DNA Nanostructures

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Biocompatible Infinite-Coordination-Polymer Nanoparticle–Nucleic-Acid Conjugates for Antisense Gene Regulation**

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Abstract: *Herein, we report the synthesis of DNA-functionalized infinite-coordination-polymer (ICP) nanoparticles as biocompatible gene-regulation agents. ICP nanoparticles were synthesized from ferric nitrate and a ditopic 3-hydroxy-4-pyridinone (HOPO) ligand bearing a pendant azide. Addition of Fe^{III} to a solution of the ligand produced nanoparticles, which were colloidally unstable in the presence of salts. Conjugation of DNA to the Fe^{III}–HOPO ICP particles by copper-free click chemistry afforded colloidally stable nucleic-acid nanoconstructs. The DNA–ICP particles, when cross-linked through sequence-specific hybridization, exhibited narrow, highly cooperative melting transitions consistent with dense DNA surface loading. The ability of the DNA–ICP particles to enter cells and alter protein expression was also evaluated. Our results indicate that these novel particles carry nucleic acids into mammalian cells without the need for transfection agents and are capable of efficient gene knockdown.*

Spherical nucleic acids (SNAs) have emerged as an interesting new class of materials that have shown promise in programmable materials synthesis,[1] biodetection,[2] and intracellular gene regulation.[3] Such structures are often comprised of a nanoparticle core functionalized with a dense layer of oligonucleotides, although hollow, core-free versions have been developed.[4] The earliest example of SNAs were gold nanoparticles modified with a dense layer of alkylthiol-functionalized DNA,[5] but iron oxide,[6] silver,[7] semiconductor-quantum-dot,[8] and organic cores have been explored as well.[9] Notably, the chemical and biological properties of SNAs are markedly different from those of their linear counterparts. SNAs exhibit cooperative binding and sharp thermal denaturation profiles, enter cells without the need for cationic transfection agents, and have the ability to bind to receptors in a polyvalent fashion.[10] Consequently, they are powerful new entities for manipulating cellular processes through gene regulation,[11] drug delivery,[12] and immunomodulatory pathways.[13] The active uptake of SNAs occurs through caveolin-mediated endocytosis, triggered by their binding to class A scavenger receptors (SR-As).[14] Although SNAs made from gold have shown commercial promise as medical diagnostic and research tools and have shown no acute toxicity in vivo,[15] there are concerns about the potential long-term toxicity of gold nanoparticles and their metabolic fate.[16] Consequently, new forms of SNAs with cores made of biocompatible materials are highly sought after. Herein, we report a strategy based on the use of infinite-coordination-polymer (ICP) nanoparticles made from ferric ions and a rigid ditopic chelating ligand to synthesize novel SNA nanoparticle conjugates. These DNA–ICPs are designed from chemical building blocks approved by the FDA for other pharmaceutical uses, exhibit cooperative binding, and can readily cross mammalian cell membranes and inhibit protein expression in a targeted fashion.

ICP nanoparticles consist of amorphous networks of organic ligands bridged by metal nodes.[17] They are promising materials for SNA construction as the ligand/metal combination that defines the ICP structure can be rationally designed

