

Self-Assembly of Poly-Adenine-Tailed CpG Oligonucleotide-Gold Nanoparticle Nanoconjugates with Immunostimulatory Activity

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Synthetic unmethylated cytosine–guanine (CpG) oligodeoxynucleotides (CpG ODNs) possess high immunostimulatory activity and have been widely used as a therapeutic tool for various diseases including infection, allergies, and cancer. A variety of nanocarriers have been developed for intracellular delivery of CpG ODNs that are otherwise nonpermeable through the cellular membrane. For example, previous studies showed that gold nanoparticles (AuNPs) could efficiently deliver synthetic thiolated CpG ODNs into cultured cells and induce expression of proinflammatory cytokines. Nevertheless, the necessity of using thiolated CpG ODNs for the modification of AuNPs inevitably complicates the synthesis of the nanoconjugates and increases the cost. A new approach is demonstrated for facile assembly of AuNP–CpG nanoconjugates for cost-effective drug delivery. It is found that non-thiolated, diblock ODNs containing a CpG motif and a poly-adenine (polyA) tail can readily self-assemble on the surface of AuNPs with controllable and tunable density. Such nanoconjugates are efficiently delivered into RAW264.7 cells and induce immune response in a Toll-like receptor 9 (TLR9)-dependent manner. Under optimal conditions, polyA–CpG–AuNPs show significantly higher immunostimulatory activity than their thiolated counterpart. In addition, the immunostimulatory activity of CpG–AuNPs can be modulated by varying the length of the polyA tail. In vivo induction of immune responses in mice is demonstrated by using polyA-tailed CpG–AuNP nanoconjugates.

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1. Introduction

Unmethylated cytosine–guanine (CpG) dinucleotide motifs, which occur frequently in viral and bacterial genomes, are considered as pathogen-associated molecular patterns (PAMPs) and recognized by the Toll-like receptor 9 (TLR9) of host cells.^[1] Activated TLR9 initiates a signaling cascade, via the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) pathway and the mitogen-activated protein kinase (MAPK) pathway, which leads to expression of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), IL-6, IL-12, etc.^[1,2] Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs can mimic the immunostimulatory activity of bacterial DNA, presumably via their interactions with endosomal TLR9.^[3] This property makes CpG ODN a promising therapeutic tool to treat infection, cancer, and allergies.^[3,4] However, free CpG ODNs have poor efficiency of cellular uptake and are prone to be degraded by nucleases, which largely prevents their practical applications as an immunostimulatory drug.^[5] Several approaches have been explored to enhance the stability or cellular uptake of CpG ODNs so that they are more accessible to endosomal TLR9.^[6] For example, the use of a nuclease-resistant phosphorothioate (PS) backbone can greatly increase the uptake of CpG ODNs.^[7] Alternatively, nanomaterials have emerged as carriers to deliver CpG ODNs. Various nanoparticles and nanotubes have been proven to enhance the cellular uptake of CpG ODNs and their immunostimulatory effects.^[8]

Gold nanoparticles (AuNPs) are well known to be nearly noncytotoxic and with minimal immunoactivity.^[9] More interestingly, DNA-AuNP nanoconjugates, in addition to their stability in physiological environments and resistance to nuclease degradation, can enter a variety of cell types without the use of auxiliary reagents, and keep the biological activity of the DNA cargo.^[10] To develop an AuNP-based strategy for the administration of CpG ODNs, we recently reported that thiolated CpG ODNs could be modified on the surface of AuNPs and these nanoconjugates could increase the delivery and enhance immunostimulatory effects of CpG ODNs.^[11] Nevertheless, the necessity of using thiolated CpG ODNs for the modification of AuNPs inevitably complicates the synthesis of the nanoconjugates and increases the cost. Herein, by taking advantage of our recent finding that AuNPs have a high affinity for poly-adenine (polyA),^[12] we develop a cost-effective approach for facile assembly of AuNP-CpG nanoconjugates with non-thiolated, diblock ODNs containing a CpG motif and a poly-adenine (polyA) tail. We test both in vitro and in vivo immune responses of these polyA-tailed CpG-AuNP nanoconjugates.

2. Results and Discussion

2.1. Characterization of CpG-polyA-AuNP Conjugates

Thiolated CpG ODN and two types of polyA-CpG ODNs with different length of polyA tails (A5 and A15) were employed to functionalize 15-nm AuNPs. Representative

transmission electron microscopy (TEM) images of AuNPs and AuNPs-CpG conjugates are shown in **Figure 1A** with graphics explaining different coating strategies. Dynamic light scattering (DLS) analysis indicated that the hydrodynamic diameter of AuNPs increased from 23.4 nm (bare AuNPs) to 30 nm (CpG-SH-AuNPs), 35.3 nm (CpG-A5-AuNPs), or 37.7 nm (CpG-A15-AuNPs) after CpG ODNs were assembled (Figure 1B), which reveals that polyA-tailed CpG ODNs were successfully attached to AuNPs. CpG ODNs with 5'-labeled carboxyfluorescein (FAM) fluorophore were used to determine the number of ODN strands assembled on a single AuNP. Mercaptoethanol (ME) was added into the solution of DNA-AuNPs conjugates to release DNA strands. The fluorescence of released ODN strands was measured and calculated in molar concentrations.^[13] We found that each AuNP was attached to 74 thiolated CpG ODNs, 50 CpG-A5 ODNs, or 24 CpG-A15 ODNs under the same conditions (Figure 1C). The surface

