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Impact of Sequence Specificity of Spherical Nucleic Acids on Macrophage Activation in Vitro and in Vivo

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Abstract

The effects of spherical nucleic acid (SNA) gold nanoparticle conjugates on the activation of macrophages in vitro and release of cytokines in vivo were explored. Herein, we show that G-quadruplexes, the formation of which is enhanced on gold nanoparticle surfaces, elicit an increase in cytokine release from mouse and human macrophages and induce the upregulation of activation receptors as well as NO₂ production in vitro. Moreover, these G-rich SNAs can induce cytokine release when injected intravenously, though there were no severe, long-term effects observed. These results further reinforce the notion that nucleic acid sequence and structure play an important role in how SNAs interact in biological milieu and highlight a key design parameter.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ASSOCIATED CONTENT

Supporting Information

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SNA nanoparticle characterization, IL-10 generation of SNAs in macrophages, and cytokine generation by G-rich or poly-T DNA (PDF)

The authors declare the following competing financial interest(s): C.A.M. has financial interest in/relative to Exicure Inc. and Aurasense LLC, companies developing SNA therapeutic platforms, which could potentially benefit from the outcomes of this research.

Keywords

G-quadruplex; macrophage activation; gold nanoparticles; spherical nucleic acids

INTRODUCTION

Spherical nucleic acids (SNAs) are an emerging class of nanomaterials consisting of a nucleic acid shell surrounding a nanoparticle core.^{1,2} Due, in part, to the dense, oriented oligonucleotide structure, SNAs behave differently than their linear counterparts and exhibit enhanced interactions with serum and cell surface proteins, resulting in higher cellular association and uptake.^{3–7} Consequently, SNAs have been developed for many gene regulation applications, including psoriasis and glioblastoma, as well as immunomodulation applications, and are in clinical trials for both.^{2,8–24}

Previously, it has been shown that immediately after SNAs are introduced to serum, serum proteins bind to the SNA and form a protein corona.^{5,7} Furthermore, the identity, number, and types of proteins that bind are dependent on the DNA sequence. Specifically, the presence of G-quadruplexes on the SNA alters protein corona formation and results in differences in cellular uptake and in vivo biodistribution.^{5,7} Evidence of enhanced uptake of guanine-rich (G-rich) SNAs by macrophages in vitro and macrophage-rich tissues, such as the liver and spleen, in vivo^{5,7} suggest that G-rich SNAs may also induce macrophage activation. Although SNAs do not inherently elicit an immune response in epithelial cells in vitro, they may be designed to modulate the immune response using oligonucleotide motifs common to pathogens.^{17,24–26} In this report, we explore for the first time how changes in nonimmunogenic nucleic acid sequences of SNAs can impact their interactions with macrophages in vitro. Understanding the role of the nucleic acid sequence on macrophage activation is crucial for the design of safe and efficacious SNAs.

Herein we explore the effect of nucleic acid sequence on macrophage activation in vitro and in vivo through the use of two SNA constructs: gold nanoparticles (AuNPs) fractionalized

with either a G-rich sequence that forms G-quadruplexes or a poly-T sequence (Scheme 1). We find that G-rich SNAs in the presence of serum proteins do indeed interact with macrophages differently than their poly-T counterparts and induce both cytokine and nitric oxide (NO) production as well as upregulate a cell surface receptor linked with early macrophage activation in vitro. Furthermore, G-rich SNAs induce increases in cytokine production in mice hours post injection, but no severe, long-term immune response was observed. These results highlight the role of the surface chemistry of nanoparticles, and nucleic acid sequence specifically, in modulating their interactions with cells both in vitro and in vivo.

EXPERIMENTAL SECTION

Synthesis of SNAs.