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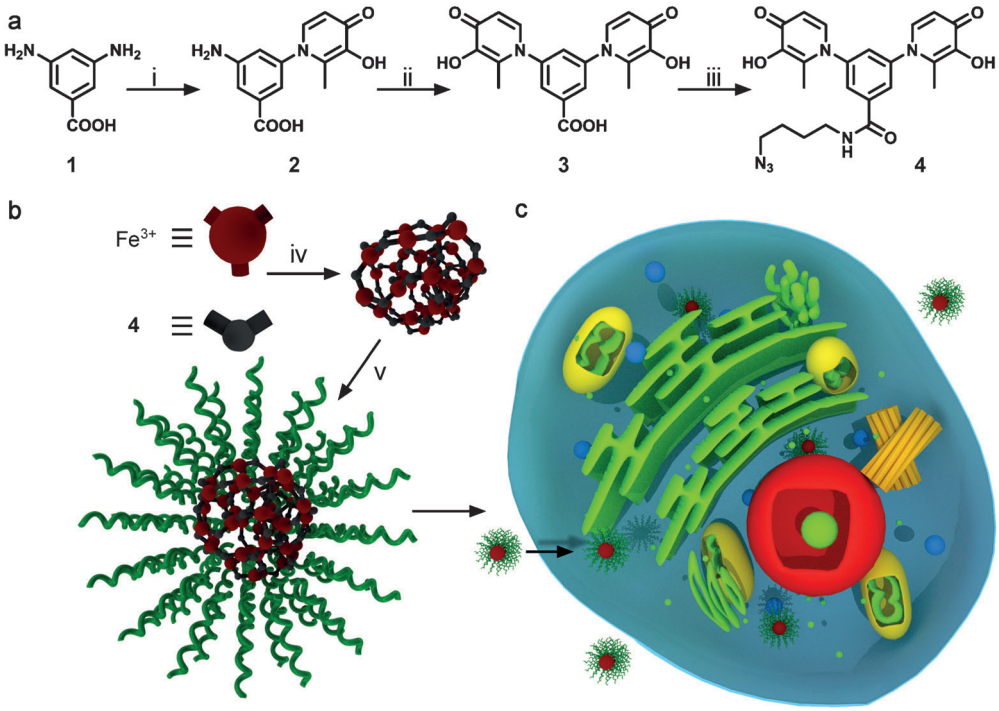
to optimize the toxicological and pharmacokinetic profiles of the DNA–ICP conjugate. One major limitation of many ICPs designed for medicinal applications is their instability in aqueous buffers. Some researchers have circumvented this limitation by encapsulating the particle core in silica[18] or a shell of lipids.[19] In contrast, we have sought to design ICP particles that could be synthesized, purified, and stored indefinitely under aqueous conditions and without specialized equipment or reagents. Furthermore, the use of relatively nontoxic metal ions is a crucial requirement for biological applications. These goals were accomplished by synthesizing ICP nanoparticles from strongly chelating 3-hydroxy-4-pyridinone (3,4-HOPO) ligands in combination with Fe^{III}, the most abundant transition metal in the body. The coordination chemistry and pharmacology of the 3,4-HOPOs have been systematically investigated,[20] and the 1,2-dimethyl derivative (deferiprone) is FDA-approved for the treatment of iron overload in humans.[21] Furthermore, the $Fe(HOPO)_3$ complex is known to dissociate below physiological pH values.[22] This dissociation provides a potential release mechanism for delivering DNA into the cytosol following cell entry, a novel property not typically associated with SNAs prepared to date.

It is known that ditopic HOPO and catechol ligands, being isoelectronic, can form insoluble coordination polymers with oxophilic metal cations, such as Fe^{III}, Cr^{III}, and Ga^{III}; however, such polymers are poorly understood and have not been well-studied.[23] These ligands have mainly been studied for metal sequestration as opposed to materials synthesis. Therefore, we saw an opportunity to construct a novel nanoparticle scaffold for modification with DNA. Specifically, we synthesized a new ditopic ligand, DABA-bis-HP-N_3 (**4**), for which we deliberately employed the inexpensive building blocks maltol and 3,5-diaminobenzoic acid (DABA, **1**; Scheme 1a). Two sequential acid-catalyzed condensations of maltol with DABA (**1**→**2**; **2**→**3**), followed by HATU-mediated amidation of the carboxylic acid, afforded the azide-bearing ditopic ligand **4**. Importantly, the carboxylic acid in **3** may be amidated with a wide variety of amine building blocks to afford ICP particles with tailorable postsynthetic chemistry dictated by the pendant functional groups.