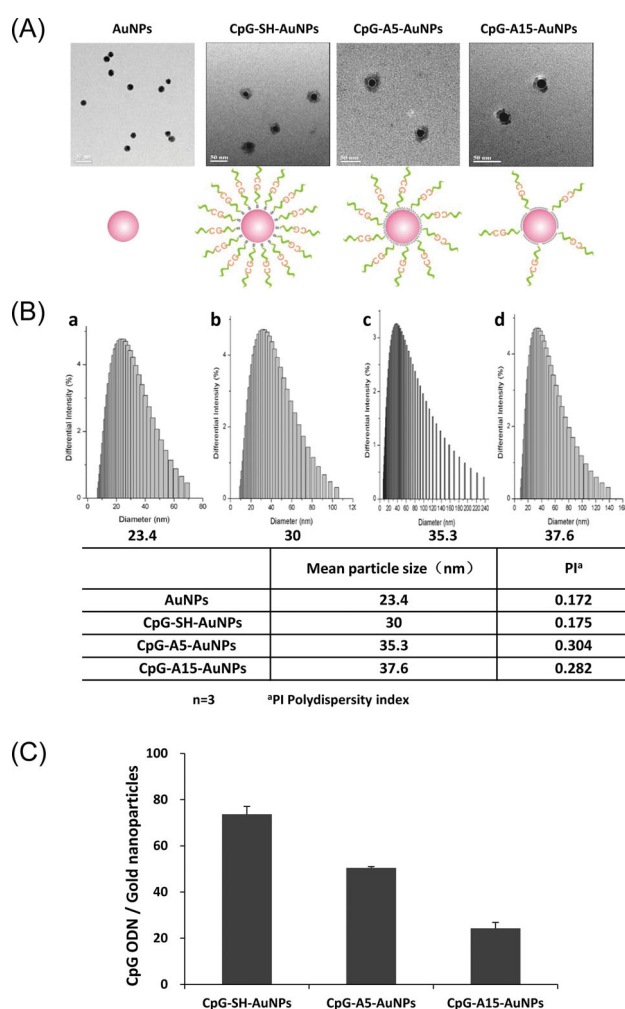


Figure 1. Design and characterization of CpG-AuNP conjugates. A) TEM images and graphics of coating strategies of indicated particle types. Scale bar: 50nm. B) DLS results of AuNPs and CpG-AuNP conjugates. C) Amount of CpG ODN strand per gold nanoparticle. Fluorescence-labeled ODN strands were released by means of ME treatment and quantified.