Citrate stabilized AuNPs (13 nm) were synthesized via the Frens method²⁷ and their size was characterized using dynamic light scattering (DLS, Malvern). Thiolated G-rich and poly-T DNA were synthesized using an MM48 Oligonucleotide synthesizer (BioAutomation) using standard solid-phase synthesis reagents (Glen Research). DNA was deprotected from its solid support and purified using high pressure liquid chromatography (HPLC) on a C18 column (Varian). DNA (30 nmol, stock solution at 100 μ M) was added to 10 mL of 10 nM AuNPs and incubated for 2 h at room temperature with agitation. NaCl was added to the mix of DNA and AuNPs gradually until the final concentrations reached 0.5 M (G-rich SNAs) and 1 M (poly-T SNAs) to ensure similar loading densities, as discussed in our previous work.²⁸ After salting, SNAs were concentrated using centrifugation (max speed = 16 873g), washed, and suspended in 1 $\times$ PBS, pH 7.4 at a concentration of 100 nM (by SNA) for all downstream in vitro and in vivo experiments. The concentration of DNA and AuNPs was measured using UV–visible spectrophotometry (Agilent) at 260 and 524 nm, respectively. Once formulated, the SNA concentration was determined using UV–vis spectrophotometry at 524 nm, with $\epsilon = 2.7 \times 10^8$ L/(mol cm). Then, the number of DNA strands per particle was determined by dissolving the AuNP cores using 40 mM KCN and measuring the DNA concentration via an Oligreen assay (Life Technologies).

Cell Culture.

THP-1 (ATCC TIB-202), Raw 264.7 (ATCC TIB-271), and Raw-Blue (invivoGen) cells were cultured in a 5% CO₂ incubator following ATCC's recommended instructions. THP-1 cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin supplemented with 0.05 mM 2-mercaptoethanol. Raw 264.7 and Raw-Blue cells were cultured in DMEM (Gibco) containing 10% FBS and 1% penicillin/streptomycin and subcultured by scraping. These cell lines were chosen to represent macrophages derived from human and mouse origin.

Cytokine Release Quantification.

A total of 100 000 THP-1 cells were seeded per well in a 96-well plate with RPMI 1640 containing 20% FBS and 100 nM phorbol myristate acetate (PMA, InvivoGen) to differentiate for 72 h. Cells were then incubated with 1 or 5 nM SNA for 4 h in OptiMEM (Gibco) with or without 10% human serum (Sigma-Aldrich) in 96-well plates (100 μ L total

volume). The media was then replaced with RPMI 1640 containing 10% FBS for 18 h. Cell supernatants were collected and analyzed using the BD Cytometric Bead Array-Human Inflammatory Cytokine Kit (BD Biosciences) to determine IL-1 β , IL-6, IL-10, and TNF α levels. A total of 100 000 Raw 264.7 cells were seeded per well in a 96-well plate with OptiMEM (100 μ L total volume) containing 10% human serum and incubated with SNAs (1 to 20 nM) for 24 h. Cell supernatants were collected and analyzed using a Luminex magnetic bead assay (R&D Biosciences) for levels of IL-6, MCP-1, RANTES, and TNF α .

Nitric Oxide Generation.

A total of 30 000 Raw 264.7 cells were seeded per well in a 96-well plate. After 24 h, cells were incubated with 1, 5, 10, or 20 nM SNAs in OptiMEM with or without 10% human serum for 1 or 4 h at a total well volume of 100 μ L. The media was then removed and replaced with DMEM containing 10% FBS for 18 h. Cell supernatants were collected and analyzed using a Griess Reagent System (Promega) to quantify NO₂⁻ release.

Flow Cytometric Analysis of Cell Surface Markers.

A total of 100 000 Raw 264.7 cells were seeded at a density of 10⁶ cells/mL in a 12-well plate for 24 h in DMEM supplemented with 10% FBS before they were incubated with SNAs (either 1 or 5 nM) in OptiMEM supplemented with 10% human serum at a total volume of 1 mL. Murine splenocytes, seeded at a density of 10⁶ cells/mL in a 12-well plate, were incubated with SNAs (1 or 5 nM) in OptiMEM supplemented with 10% human serum. After a 24 h incubation, cells were washed three times with PBS and stained with antibodies for CD69, CD86, and MHC-II (BD Biosciences) for 30 min at 4 °C and then fixed using 4% paraformaldehyde at 4 °C for 30 min. For murine splenocytes, F4/80 antibody was also incubated alongside the other antibodies to identify macrophages via flow cytometry. Samples were then analyzed on an LSRII flow cytometer (BD Biosciences). The percent of upregulated CD69, CD86, and MHC-II for each sample was plotted following dead cell exclusion.