To synthesize ICP nanoparticles from ligand **4**, we prepared a dilute NaOH solution of ligand **4** (1.07 mM ligand, 1877 μL) and injected a solution of ferric nitrate (10.8 mM, 123 μL) into it (Scheme 1b). Particle formation occurred instantaneously, and the color of the solution changed from clear to red as a result of ligand–metal charge transfer (LMCT) in the $Fe(HOPO)_3$ complex ($\lambda_{max} \approx 460$ nm).[24] The resulting ICP-N_3 nanoparticles were colloidally unstable in the presence of low concentrations of salts (NaCl, Tris·HCl; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol), thus leading to gradual precipitation of a red, insoluble material. The crude ICP-N_3 particles were purified by centrifugal filtration (100 kDa molecular-weight cutoff) and resuspended in H_2O. The particles were retained on the filter, as they were too large to pass through. Minimal loss of material through the filter indicated that a colloidal dispersion of high-molecular-weight species was obtained. In deionized H_2O, the as-synthesized particles were stable, with a mean hydrodynamic diameter of 10–20 nm, as determined by dynamic light scattering (DLS; Figure 1). TEM and AFM imaging revealed aggregates of small nanoparticles, with some degree of fusion occurring upon drying (see Figure S4 in the Supporting Information). The composition of the ICP-N_3 particles was also probed spectroscopically. Aliquots containing a fixed concentration of the DABA-bis-HP-N_3 ligand in H_2O were prepared and treated with increasing amounts of iron ranging from 0 to 1.1 equivalents. The absorbance at 460 nm increased until 0.66 equivalents of Fe^{III} had been added, consistent with a metal–ligand stoichiometry of Fe_2L_3 (see Figure S1).



Scheme 1. Synthesis and assembly of ICP particles and their cellular uptake. a) Synthetic route to the bis-3,4-HOPO azide **4**: i) maltol, *n*-propanol, reflux, 16 h; ii) maltol, ethoxyethanol, 64 h, reflux; iii) 4-azidobutan-1-amine, HATU, diisopropylethylamine, DMSO, room temperature, 4 h. b) Assembly of ICP particles from $Fe(NO_3)_3$ and compound **4**, followed by conjugation with DNA by a copper-free "click" reaction: iv) $Fe(NO_3)_3 \cdot 9H_2O$, NaOH (aq), room temperature, 10 min; v) dibenzocyclooctyne–DNA, 0.5 M NaCl, room temperature, 16 h. c) Cellular uptake of the DNA–ICP conjugates. DMSO = dimethyl sulfoxide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

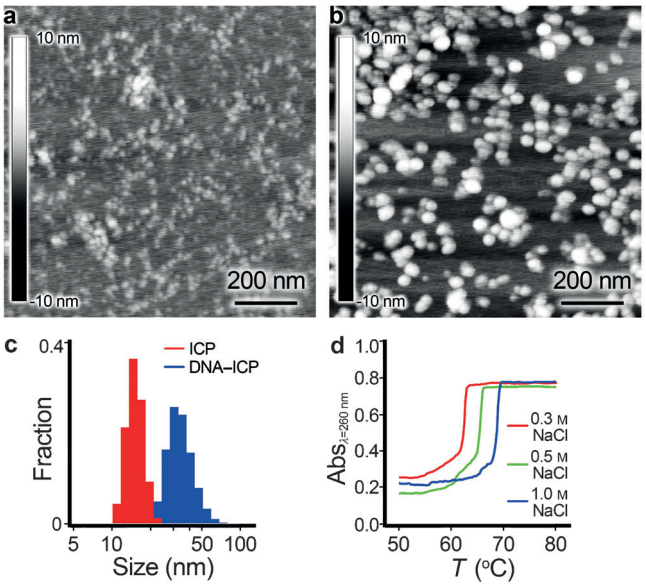


Figure 1. Characterization of the DNA–ICP particles. a) AFM image of bare ICP particles drop-cast and dried on mica. b) AFM image of DNA-functionalized ICP particles drop-cast and dried on mica. c) DLS histograms comparing the size distribution of bare and DNA-functionalized ICPs. d) Cooperative melting of ICP–DNA aggregates.