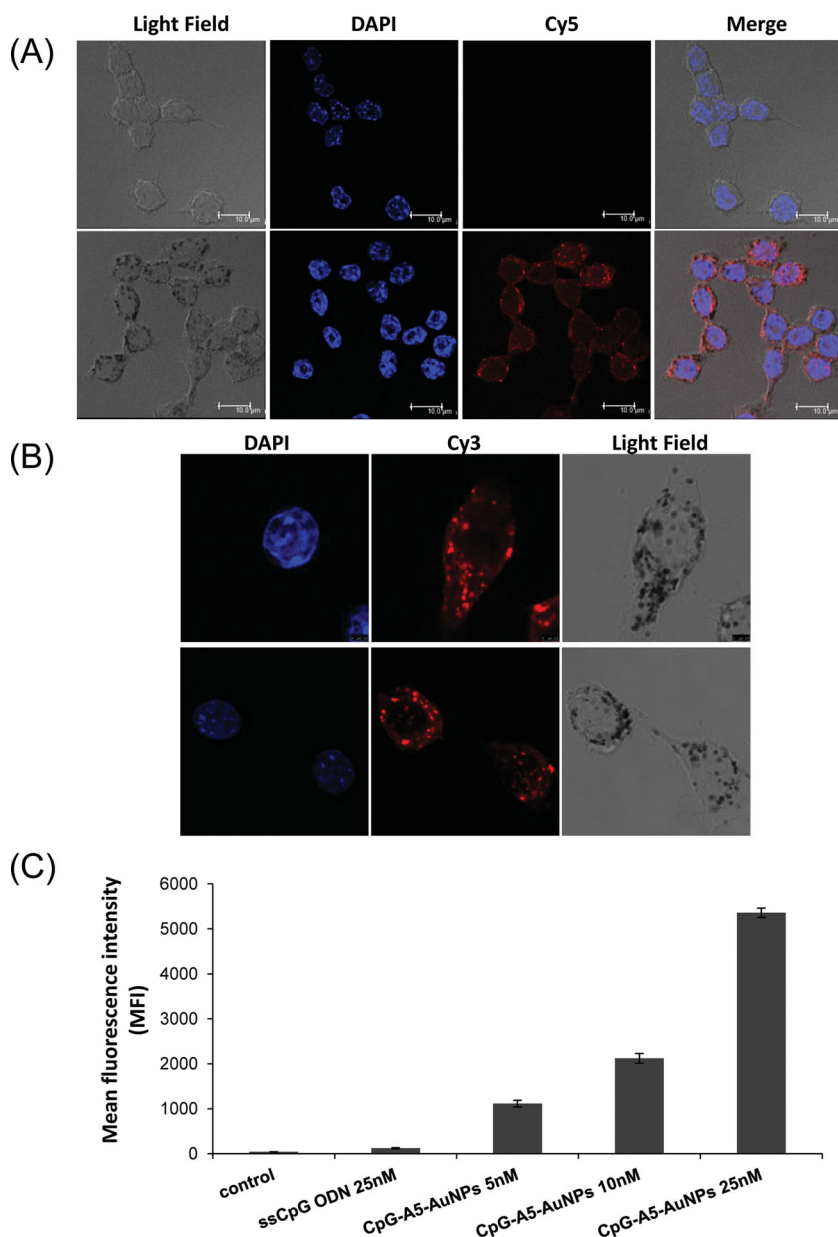


Figure 2. Cellular uptake of CpG-A5-AuNPs. A) Light-field and fluorescence microscopy images of RAW264.7 cells treated with Cy5-ss CpG ODN (upper) or Cy5-CpG-A5-AuNPs (lower) for 4 h. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar: 10 μm. B) Representative cell images at high magnification showed cytoplasmic distribution of Cy5-CpG-A5-AuNPs. C) Flow cytometric analysis of the mean fluorescence intensity in RAW264.7 cells incubated with Cy5-ss CpG DNA or Cy5-CpG-A5-AuNPs for 4 h. Results are expressed as the mean ± SD of two determinations.

density of polyA-tailed ODNs on the AuNP was inversely related to the length of polyA, which suggests that all A bases in the polyA tail are completely adsorbed on AuNPs to enable full surface coverage,^[12] while thiolated ODN has only one contact point for AuNPs. Therefore, polyA tails could provide anchors as well as density control for non-thiolated ODNs. Since the surface density of CpG ODNs varies in different conjugates, the concentration of CpG-AuNP conjugates in this study refers to that of CpG ODNs unless otherwise indicated.

2.2. Efficient Cellular Uptake of CpG-polyA-AuNP Conjugates

To examine whether AuNPs can efficiently deliver CpG-polyA into the mouse RAW264.7 cell, we assembled Cy5-labeled CpG-A5 ODNs on AuNPs. After incubation, small granules were localized in RAW264.7 cells and fluorescent signals were observed from the cytoplasm (Figure 2B). As a control, neither granules nor fluorescent signals were observed in cells incubated with free Cy5-ssCpG-A5 ODNs (Figure 2A). We further quantified the cellular uptake of CpG-A5-AuNPs by using flow cytometric analysis. Cy5-CpG-A5-AuNPs were localized in RAW264.7 cells in a dose-dependent manner, while free Cy5-ssCpG-A5 ODNs were hardly detected in these cells (Figure 2C). Together, these two pieces of evidence demonstrate that CpG-polyA ODNs could be efficiently delivered into cells through the interaction between polyA tails and AuNPs.

Subsequently, the subcellular localization of CpG-polyA-AuNP conjugates was examined by using TEM. CpG-polyA15-AuNP conjugates were readily observed in RAW264.7 cells and stably localized in endosomal vesicles of the cytoplasm at various time points. In contrast, the conjugates were not detected in the nucleus (Figure 3, S1). Within the endosome, the CpG-polyA-AuNP conjugates appeared to be monodisperse. We speculate that this conformation is in favor of an interaction with the TLR9. The overall morphology of RAW264.7 cell was not affected after treatment with 25 nM CpG-polyA15-AuNP conjugates, which was the highest concentration used in this study (Figure S2A). Consistently, thiazolyl blue tetrazolium bromide (MTT) assays showed that various concentrations of CpG-polyA-AuNP conjugates did not trigger cellular toxicity (Figure S2B). These results indicate that internalized CpG-polyA-AuNPs accumu-

lated within the endosomal vesicles and did not exhibit cytotoxicity to RAW264.7 cells.

