Oligodeoxynucleotides

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Polyvalent Immunostimulatory Nanoagents with Self-Assembled CpG Oligonucleotide-Conjugated Gold Nanoparticles**

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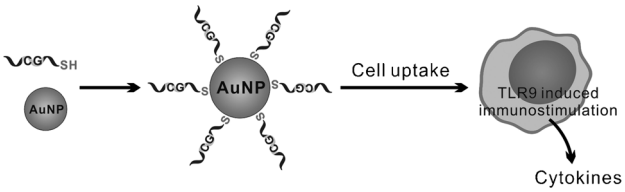
During the past decade, nucleic acid based therapeutics has developed from experimental techniques to preclinically practical strategies. Compared to conventional plasmid-containing transgenic methods, synthetic oligodeoxynucleotides (ODNs), including antisense DNA, aptamers, and small interfering RNAs (siRNAs), have emerged as highly attractive candidates for the treatment of various human diseases.[1] These ODNs are generally water soluble and stable with extremely low in vivo toxicity, and often interact with their targets with high specificity and sensitivity. Despite these advances, drug applications of ODNs are largely limited by delivery approaches. Naked ODNs cannot penetrate through the cell membrane and are prone to be cleared by nucleases in serum or cytoplasm.[1] The emergence of nanobiotechnology has provided unprecedented opportunities for biocompatible, low-toxicity, and highly efficient approaches for exogenous ODN administration in target cells.[2] Several promising nanomaterials, including gold nanoparticles (AuNPs), mesoporous silica nanoparticles, quantum dots, and carbon nanomaterials, have shown great promise as intracellular delivery nanoagents for imaging and gene regulation purposes.[3] In this work, we develop an AuNP-based polyvalent immunostimulatory nanoagent by using self-assembled cytosine–phosphate–guanosine (CpG) oligonucleotide-conjugated AuNPs (see Scheme 1).

Unmethylated CpG motifs are widely present in the genomic DNA of invading bacteria and viruses, while most of the CpG sequences are methylated in the vertebrate genome.[4] Hence, the mammalian immune system can sense and react upon stimulation by microbe DNAs containing unmethylated CpG motifs or synthetic CpG ODNs.[4,5] This immune response is mainly mediated through a subpopulation of pattern recognition receptors, toll-like receptor 9 (TLR9).[6] Stimulation of TLR9 by the CpG motif activates MyD88-dependent NF-κB and MAPK signaling pathways,[6,7] and subsequently induces expression of the proinflammatory cytokines necessary for Th1-like innate immune response as well as adaptive immunity.[8] Therefore, synthetic CpG ODNs have become a promising tool in immunotherapeutic applications for the treatment of various diseases, including cancer and infectious and allergic diseases.[5,9]



Scheme 1. Assembly of CpG-conjugated AuNPs and the immunostimulatory effects.

Due to their growing clinical significance, various methods to improve delivery of CpG ODNs into target cells have been developed.[10] For example, sequences and modifications of synthetic CpG ODNs have been designed to achieve optimal stability and immunostimulatory activities.[11] Replacement of the native phosphodiester (PO) bond with a nuclease-resistant phosphorothioate (PS) backbone can greatly increase the stability of synthetic CpG ODNs in vivo.[1,12] Moreover, a variety of transfection agents have been utilized in an attempt to increase cellular uptake and reduce the cellular toxicity of CpG ODNs.[10b,13] While these methods have significantly improved the applicability of CpG ODNs in biological studies and even clinical trials, it is still highly demanding to develop a simple and cost-efficient approach that can simultaneously address the challenges including efficiency of cellular internalization, DNA stability against nuclease degradation, bioactivity of CpG ODNs, and potential cellular toxicity upon complexation with transfection agents.[14] Since AuNPs are nearly noncytotoxic and their synthesis and surface modification have been well established with high reproducibility,[15] we herein explore the possibility of using AuNPs as a vehicle for intracellular delivery of CpG ODNs and interrogate the immunological effects of CpG ODN–gold nanoparticle conjugates (CpG-AuNPs) in cells.