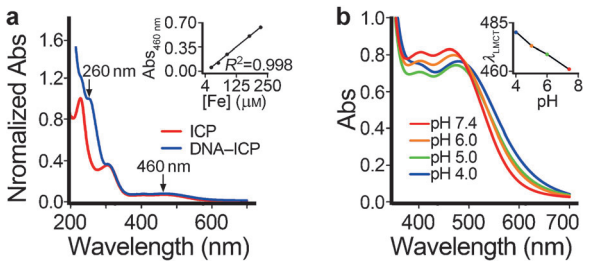


Figure 2. UV/Vis spectroscopic analysis of the DNA–ICP particles. a) Comparison of bare ICP particles with DNA–ICP particles showing DNA absorbance at 260 nm. Inset: determination of LMCT extinction coefficient (ε_{460}). b) pH dependence of the LMCT absorbance. The red-shift of λ_{max} as the pH value decreases is indicative of complex dissociation (see inset).

For conjugation to bare ICP-N_3 particles, all oligonucleotides were made on an automated DNA synthesizer, purified by reverse-phase HPLC, and characterized by MALDI-TOF MS. Dibenzocyclooctyne (DBCO) phosphoramidites are commercially available and were readily incorporated at the 5′ termini of the oligonucleotides. DNA strands modified with a Cyanine 5 (Cy5) dye were used for intracellular imaging studies. DNA strands modified at the 5′ end with an alkylthiol were used to construct gold-nanoparticle SNAs (AuNP-SNAs) for comparison with DNA–ICP particles (see Table S1 in the Supporting Information). DBCO-bearing oligonucleotides were conjugated to ICP-N_3 particles by simply mixing the two reactants in aqueous NaCl (0.5 M), followed by repeated filtration to remove unreacted DNA. The resulting DNA–ICP particles were suspended in Tris·HCl buffer (100 mM, pH 8.0) and remained colloidally stable when stored at 5 °C or when heated up to 80 °C over the course of a melting experiment.

In addition to increasing colloidal stability, the conjugation of DNA to the surface of ICP-N_3 particles resulted in changes to particle size, surface charge, and morphology (Figure 1). DLS and ζ-potential measurements showed a consistent increase in hydrodynamic diameter and surface charge, respectively. Particles were imaged by AFM to visualize changes in size and morphology. UV/Vis spectroscopy was used to calculate the relative contribution of DNA to the absorbance at 260 nm, and hence the DNA concentration was determined. Inductively coupled plasma mass spectrometry (ICPMS) was used to calculate directly the extinction coefficient ε_{460} of the ICP particles (Figure 2a). Finally, incubation of DNA–ICP particles in aqueous buffers ranging from a physiological pH value (7.4) to a low lysosomal pH value (4.0) showed a clear red-shift in the LMCT λ_{max} value, thus indicating partial dissociation of the triscoordinated Fe^{III} nodes of the particle (Figure 2b). Other supramolecular systems based on HOPOs and catechols exhibit similar pH dependence.[25]

To probe the surface density of oligonucleotides on the DNA–ICP particles, thermal denaturation experiments were carried out wherein ICPs with complementary sequences (**A-ICP** and **B-ICP**) were mixed, allowed to hybridize, and then heated above the melting transition of the duplex. The free double-stranded DNA duplex possesses a 17 base pair overlap with $T_m = 54.0$ °C in 0.3 M NaCl. In contrast, the same complementary strands form duplexes with $T_m = 66.9$ °C when conjugated to ICP-N_3 particles: an increase of nearly 13 °C. The melting transition of the DNA–ICP particle aggregates is extremely narrow, which is an indication of cooperativity; the full width at half-maximum of the melting curve is typically < 2 °C, as compared to 10–20 °C for free double-stranded DNA (Figure 1d). **A-ICP** particles alone exhibited no aggregation or melting under the experimental conditions, nor did **A-ICP** particles mixed with noncomplementary particles (**NonTarget-ICP**). We also studied the interaction of DNA–ICP particles with conventional AuNP-SNAs (**A-AuNP**) that were prepared and purified according to established protocols.[26] Similar aggregation and melting behavior was observed between **A-AuNP** and **B-ICP** particles mixed in a 1:1 ratio (see Figure S3). Overall, these studies suggest high DNA surface loading on the ICP-N_3 particles.[6a]