2.3. CpG-polyA-AuNP Conjugates Stimulate Secretion of Cytokines

CpG 1826 was identified as an optimal sequence with potent immunostimulatory effects in murine cells.^[14] All CpG ODNs in this study contained this core sequence. After

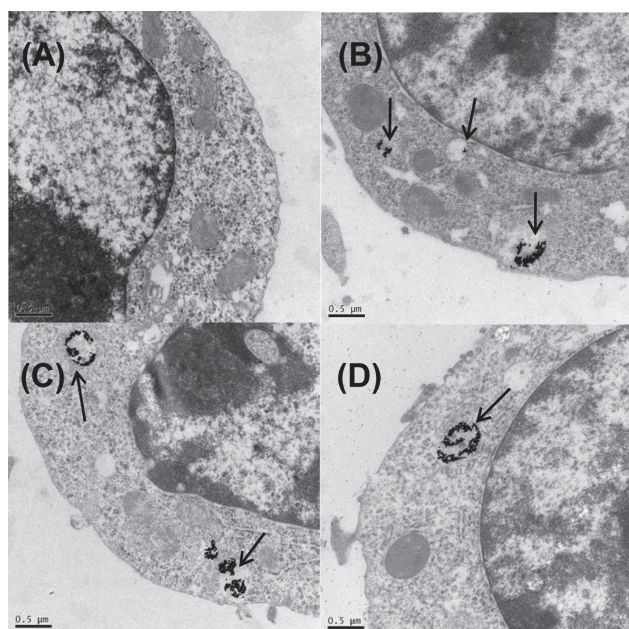


Figure 3. TEM images of internalized CpG-A15-AuNPs. RAW264.7 cells were incubated with 25 nM CpG-A15-AuNPs at indicated time point: A) untreated, B) 4, C) 8, D) 24 h. Black arrow indicates the location of the internalized CpG-A15-AuNPs. The scale bar represents 0.5 μm .

incubation with CpG-A15-AuNPs or S-CpG ODNs, we detected secretion of TNF- α . The levels of secretion were proportional to the dosage of CpG-AuNPs conjugates or S-CpG ODNs. Significantly, CpG-A15-AuNPs had significantly higher immunostimulatory activity than S-CpG ODNs at various concentrations (**Figure 4**). In a previous study, we showed that thiol-modified CpG-AuNPs (CpG-SH-AuNPs) also enhanced the secretion of proinflammatory cytokines.^[11]

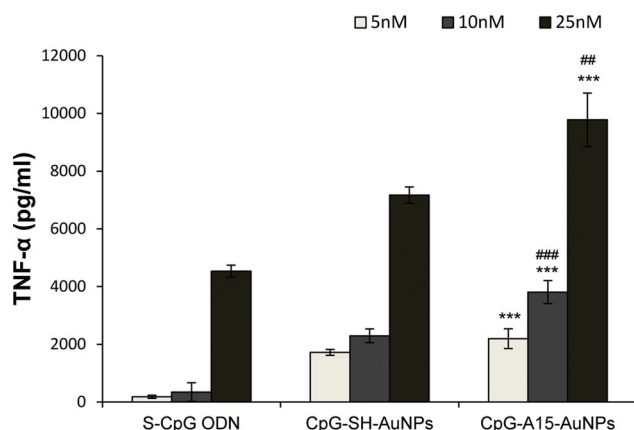


Figure 4. TNF- α production from RAW264.7 cells. Indicated concentrations of S-CpG ODN, CpG-SH-AuNPs, and CpG-A15-AuNPs were incubated with RAW264.7 cells for 8 h. The concentrations TNF- α in culture media were measured. Results are expressed as the mean $\pm$ SD of three determinations. *** P < 0.001, significantly different from S-CpG ODN; ## P < 0.01, ### P < 0.001, significantly different from CpG-SH-AuNPs.

We compared the immunostimulatory activity of these two types of CpG-AuNPs. At 5 nM, CpG-A15-AuNPs and CpG-SH-AuNPs showed similar immunostimulatory activity. In contrast, CpG-A15-AuNPs had significantly higher immunostimulatory activity than CpG-SH-AuNPs at 10 or 25 nM (**Figure 4**), which suggests that polyA-tailed CpG-AuNPs could be more efficient at inducing host immune response than CpG-SH-AuNPs. We also measured the secretion levels of other cytokines (IL6 and IL12) in response to the stimulation of CpG-A15-AuNPs and CpG-SH-AuNPs (**Figure S4**). At lower concentrations, CpG-A15-AuNPs induced higher levels of IL6 than CpG-SH-AuNPs. For the stimulation of IL12, CpG-A15-AuNPs showed slightly higher efficiency, but the difference is not significant.

Since CpG-A15-AuNPs have fewer ODN strands per particle than CpG-SH-AuNPs, more AuNPs are present in CpG-A15-AuNP conjugates than in CpG-SH-AuNP conjugates when the same concentration of CpG ODNs is delivered. However, the AuNP itself does not contribute to the observed immunostimulatory effects (**Figure S3**), which is also confirmed in other studies.^[15] Hence, we speculate that the observed difference in immunostimulatory activity between CpG-A15-AuNPs and CpG-SH-AuNPs might come from the surface properties of these nanoconjugates. Given that target accessibility of these two nanoconjugates is markedly different,^[12] it is possible that CpG ODNs are more biologically active at the surface of CpG-A15-AuNPs than at that of CpG-SH-AuNPs.