In Vivo Cytokine Determination.

The in vivo experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee at Northwestern University. C57BL/6 mice (6–8 weeks old) were injected with 200 μ L of 100 nM G-rich SNA, poly-T SNA, or 1 $\times$ PBS intravenously through the tail vein. Blood samples were collected from mice at 8 and 24 h post injection via a retro-orbital blood draw. Samples were spun down, and serum was used to conduct cytokine analysis using a Cytometric Bead Array-Mouse Inflammatory Cytokine Kit (BD Biosciences). Livers and spleens were collected from mice that were transcardially perfused and sacrificed at 8 h, 24 h, and 28 days post injection. Tissues were immediately fixed using a 10% neutral buffered formalin solution, paraffin-embedded and sectioned, and stained using hematoxylin and eosin (H&E) or terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Roche).

RESULTS

Macrophage activation leads to secretion of nitric oxide (NO), a whole host of cytokines (i.e., TNF α , IL-12, IL-1, IL-1 β , IL-6, IL-10) and chemokines (i.e., IP-10, MIP-1 α , MCP-1), as well as upregulation of biological markers (i.e., MHC- II, CD86).^{29–31} To determine the extent of macrophage activation via the secretion of proinflammatory cytokines and chemokines, human (PMA-differentiated THP-1) and mouse (Raw 264.7) macrophages were treated with G-rich and poly-T SNAs and analyzed for cytokine secretion. The results of a 4 h SNA incubation followed by an 18 h media replenishment period (with 10% FBS) in THP-1 macrophages indicate that G-rich SNAs, more so than poly-T SNAs, cause cells to generate the proinflammatory cytokines IL-1 β , IL-6, and TNF α in the presence of serum proteins at higher treatment concentrations (5 nM) (Figure 1A). Specifically, although baseline levels of TNF α and IL-1 β secretion were detected after treatment with low concentrations of G-rich and poly-T SNAs (1 nM), the presence of serum proteins in addition to both SNAs induced a minor amount of IL-6 secretion. In addition, the levels of IL-1 β and IL-6 secretion were higher for cells treated with G-rich SNAs with and without the addition of serum proteins as compared to their poly-T SNA counterparts, whereas TNF α secretion was especially pronounced for cells treated with G-rich SNAs in the presence of serum proteins. Interestingly, levels of IL-10, an anti-inflammatory cytokine that can activate macrophages through the alternative, or type II, pathway were indistinguishable between control and experimental samples (Figure S1). Experimental samples were run alongside a bacterial lipopolysaccharide (LPS, 200 ng/mL) positive control, which exhibited similar IL-1 β and TNF α production but much higher IL-6 production (Figure 1A). This indicates that while G-rich SNAs can induce cytokines, it is less severe than the induction achieved by LPS.

In mouse macrophages, the trend is similar: G-rich SNAs coated with serum proteins after a 24 h incubation induce proinflammatory cytokines and chemokines (IL-6, MCP-1, RANTES, and TNF α) at higher levels than that of poly-T SNAs (Figure 1B). Interestingly, upon exploring higher SNA concentrations, a dose dependency arises. Meanwhile, cytokine production remains unchanged for both linear G-rich and poly-T DNA (Figure S2) for cells treated with and without serum, suggesting that the serum protein interaction with the nucleic acid shell on the surface of the SNA is responsible for the observed results. One explanation for the enhanced macrophage activation from serum-coated G-rich SNAs could be due to the fact that they have a higher affinity for and bind more opsonin proteins (such as complement C3b) compared to poly-T SNAs, flagging themselves for macrophage uptake and eliciting activation.^{5,7} It is of note that when compared to a similar dose of immunostimulatory DNA (CpG, 1826) at 1 μ M by DNA (equivalent in DNA amount to about 15 nM of SNA), SNA activation levels are significantly lower (Figure 1B).