AuNPs of size 15 and 30 nm were employed to conjugate CpG ODNs. We employed a specific B-type sequence of CpG ODN that induced optimal immunostimulatory effects in

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mammalian cells.[10b] Since previous studies showed that a free 5′ end of the ODN was essential for its immunostimulatory functions,[16] the 3′ end of the CpG ODN was modified with a thiol group for subsequent self-assembled conjugation with AuNPs. Dynamic light scattering (DLS) studies revealed that the hydrodynamic diameters of the AuNPs were significantly increased after conjugation with CpG (from 23 to 41 nm and 30 to 103 nm), which suggests that thiolated CpG ODNs have been loaded on the surface of AuNPs (see Table S1 in the Supporting Information). Transmission electron microscopy (TEM) studies confirmed that a layer of soft material (DNA strands) surrounds the AuNP surface (Figure 1).

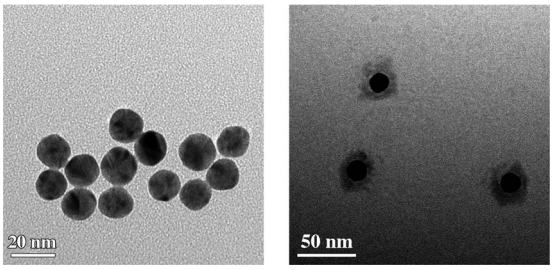

Figure 1. Characterization of AuNPs. TEM images of 15 nm AuNPs (left) and CpG-AuNP conjugates (right) after phosphotungstic acid staining.

The potential cellular toxicity of CpG-AuNP conjugates was evaluated by a conventional MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The viability of RAW264.7 cells was measured in the presence of CpG-AuNPs at various concentrations (0.01–0.1 μM). No cellular toxicity of either unmodified AuNPs or CpG-AuNPs was observed, even at the highest concentration (0.1 μM; see Figure S2 in the Supporting Information). These results indicate that AuNPs exhibit no apparent cytotoxicity to RAW264.7 cells and are favorably biocompatible.

Next, the cellular uptake efficiency of CpG-AuNPs was measured by confocal fluorescence microscopy using CpG ODN modified with a fluorophore (Cy5) at the 5′ end. Cy5-CpG-AuNPs or single-stranded (ss) Cy5-CpG ODNs were incubated with RAW264.7 cells. No fluorescence signal was detected in cells incubated with Cy5-CpG ODNs alone, which is consistent with the facts that naked ODNs have difficulty passing the cytoplasmic membrane and are also prone to degradation by nucleases. Significantly, a bright fluorescence signal was observed throughout the cytoplasm of cells incubated with Cy5-CpG-AuNPs (Figure 2). In addition, small dots that might represent clustered AuNPs were observed inside Cy5-CpG-AuNP-treated cells in bright-field microscopy, but not in control cells. Hence, CpG-AuNP conjugates can strongly enhance the efficiency of cellular uptake of CpG ODNs.

Subsequently, we tested the biological activities of CpG-AuNP conjugates by measuring secreted cytokine (tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)) levels of treated RAW264.7 cells. We found that both 15 and 30 nm AuNPs functionalized with CpG ODNs could stimulate the secretion of TNF-α in a dose-dependent manner (Figure 4 A).

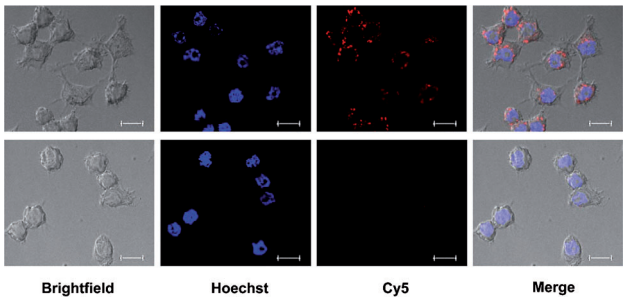

Figure 2. Cellular internalization of Cy5-CpG-AuNPs. Confocal images of RAW264.7 cells treated with Cy5-ssCpG (bottom) and Cy5-CpG-AuNPs (top) for 4 h. The cell nucleus was indicated using Hoechst 33258. Overlay images show the relative uptake and localization of CpG. Scale bar = 10 μm.