2.4. CpG-polyA-AuNPs Induce Immune Response via TLR9

Invading viruses or bacteria containing unmethylated CpG motifs are recognized by endosomal TLR9, which in turn activates the downstream immune pathways. Since CpG-A15-AuNPs and CpG-SH-AuNPs induce different levels of immune responses in RAW264.7 cells, it is intriguing to know whether they function through the TLR9 pathway. To test this, we applied chloroquine, a well-established TLR9 inhibitor, to our assays in RAW264.7 cells. The induction of proinflammatory cytokines by CpG-A15-AuNPs was abolished in the presence of chloroquine, which confirms that the TLR9 pathway was required for the immunostimulatory effects of CpG-A15-AuNPs (**Figure 5**).

CpG-A15-AuNPs and CpG-SH-AuNPs differ in their 3' modifications as well as their surface densities. To distinguish which factor might have an effect on the immunostimulatory activity, we next compared the activity of CpG-polyA-AuNPs with different lengths of polyA tail. Interestingly, CpG-A5-AuNPs, which has a higher surface density of CpG ODNs, is less efficient than CpG-A15-AuNPs in inducing the secretion of TNF- α , IL-6, and IL-12 at various concentrations (**Figure 6**). To exclude any possible interference caused by the different amount of AuNPs, we adjusted the total amount of nanoparticles in these two samples to comparable levels by supplementing with additional AuNPs, which caused no difference in cytokine induction. (**Figure S5**). These results suggest that less populated CpG motifs on the surface of AuNPs may be in favor of the recognition and

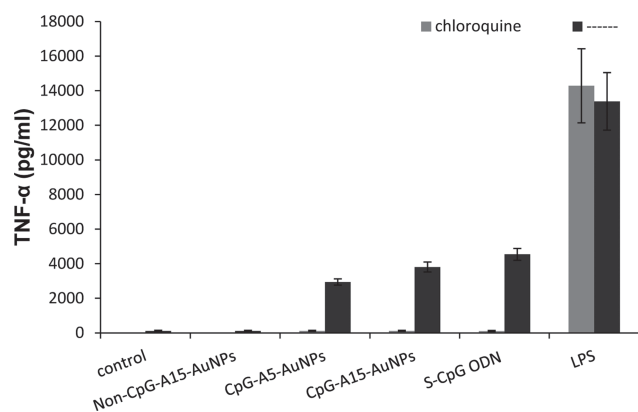


Figure 5. CpG-polyA-AuNPs-mediated cytokine production are TLR-9-dependent. The TLR-9 inhibitor, chloroquine was added to the cells 1 h prior to stimulation. Cells were stimulated for 8 h with CpG-polyA-AuNPs (10 nM), S-CpG ODN (25 nM) or lipopolysaccharide LPS (10 ng mL⁻¹) in the presence or absence of chloroquine. Results are expressed as the mean $\pm$ SD of three determinations.

interaction by TLR9, and thus enhance their immunostimulatory effects.

2.5. In Vivo Immunostimulatory Effects of CpG-polyA-AuNPs

Both CpG-polyA-AuNPs and CpG-SH-AuNPs can effectively stimulate the production of proinflammatory cytokines *in vitro*. To examine whether these nanoconjugates conserve their immunostimulatory activity *in vivo*, CpG ODN containing materials were administered to ICR mice by injection into the tail vein at 60 μ g kg⁻¹ (ODN per body weight), and serum IL-12 and MCP-1 levels were determined 3 h postadministration. As shown in **Figure 7**, control ssCpG ODN, bare AuNPs, and non-CpG-A5-AuNPs did not induce cytokine production, whereas CpG-A5-AuNPs and CpG-SH-AuNPs resulted in elevated levels of both cytokines. These data confirmed the *in vitro* stimulation of murine RAW264.7 cells. At tested concentrations our positive control, S-CpG ODN, appears to be the most potent inducer for both IL-12 and MCP-1. This result is not in agreement with the *in vitro* results. One possible explanation is the administrated concentration is more suitable for S-CpG ODN function. As shown in Figure 4, CpG-polyA-AuNPs and CpG-SH-AuNPs exhibited higher immunostimulatory activity than S-CpG ODN at lower concentrations. The difference in immunostimulatory activity between 10 and 25 nM S-CpG ODN was 13-fold, much larger than that with CpG-A15-AuNPs (threefold). Therefore, in future study, it is important to optimize the injected concentrations and determine a lower, safer dosage with reasonable activity for CpG-polyA-AuNPs as nanoagents.