Although cytokine production in both human and mouse macrophages indicates a greater extent of macrophage activation upon treatment with G-rich SNAs in the presence of serum proteins, a second measure of activation was also probed: degree of nitric oxide (NO) production.^{29–33} Raw 264.7 mouse macrophages were incubated for 1 or 4 h with G-rich and poly-T SNAs in the absence and presence of serum before the media (containing 10% FBS) was replenished for 18 h and analyzed for nitrite (NO₂[−]), the primary oxidation product of

NO under physiological conditions (Figure 2A). A Griess reagent assay (to quantify nitrite concentration in the media) indicated that after a 1 h incubation, cells treated with protein-coated G-rich SNAs induced significantly more NO secretion than G-rich SNAs given without human serum or poly-T SNAs at 1 or 5 nM, with or without the presence of human serum. At 10 and 20 nM, however, both types of SNAs induced NO generation, suggesting macrophage activation, an observation that is consistent with the increase in proinflammatory cytokines and chemokine production at higher SNA concentrations (Figure 1). After a 4 h incubation, all cells exhibit enhanced NO production compared to cells treated for 1 h. Interestingly, incubation with protein-coated G-rich SNAs for 4 h exhibits near-saturation levels at a concentration as low as 1 nM, a similar level that was observed at 20 nM after 1 h (Figure 2A).

Although enhanced NO production was seen across all constructs with increasing nanoparticle concentration and incubation time, protein-coated G-rich SNAs induced the highest levels of NO production across all tested groups at each concentration, a conclusion that is consistent with the cytokine profile results (Figure 1). Moreover, upon a deeper analysis into NF- κ B, a transcription factor for proinflammatory genes, expression levels in Raw 264.7 mouse macrophages also reach the same conclusion: G-rich SNAs induce higher levels of macrophage activation than poly-T SNAs, or G-rich or poly-T DNA (Figure S3). This is to be expected, as increased NF- κ B expression can be triggered by TNF α and NO production.^{31,32} These results further reinforce the notion that changes in nucleic acid sequence can alter SNA–protein interactions and consequently affect SNA–cell interactions.

Activated macrophages can also exhibit changes in the expression levels of cell surface receptors.^{29–31} To test this, the upregulation of cell surface receptors CD69, CD86, and MHC-II was measured in mouse macrophages (Raw 264.7, Figure 2B) using flow cytometry. Interestingly, neither G-rich nor poly-T SNAs induce enhanced expression of CD86 or MHC-II in mouse macrophages, typical markers of macrophage activation.^{29–31} However, G-rich SNAs do exhibit enhanced CD69 activation, a marker of early activation,³⁴ at higher dosages (5 nM), with even higher levels than that induced by LPS dosed at 200 ng/mL (Figure 2B). Although CD69 is not constitutively active in murine macrophages, it can be activated through TNF α ,³⁴ which is secreted in a dose dependent manner from Raw 264.7 macrophages upon interaction with G-rich SNAs in the presence of human serum (Figure 1A).

To further explore the impact of G-rich SNAs on CD69 activation, G-rich and poly-T SNAs and DNA were added to mouse splenocytes (Figure 3). Flow cytometry was used to look at the upregulation of cell surface receptors of primary macrophages (F4/80⁺): CD40, CD86, and MHC-II and CD69, in which G-rich SNAs induced CD69 and CD40 upregulation while poly-T SNAs did not. These results indicate that although the mouse macrophages show early stages of activation, they are not fully activated through canonical pathways because of the absence of commonly upregulated cell surface receptors, further emphasizing that SNAs will not cause long-term, nonspecific side effects, which are commonly seen when delivering nucleic acid therapies by other means.^{29–31,34}

Thus far, all the results have indicated that G-rich SNAs, likely because of the composition of the serum proteins in the protein corona, can induce higher levels of macrophage activation than poly-T SNAs. Next, it was important to determine if a similar phenomenon occurs in vivo. To test this, G-rich and poly-T SNAs were injected intravenously in mice at a dosage of approximately 20 pmol of SNA (200 μ L at 100 nM SNA) or approximately 1.5 mg of DNA per kg of body weight. Cytokine analysis of the blood after 8 and 24 h shows that G-rich SNAs induced little inflammatory, MCP-1 and IL-12p70, or anti-inflammatory, IL-10, cytokine production over a PBS control injection, except for statistically significant higher levels of IL-6 (Figure 4). This level of IL-6, however, is orders of magnitude lower than if mice were given true immune stimulatory agents, such as bacterial LPS,^{35,36} or even SNAs designed to activate TLR9.¹⁷ Meanwhile, the level of cytokine secretion from poly-T SNAs is not statistically significant compared to a PBS control. These results suggest that although by 8 h, G-rich SNAs are interacting with serum proteins and exhibit an increase in IL-6 production, by 24 h post injection, all cytokine levels have returned to the baseline, suggesting no long-term macrophage activation. This correlates well with previous studies that have probed the longer-term effects of SNA treatment in mice.^{25,26} Furthermore, neither G-rich nor poly-T SNAs exhibit gross histological changes in macrophage-rich tissues, such as the liver or spleen, as determined through hematoxylin and eosin (H&E) staining (Figure 5) at 24 h post injection, again confirming that SNAs do not inherently elicit immune responses that lead to long-term, negative consequences in vivo.

