparticle Assemblies? *J. Am. Chem. Soc* 2003, 125, 1643–1654. [PubMed: 12568626]
- (11). Xu X; Rosi NL; Wang Y; Huo F; Mirkin CA Asymmetric Functionalization of Gold Nanoparticles with Oligonucleotides. *J. Am. Chem. Soc* 2006, 128, 9286–9287. [PubMed: 16848436]
- (12). Kim Y; Macfarlane RJ; Jones MR; Mirkin CA Transmutable Nanoparticles with Reconfigurable Surface Ligands. *Science* 2016, 351, 579–582. [PubMed: 26912697]
- (13). Nam JM; Stoeva SI; Mirkin CA Bio-Bar-Code-Based DNA Detection with PCR-Like Sensitivity. *J. Am. Chem. Soc* 2004, 126, 5932–5933. [PubMed: 15137735]
- (14). Prigodich AE; Lee OS; Daniel WL; Seferos DS; Schatz GC; Mirkin CA Tailoring DNA Structure to Increase Target Hybridization Kinetics on Surfaces. *J. Am. Chem. Soc* 2010, 132, 10638–10641. [PubMed: 20681682]
- (15). Prigodich AE; Randeria PS; Briley WE; Kim NJ; Daniel WL; Giljohann DA; Mirkin CA Multiplexed Nanoflares: mRNA Detection in Live Cells. *Anal. Chem* 2012, 84, 2062–2066. [PubMed: 22288418]
- (16). Ming X; Laing B Bioconjugates for Targeted Delivery of Therapeutic Oligonucleotides. *Adv. Drug Delivery Rev* 2015, 87, 81–89.
- (17). Krieg AM Therapeutic Potential of Toll-Like Receptor 9 Activation. *Nat. Rev. Drug Discovery* 2006, 5, 471–484. [PubMed: 16763660]
- (18). Klinman DM Immunotherapeutic Uses of CpG Oligodeoxynucleotides. *Nat. Rev. Immunol* 2004, 4, 249–258. [PubMed: 15057783]

- (19). Vollmer J; Weeratna R; Payette P; Jurk M; Schetter C; Laucht M; Wader T; Tluk S; Liu M; Davis HL; Krieg AM Characterization of Three CpG Oligodeoxynucleotide Classes with Distinct Immunostimulatory Activities. *Eur. J. Immunol* 2004, 34, 251–262. [PubMed: 14971051]
- (20). Blazar BR; Krieg AM; Taylor PA Synthetic Unmethylated Cytosine-Phosphate-Guanosine Oligodeoxynucleotides Are Potent Stimulators of Antileukemia Responses in Naive and Bone Marrow Transplant Recipients. *Blood* 2001, 98, 1217–1225. [PubMed: 11493473]
- (21). Ashley CE; Carnes EC; Phillips GK; Padilla D; Durfee PN; Brown PA; Hanna TN; Liu J; Phillips B; Carter MB; Carroll NJ; Jiang X; Dunphy DR; Willman CL; Petsev DN; Evans DG; Parikh AN; Chackerian B; Wharton W; Peabody DS; et al. The Targeted Delivery of Multicomponent Cargos to Cancer Cells by Nanoporous Particle-Supported Lipid Bilayers. *Nat. Mater* 2011, 10, 389–397. [PubMed: 21499315]
- (22). Irvine DJ Drug Delivery: One Nanoparticle, One Kill. *Nat. Mater* 2011, 10, 342–343. [PubMed: 21499312]
- (23). Kerkmann M; Costa LT; Richter C; Rothenfusser S; Battiany J; Hornung V; Johnson J; Englert S; Ketterer T; Heckl W; Thalhammer S; Endres S; Hartmann G Spontaneous Formation of Nucleic Acid-Based Nanoparticles Is Responsible for High Interferon-Alpha Induction by CpG-A in Plasmacytoid Dendritic Cells. *J. Biol. Chem* 2005, 280, 8086–8093. [PubMed: 15591070]
- (24). de Titta A; Ballester M; Julier Z; Nembrini C; Jeanbart L; van der Vlies AJ; Swartz MA; Hubbell JA Nanoparticle Conjugation of CpG Enhances Adjuvancy for Cellular Immunity and Memory Recall at Low Dose. *Proc. Natl. Acad. Sci. U. S. A* 2013, 110, 19902–19907. [PubMed: 24248387]
- (25). Basu S; Wickstrom E Temperature and Salt Dependence of Higher Order Structure Formation by Antisense C-Myc and C-Myb Phosphorothioate Oligodeoxyribonucleotides Containing Tetraguanylate Tracts. *Nucleic Acids Res.* 1997, 25, 1327–1332. [PubMed: 9060425]
- (26). Förster T. Zwischenmolekulare Energiewanderung Und Fluoreszenz. *Ann. Phys* 1948, 437, 55–75.
- (27). Meckes B; Banga RJ; Nguyen ST; Mirkin CA Enhancing the Stability and Immunomodulatory Activity of Liposomal Spherical Nucleic Acids through Lipid-Tail DNA Modifications. *Small* 2018, 14, 1702909.
- (28). Dearman RJ; Cumberbatch M; Maxwell G; Basketter DA; Kimber I Toll-Like Receptor Ligand Activation of Murine Bone Marrow-Derived Dendritic Cells. *Immunology* 2009, 126, 475–484. [PubMed: 18778283]
- (29). Lee BL; Barton GM Trafficking of Endosomal Toll-Like Receptors. *Trends Cell Biol.* 2014, 24, 360–369. [PubMed: 24439965]
- (30). Majer O; Liu B; Barton GM Nucleic Acid-Sensing TLRs: Trafficking and Regulation. *Curr. Opin. Immunol* 2017, 44, 26–33. [PubMed: 27907816]
- (31). Schwartz RH A Cell Culture Model for T Lymphocyte Clonal Anergy. *Science* 1990, 248, 1349–1356. [PubMed: 2113314]
- (32). Lancaster GI; Khan Q; Drysdale P; Wallace F; Jeukendrup AE; Drayson MT; Gleeson M The Physiological Regulation of Toll-Like Receptor Expression and Function in Humans. *J. Physiol* 2005, 563, 945–955. [PubMed: 15661814]
- (33). June CH; Bluestone JA; Nadler LM; Thompson CB The B7 and CD28 Receptor Families. *Immunol. Today* 1994, 15, 321–331. [PubMed: 7522010]
- (34). Madaan A; Verma R; Singh AT; Jain SK; Jaggi M A Stepwise Procedure for Isolation of Murine Bone Marrow and Generation of Dendritic Cells. *J. Biol. Methods* 2014, 1, No. 1.
- (35). Borlinghaus RT; Kappel C Hyvolution-the Smart Path to Confocal Super-Resolution. *Nat. Methods* 2016, 13, I–III.
- (36). Manders EM; Stap J; Brakenhoff GJ; van Driel R; Aten JA Dynamics of Three-Dimensional Replication Patterns during the S-Phase, Analysed by Double Labelling of DNA and Confocal Microscopy. *J. Cell Sci* 1992, 103, 857–862. [PubMed: 1478975]
- (37). Manders EMM; Verbeek FJ; Aten JA Measurement of Co-Localization of Objects in Dual-Colour Confocal Images. *J. Microsc* 1993, 169, 375–382.