3. Conclusion

We employed gold nanoparticles as the delivery vehicle for polyA-tailed CpG in RAW264.7 cells. This work proves that polyA-DNA-modified AuNPs are efficient in cellular uptake

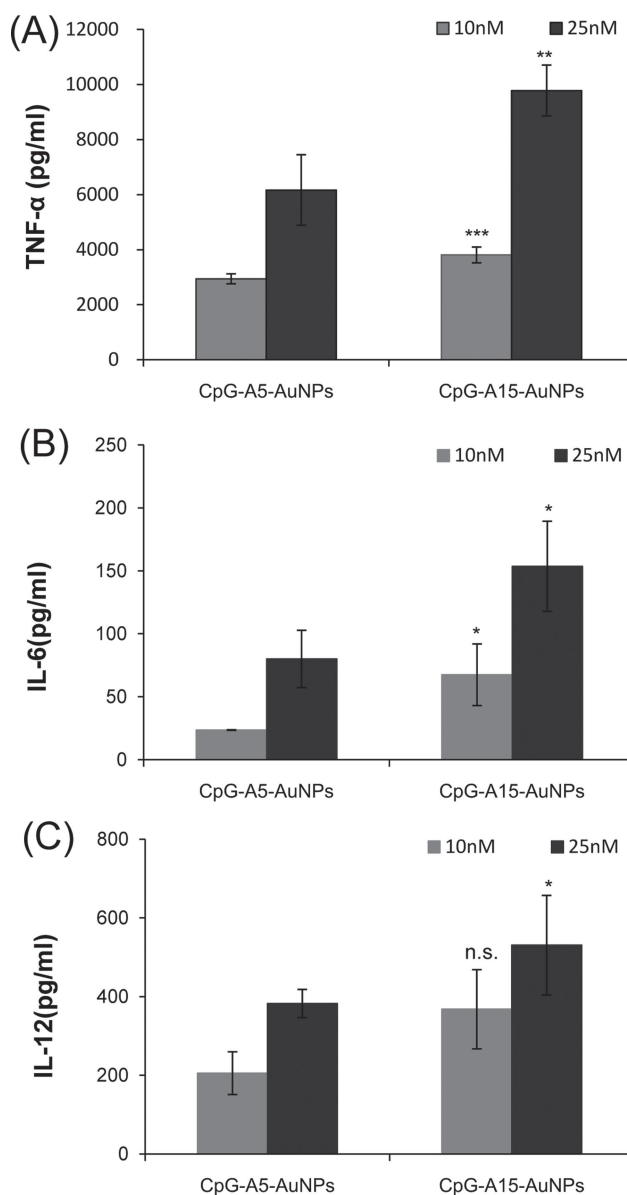


Figure 6. Comparison of the immunostimulatory activity of CpG-A5-AuNPs and CpG-A15-AuNPs. The concentrations of A) TNF- α , B) IL-6, and C) IL-12 in culture media were measured at 8 (TNF- α) or 24 h (IL-6, IL-12) after treatment with indicated concentrations of CpG-polyA-AuNPs. Results are expressed as the mean $\pm$ SD of three determinations. *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.

and the conjugated DNA is stable inside the cell. Similar to thiol-modified CpG-AuNPs, polyA-CpG-AuNP conjugates also enhanced their immunostimulatory activity. In contrast to bare AuNPs, free CpG ODN, or non-CpG-polyA-AuNPs, which had no immunostimulatory effect, we can demonstrate that AuNPs have no nonspecific immunostimulatory activity and CpG-polyA-AuNPs reserved the biological function of CpG ODN via the TLR9 pathway. Furthermore, our results indicate that, by controlling the length of polyA anchors, we can easily regulate the surface density of CpG ODNs as well as the immunostimulatory capacity of polyA-CpG-AuNPs nanoconjugates. Our approach avoids the requirement for

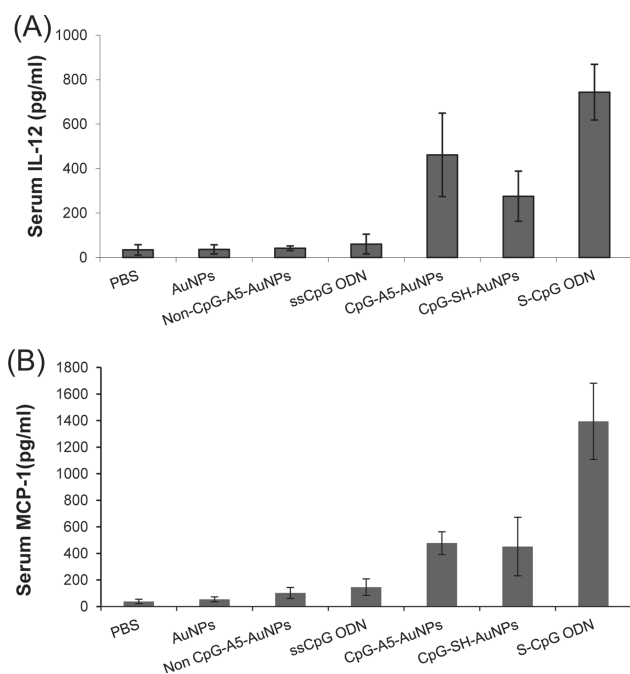


Figure 7. CpG-polyA-AuNPs have immunostimulatory activity in vivo. Indicated materials were administered intravenously (i.v.) to ICR mice at $60 \mu\text{g kg}^{-1}$ dose. Blood samples were obtained 3 h after injection, and serum IL-12 levels were analyzed by sandwich ELISA; MCP-1 levels were analyzed by CBA.