DISCUSSION

This work probes the impact of sequence on an SNA's interaction with macrophages in biological milieu. These observations are important for several reasons. The data suggests that changes in sequence, which are known to lead to differences in the SNA's protein corona composition,^{5,7} can also induce acute macrophage activation. Furthermore, these changes in sequence also translate to an increase in cytokine release shortly after administration in vivo. Taken together, these results might hint at unwanted side effects; however, although sequence can alter the SNA's interactions with macrophages, there is no lasting observable cellular activation as determined through systemic cytokine release at later time points and macrophage-rich tissue histology. Finally, this work highlights the importance of nucleic acid sequence in designing more efficacious SNAs, because the formation of nucleic acid tertiary structures can change the short-term extent of macrophage activation in vitro and cytokine release in vivo. For example, these results could be particularly advantageous for immune modulation purposes, that is, the adjuvancy of a TLR9 activating SNA is likely to be enhanced by either incorporation of G-rich sequences on the surface of the SNA (in addition to the TLR9 activating DNA) or by enriching the TLR9 activating DNA with additional guanine tails at the SNA surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

SNA	spherical nucleic acid
AuNP	gold nanoparticle
PMA	phorbol myristate acetate
H&E	hematoxylin and eosin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
LPS	lipopolysaccharide

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Figure 1.

(A) Cytokine production from human macrophages (PMA-differentiated THP-1) after a 4 h treatment with G-rich or poly-T SNAs in the presence and absence of 10% human serum (HS), followed by complete media replenishment for 18 h, with 10% FBS, as analyzed using a BD Cytometric Bead Array. (B) Cytokine production from mouse (Raw 264.7) macrophages treated with G-rich or poly-T SNAs at varying concentrations for 24 h, in media containing 10% human serum, as analyzed using a Luminex magnetic bead assay.

Statistical analysis was conducted using Tukey's multiple comparison test (* for $p < 0.05$, ** for $p < 0.01$, and **** for $p < 0.0001$).

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Figure 2.

(A) Griess reagent quantifying the levels of NO_2^- secretion by Raw 264.7 mouse macrophages incubated with G-rich or poly-T SNAs in the presence and absence of human serum (HS) after 1 or 4 h. (B) Flow cytometry analysis of cell surface receptor (CD69, CD86, and MHC-II) expression levels in Raw 264.7 macrophages after a 24 h incubation with G-rich or poly-T SNAs. Tukey's multiple comparison test was used to determine statistical significance, where *** denotes $p < 0.001$, and **** denotes $p < 0.0001$.

Figure 3.

Upregulation of cell surface receptors (CD40, CD69, CD86, and MHC-II) in macrophages (F4/80+) after primary splenocytes were treated with a 24 h incubation with G-rich or poly-T DNA or SNAs (at 1 and 5 nM) in the presence of human serum, as determined using flow cytometry. ** denotes $p < 0.01$, and **** denotes $p < 0.0001$ for G-rich SNAs in relation to untreated, as determined using Tukey's multiple comparison test.

Figure 4.

Cytokine release in vivo in response to intravenously injected G-rich or poly-T SNAs. * denotes $p < 0.05$, ** denotes $p < 0.001$, and **** denotes $p < 0.0001$ for all groups compared to PBS control.

Figure 5.
H&E stained spleen and liver 24 h post injection. Scale bar represents 100 μm .

Scheme 1.

Gold Nanoparticles Are Coated with Thiolated G-Rich and Poly-T DNA Strands To Form SNAs