Also of note, AuNPs of 30 nm were less effective than smaller ones (15 nm) at all concentrations (0.01–0.1 μM; Figure 4 A), which is consistent with previous reports that smaller particles are more cell permeable than larger ones.[17] The secreted TNF-α levels induced by 15 nm CpG-AuNPs were compared with those of lipopolysaccharide (LPS), a well-established strong immunostimulatory factor inducing the upregulation of a variety of cytokines.[18] In addition, an equal amount of CpG ODNs bearing a PS backbone (S-CpG) was also included as another positive control (see Figure S3 in the Supporting Information). The levels of TNF-α induced by LPS, S-CpG, and CpG-AuNPs were comparable, which suggests that CpG-AuNPs are an efficient stimulus for cells.

By measuring the levels of secreted TNF-α and IL-6, we compared the immunostimulatory activity of 15 nm CpG-AuNPs with that of naked ssCpG ODNs, as well as CpG ODNs transfected with a commercial reagent, lipofectamine 2000 (Figure 3). Significantly, the amount of secreted TNF-α and IL-6 stimulated by CpG-AuNPs was approximately 20-fold higher than that of naked CpG ODN, thus suggesting that high cellular uptake of CpG-AuNPs is critically important for the observed immunostimulatory activity. CpG-AuNP conjugates also exceeded lipofectamine-transfected CpG by 11.58/5.41-fold in producing TNF-α and IL-6. This remarkable immunostimulatory activity of CpG-AuNPs might come from a combination of nanoscale effects, the high intracellular delivery ability of AuNPs, large polyvalence due to high-density loading of CpG ODNs at the AuNP surface, and increased stability of surface-confined CpG ODNs. In addition, unmodified AuNPs or AuNPs conjugated with a control non-CpG sequence had nearly no effect on cytokine secretion, thus indicating that the observed immunostimulatory activities were indeed caused by the CpG sequence within the ODN (Figure 3). Such specificity is important for potential immunotherapeutic applications.

Since the use of AuNPs provides high flexibility in designing efficient CpG ODN carriers, it is possible to further increase the immunostimulatory efficiency of CpG-AuNPs. For this purpose, we prepared bi-CpG-AuNPs by assembling ODNs containing two copies of CpG sequences on 15 nm AuNPs (see Table S1 in the Supporting Information). We found that both CpG-AuNP and bi-CpG-AuNP treatment

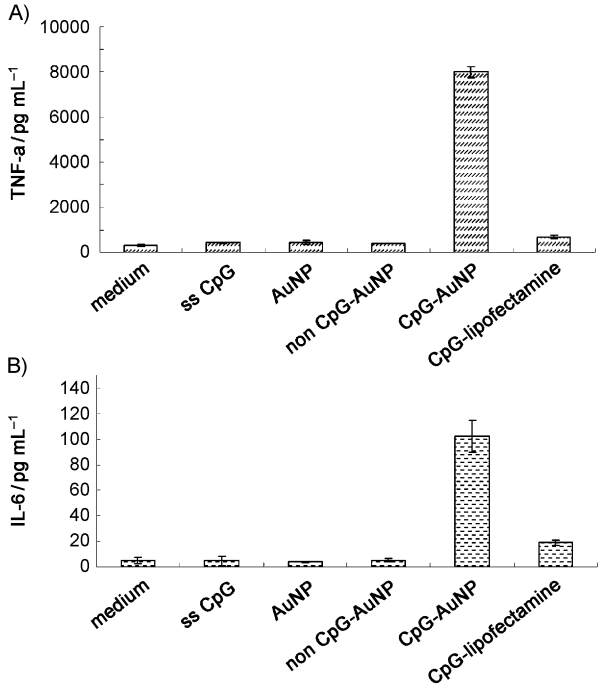

Figure 3. CpG-AuNP conjugates stimulate the secretion of cytokines. RAW264.7 cells were treated with the indicated materials at a DNA concentration of 0.05 μM. An equal molar concentration of AuNPs was used as control. The concentrations of A) TNF-α and B) IL-6 in culture media were measured at 8 h (TNF-α) or 24 h (IL-6) by an ELISA method. Results are expressed as the mean ± standard deviation (SD) of three determinations.