thiol modification, greatly simplifies the procedure, and reduces the cost of DNA synthesis. Under optimal conditions, polyA-CpG-AuNPs show significantly higher immunostimulatory activity than their thiolated counterpart. Finally, we show that CpG-polyA-AuNPs exhibit a potent immunostimulatory activity in vivo, which suggests the suitability of using this simple approach for therapeutic applications of CpG ODNs.

4. Experimental Section

Chemicals and Materials: The mouse leukemic monocyte macrophage cell line RAW264.7 was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai). Anti-mouse TNF- α , biotin anti-mouse TNF- α cocktail, recombinant mouse TNF- α , anti-mouse IL-6, biotin anti-mouse IL-6, recombinant mouse IL-6, and avidin horseradish peroxidase (AVHRP) were purchased from eBioscience. Murine IL-12 ELISA development kit was purchased from Peprotech. K-blue TMB substrate was purchased from Neogen Co. Cytometric bead array (CBA) mouse inflammation kit was purchased from BD. Thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), DAPI, LPS (E.coli 055:B5), and chloroquine were purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was purchased from Aladdin. All other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co. HPLC-purified oligonucleotides (Table 1) were synthesized by Invitrogen Biotechnology Co., Ltd.

Synthesis of Gold Nanoparticles: Citrate-stabilized gold nanoparticles were prepared using a previously published method.^[16]

Table 1. Sequences of oligonucleotides employed in this work.^{a)}

ODN Name	Sequence (5' to 3')
CpG-poly A5	TCCATGACGTTCTGACGTTTTTTTAAAAA
CpG-polyA15	TCCATGACGTTCTGACGTTTTTTTAAAAA
CpG-SH	TCCATGACGTTCTGACGTTTTTTT-SH
FAM-CpG-poly A5	FAM-TCCATGACGTTCTGACGTTTTTTTAAAAA
FAM-CpG-poly A15	FAM-TCCATGACGTTCTGACGTTTTTTTAAAAA
Cy5-CpG-poly A5	Cy5-TCCATGACGTTCTGACGTTTTTTTAAAAA
Non CpG-poly A5	TCCATGAGCTTCTGACGTTTTTTTAAAAA
Non CpG-poly A15	TCCATGAGCTTCTGACGTTTTTTTAAAAA
S-CpG	TCCATGACGTTCTGACGTT(phosphorothioate backbone)
ss CpG ODN	TCCATGACGTTCTGACGTT ^{b)} (CpG 1826)

^{a)}All oligonucleotides have a phosphodiester backbone except for S-CpG. ; ^{b)} Denotes the optimal murine sequence of immunostimulatory CpG ODN.

Briefly, 3.5 mL trisodium citrate solution (10 mg mL^{-1}) was added into 100 mL boiling, rapidly stirred 0.01% HAuCl_4 aqueous solution to produce 15 nm gold nanoparticles. The resulting solution was kept boiling and stirred for 20 min before being cooled down to room temperature and filtered through a $0.22 \mu\text{m}$ membrane. Synthesized AuNPs were stored at 4°C .

Functionalization of AuNPs with ODNs: First, 5 mL of AuNPs was concentrated to 1 mL (about 10 nm) by centrifugation (12 000 rpm, 20 min, 4°C). Subsequently, polyA-tailed or thiolated DNA were added to AuNPs to a final concentration of $3 \mu\text{M}$. The mixture was gently shaken at room temperature overnight. Next, the concentration of NaCl in the solution was slowly increased to 0.1 M by stepwise addition of 1 M phosphate-buffered saline (PBS) (pH 7.4) and adjusted to 0.1% SDS. The final mixture was further incubated with constant shaking for 24 h, centrifuged (13 000 rpm, 20 min, 12°C , supernatant decanted) and rinsed with 500 μL of 0.1 M PBS (pH 7.4). This procedure was repeated three times to remove unbound DNA. The final deposition was resuspended in 0.1 M PBS and stored at 4°C for further use. The concentration of AuNPs was calculated from UV absorbance via Beer's law ($A = \epsilon bc$, $\epsilon \approx 4 \times 10^8 \text{ L mol}^{-1} \text{ cm}^{-1}$). UV absorbance values were measured by UV-vis spectroscopy (U-3010 UV-vis spectroscopy, Hitachi, Japan). The density of fluorescence-labeled ODN strands was quantified according to the published protocol.^[13] Mercaptoethanol (ME) was added to the solution of DNA-AuNPs conjugates to a final concentration of 20 mM and the solutions was incubated overnight with shaking at room temperature. After centrifugation, fluorescence of released ODN strand was measured using a fluorescence spectrometer (F-4500, Hitachi, Japan). The fluorescence was then calculated to molar concentrations.