Figure 1. Characterization of CpG-A SNAs. (a) Agarose gel electrophoresis of liposomes functionalized with different amounts of cholesterol-labeled CpG-A ODN; the symbol ∞ indicates free cholesterol-labeled CpG-A ODNs. (b) Hydrodynamic diameter of CpG-A SNAs as measured by DLS. CryoTEM images of (c) bare liposomes and (d) SNAs functionalized with 150 CpG-A ODNs. Scale bars: 100 nm. Insets: schematics of the corresponding constructs.

Figure 2.

Characterization of dual-CpG SNAs (50:50 mol ratio CpG-A to CpG-B). (a) FRET efficiency comparison between fluorescein-CpG-A SNA/Cy3-CpG-B SNA mixture and dual-CpG SNA. Fluorescence signals of (b) fluorescein-CpG-A and (c) Cy5-CpG-B after 30 min treatments in single-component or dual-CpG form. (d) Pearson correlation coefficient (ρ) between CpG-A and CpG-B uptake in dual-CpG SNA and linear CpG mixture forms. Representative flow cytometry data of (e) dual-CpG SNA and (f) linear CpG mixture uptake. (g) Manders overlap coefficients between CpG-A and CpG-B in dual-CpG SNA and linear CpG mixture forms over time after cellular uptake. (h) Representative confocal microscopy images of dual-CpG SNA 30 min, 4 h, and 24 h after initial uptake. Color assignments: fluorescein-CpG-A (green), Cy5-CpG-B (red), nucleus (blue), actin (gray). Scale bars: 5 μ m.

Figure 3.

Cytokine and IFN production following intracellular localization. Production of (a) TNF- α , (b) IL-1 α , and (c) IFN- α (#, signal below the lower limit of detection for untreated samples) after 24 h of treatment. (**, $p < 0.01$; *, $p < 0.05$). Manders colocalization coefficients of (d) CpG-SNAs and (e) CpG ODN in intracellular markers over time. Dual-CpG data shown in (d,e) incorporated a 1:1 ratio of CpG-A to CpG-B.

Figure 4.

DC costimulation marker activation. (a) Representative flow cytometry data for CD80 and CD86 expression after 24 h treatment of CpG SNAs or linear CpG ODNs. Mean fluorescence intensity (MFI) of (b) CD80 and (c) CD86 expression. (d) Percentage of CD80⁺/CD86⁺ double-positive cell population after treatment. (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

Scheme 1. SNA Designs with Sequence Multiplicity^a

^aUpper case letters: nucleobases with PO backbone; lower case letters: nucleobases with PS backbone; chol = cholesterol anchor.