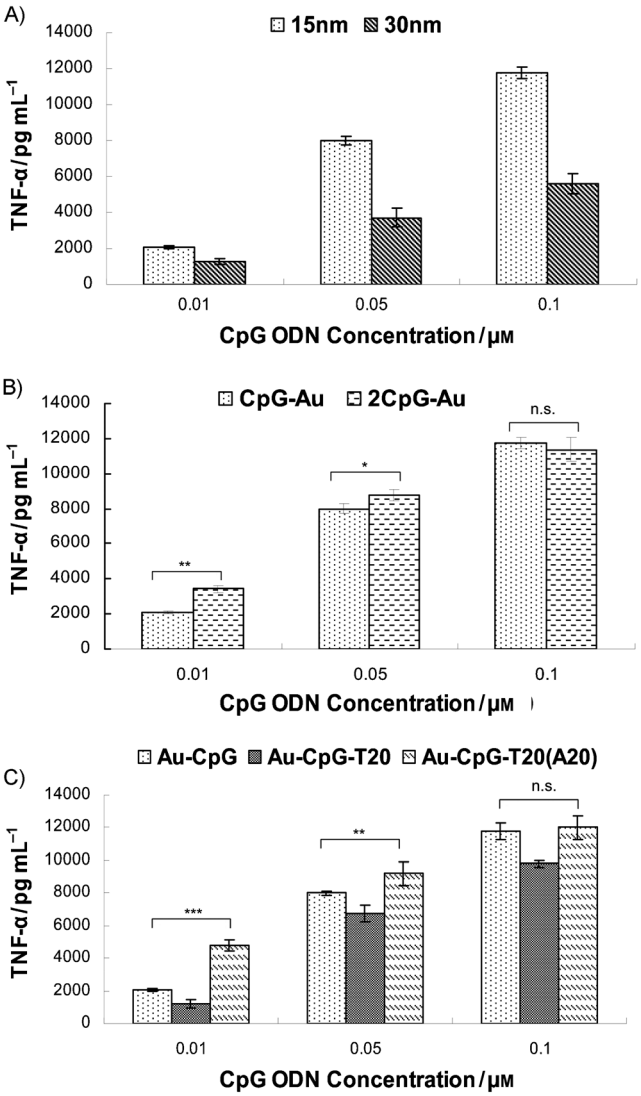

Figure 4. Concentrations of TNF-α in culture media were measured at 8 h after incubation with the indicated CpG-ODN-AuNP conjugates to RAW264.7 cells at the indicated concentrations. Results are expressed as the mean ± SD of three determinations. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ by Student's *t* test).

resulted in high levels of TNF-α secretion in a concentration-dependent manner (Figure 4B). Significantly, bi-CpG-AuNPs at 10 nM induced higher levels of TNF-α secretion (about 1.67-fold) than CpG-AuNPs (10 nM), thereby implying that the immunostimulatory activity was dependent on the density of CpG motifs at the surface of AuNPs. It is worthwhile noting that bi-CpG-AuNPs at 100 nM had a similar effect to CpG-AuNPs (Figure 4B), possibly due to saturated intracellular concentration of CpG at this high dosage of the nanoconjugates.

We also found that the structure of ODN on the surface of AuNPs played an important role in the immunostimulatory effect of CpG-AuNPs. We initially designed CpG-T_{20} ODN conjugated AuNPs, in which the inserted T_{20} spacer increased the distance between CpG motifs and AuNPs, to reduce the steric hindrance effect for the access of TLR9. Nevertheless, we did not observe the expected increase in stimulatory activities in TNF-α secretion at all the tested concentrations (0.01–0.1 μM; Figure 4C). We reason that the floppy T_{20} spacer does not provide sufficient rigidity to extend the CpG motif into the solution. Consequently, we hybridized CpG-T_{20}-AuNPs with an A_{20} oligonucleotide, which was known to form a rigid duplex between the CpG motif and AuNPs. As expected, the CpG-T_{20}/A_{20}-AuNP conjugates (10 nM) exhibited significantly (2.32/4.05-fold) higher activity to stimulate TNF-α secretion than CpG-AuNPs and CpG-T_{20}-AuNPs (Figure 4C). Similar to the case of bi-CpG-AuNPs, the immunostimulatory activity saturated at increased use of CpG-T_{20}/A_{20}-AuNPs (100 nM), and the difference became indistinguishable at this high dosage.