Dynamic Light Scattering Analysis: The apparent hydrodynamic size of the DNA-Au samples was measured by using laser light scattering with a Delsa NanoC particles Analyzer (Beckman Coulter, America).

TEM Image Analysis: For TEM images of cells, RAW264.7 cells were seeded in 10-cm wells at the density of 4×10^6 cells well^{-1} , cultured at 37°C for 24 h, and incubated with 25 nm CpG-A15-AuNPs for 4, 8, and 24 h before collection. Cells were washed with PBS before collected by centrifugation, fixed with 2.5% glutaraldehyde in PBS, then fixed in 1% osmium tetroxide, dehydrated

with ethanol and embedded in Quetol-Spurr resin. Sections were 100 nm thick and stained with uranyl acetate and lead citrate. Samples were placed on a TEM grid and imaged by using TEM (Jeol, JEM 1230, 80 KV).

Cell Culture and Cytotoxicity Analysis: RAW264.7 cells were grown at 37 °C with humidified atmosphere (5% CO₂), in DMEM supplemented with 10% heat-inactivated FBS, 1.5 g L⁻¹ NaHCO₃, 100 units mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, 4.5 g L⁻¹ glucose, and 4 mm L-glutamine. Cytotoxicity was estimated using an MTT assay. Briefly, cells were seeded in 24-well plates and cultured overnight to reach about 80% confluency. Fresh media containing AuNPs or CpG-AuNPs at indicated concentrations were incubated with cells for 24 h. 50 µL of 5 mg mL⁻¹ MTT solution was then added to each well, followed by 4 h incubation at 37 °C. Next, cells were lysed with 10% acid SDS solution (pH ca. 2–3). After centrifugation, the absorbance of supernatant was measured at 570 nm by using a microplate reader (Bio-Rad 680, USA).

Confocal Microscopy: Cellular uptake of a fluorophore-labeled CpG ODN was observed by confocal microscopy. Cy5-labeled CpG-A5 ODN was used for the preparation of fluorophore-labeled CpG-A5-AuNPs. RAW264.7 cells were seeded on glass coverslips and incubated at 37 °C. 24 h later, cells were washed with PBS and exposed to fresh culture medium containing either Cy5-CpG-A5-AuNPs or naked Cy5-CpG for 4 h. Fluorophore-labeled CpG was then washed off with PBS. Cells were fixed with 3% paraformaldehyde/sucrose and the nuclei were stained using 3 µg mL⁻¹ Hoechst 33258. The coverslips were mounted on a glass slide. All images were obtained using a laser confocal microscope (Leica TCS SP5).

Flow Cytometry Analysis: RAW264.7 cells were seeded on 24-well culture plates at a density of 4 × 10⁵ cells mL⁻¹ (0.5 mL) and cultured for 24 h, then fresh culture medium containing different concentrations of Cy5-CpG-A5-AuNPs were added to cells and incubated for 4 h at 37 °C. Cells were harvested, washed three times with PBS, and the mean fluorescence intensity of the cells was determined by flow cytometry (FACS array, BD Bioscience).

Cytokine Secretion from RAW264.7 Cells: RAW264.7 cells were seeded on 24-well culture plates at a density of 2 × 10⁵ cells well⁻¹. After 24 h incubation, cells were washed with 0.5 mL PBS before treatment with indicated conditions for 8 (TNF-α) or 24 h (IL-6 and IL-12). The media were collected and centrifuged at 12 000 rpm for 20 min at 4 °C. The supernatants were stored at -80 °C. The levels of cytokines were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) method using protocols recommended by the manufacturer.

Cytokine Secretion from Mice: 4–6 week-old male ICR mice were purchased from Shanghai SLAC laboratory animal Co. LTD (China). For in vivo immunostimulation, ODNs, AuNPs, or CpG-ODN-AuNP conjugates were injected into the mice via tail vein (i.v.) at 60 µg kg⁻¹ (ODN per body weight); the same volume of PBS was injected as a control. Blood samples were collected 3 h postinjection. Serum was prepared by centrifugation at 3000 rpm for 15 min and stored at -80 °C until assay. Serum IL-12 levels were determined by sandwich ELISA. MCP-1 levels were tested by using a CBA kit using protocols recommended by the manufacturer and then data acquired on a flow cytometer. Data were analyzed using the FCAP Array software.

Statistical Analysis: In all experiments, significance was determined using the Student's t-distribution (two-tailed; two-sample equal variance). *** equals P < 0.001; ** equals P < 0.01; * equals P < 0.05; ns: not significant.

Supporting Information

Supporting information is available from the Wiley Online Library or from the author.

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