TLR9 activation and subsequent production of Th1 or proinflammatory cytokines induced by CpG ODN is part of the defensive immune response mediated by the TLR family members. Many vertebrate TLRs have been identified,[18a] which are localized on the cell surface to detect pathogen-specific molecules, for example, LPS (TLR4), lipoprotein (TLR1, TLR2, and TLR6), and flagellin (TLR5). A subset of TLRs, including TLR3, TLR7, TLR8, and TLR9, are expressed intracellularly and detect nucleic acids, such as double-stranded (ds) RNA (TLR3), ssRNA (TLR7 and TLR8), and DNA-containing unmethylated CpG motifs (TLR9).[19] Hence, to interrogate the possibility that CpG-AuNP functions through other TLRs, we compared the

induction of interferon beta (IFN-β) by CpG-AuNPs and LPS, which is an activator of TLR4 and known to induce high levels of IFN-β (see Figure S4 in the Supporting Information). As shown in Figure S4B, RAW264.7 cells showed high levels of upregulation of IFN-β mRNA (> 200-fold) in response to LPS and moderate increase (about 20-fold) of IFN-β in response to both CpG-AuNPs and S-CpG ODNs. As a quality control of our reverse transcription PCR analysis, we also monitored the mRNA levels of TNF-α (see Figure S4 A in the Supporting Information), which correlated well with the protein levels measured by ELISA assays (see Figure S3 in the Supporting Information). Our observation of different induction levels of IFN-β by LPS and CpG ODN is consistent with previous reports.[18b,20] Therefore, we conclude that the immunostimulatory effect of CpG-AuNPs is mediated mainly by TLR9 and is unlikely to have a crosstalk with TLR4 pathways.

We have shown that CpG-AuNPs induced production of proinflammatory cytokines (TNF-α and IL-6). While the initial cellular response to stimuli (CpG ODN or LPS) is an increased production of proinflammatory cytokines, there is a subsequent induction of immunoregulatory cytokine IL-10, which has potent anti-inflammatory properties and can downregulate the CpG effect.[21] Therefore we determined the induction of IL-10 by CpG-AuNPs (see Figure S5 in the Supporting Information). Similar to previous reports,[18b,22] LPS and S-CpG ODNs induced significant production of IL-10 after 24 h of treatment; CpG-AuNPs and CpG-$T_{20}A_{20}$-AuNPs behave just like S-CpG ODNs. Importantly, naked AuNPs or non-CpG-AuNPs have no effect on IL-10 production. Again, these results confirmed that the immunostimulatory effects of CpG-AuNPs are comparable to those of the clinically tested S-CpG ODNs.

Since TLR9 is one of the essential regulators of both innate immunity and adaptive immunity, synthetic CpG ODN can be utilized as an agonist of TLR9 receptor to boost the immune response, which is favorable for the treatment of diseases including cancer and allergic diseases.[9a] Therefore, CpG-related agonists have been developed and tested in preclinical trials as vaccine adjuvants.[5,9b] Here we have demonstrated that polyvalent CpG-AuNP conjugates are a type of highly efficient nanoagent for intracellular delivery of CpG motifs and stimulating immunological reactions in cells. CpG-AuNP conjugates are more efficient than most reported ssCpG ODN or CpG-derived stimuli. Strikingly, at a DNA level of 0.1 μM (ca. 0.3 μg), CpG-AuNPs induced TNF-α secretion up to approximately 12000 pg mL^{-1}, while it was reported that naked ssCpG ODN has a much lower efficiency (10 μg CpG ≈ 800 pg mL^{-1}),[22] thus indicating that gold nanoparticles are a promising material to deliver CpG ODN into target cells.

The use of AuNPs provides several unprecedented advantages for intracellular delivery of functional nucleic acids. First, AuNPs are low cost, relatively homogeneous in size, and have minimal immunogenicity and cytotoxicity,[23] which make them a kind of biocompatible nanomaterial for biomedical applications. Second, the nanoscale surface of AuNPs with high surface-to-volume ratios provides a versatile platform for conjugation of ODNs with flexible structure design,[24] which has proven effective to tune the activity of CpG-AuNPs. Third, DNA-AuNP conjugates can be easily internalized by cells[15] and, in contrast to many polymer transfection agents, AuNPs minimally influence the bioactivity of ODNs. More significantly, conjugation of ODNs on AuNPs effectively protects them from nuclease degradation, an effect that is critically important for the bioactivity of ODNs.[25] Collectively, ODN-AuNP conjugates can satisfactorily address the main challenges for intracellular delivery of ODNs. Our studies provide strong evidence that CpG-AuNP conjugates can be utilized as proinflammatory stimuli in vitro, and potentially as a promising therapeutic tool in animals.

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