Owing to the high apparent oligonucleotide density on the DNA–ICP surface, we hypothesized that these particles would function as efficient gene delivery agents, much like their gold predecessors.[3] To test this assumption, we functionalized ICP-N_3 particles with the poly(CCT) oligonucleotide **Cy5-DBCO** bearing an internal fluorophore label to afford **Cy5-ICP** particles. Likewise, gold nanoparticles (15 nm) were functionalized with the analogous **Cy5-SH** oligonucleotide to afford **Cy5-AuNP** particles with a loading of approximately 113 strands per AuNP, as determined by fluorescence measurements (see the Supporting Information). To test our hypothesis, uptake was examined in HeLa cervical cancer cells (Figure 3). The DNA–ICP particles were found to cross cell membranes more efficiently than the free DNA strands and exhibited comparable uptake to AuNP-SNA nanoparticles[27] with the same sequence.

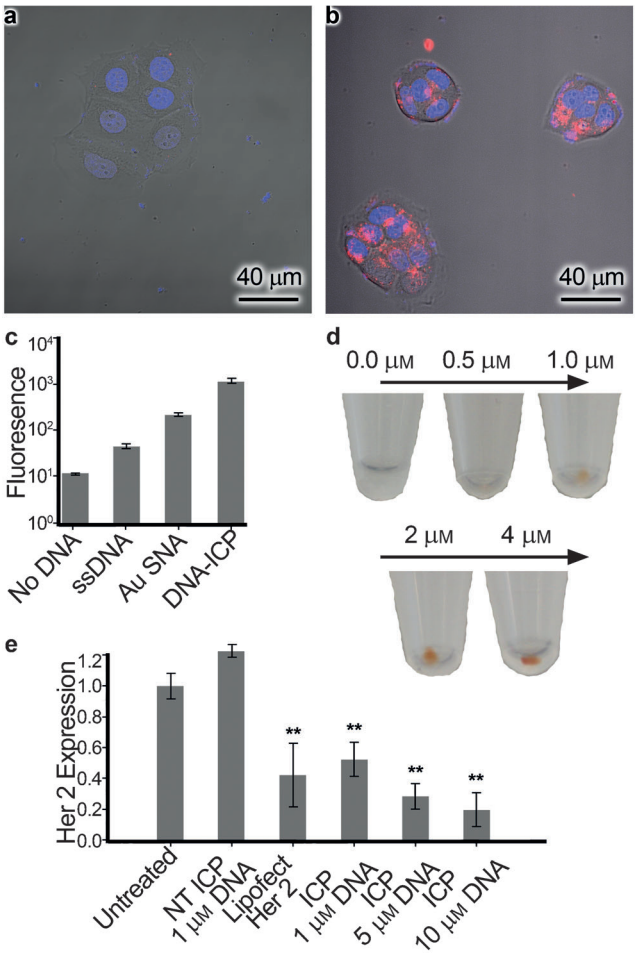


Figure 3. Cellular uptake and gene knockdown. a, b) Confocal microscopy images of HeLa cells treated with Cy5-ssDNA (a) and DNA–ICP particles (b; 100 nM in DNA in each case). Hoechst stain denotes the nucleus in blue, whereas the Cy5 dye attached to the DNA is red. c) Fluorescence intensity of the Cy5 dye as quantified by flow cytometry. d) Naked-eye visualization of DNA–ICPs taken up in pelleted SKOV-3 ovarian-cancer cells. e) Expression of HER2 protein in SKOV-3 cells treated with nontargeting (NT) DNA–ICPs, HER2-targeting ssDNA + Lipofectamine (25 nM DNA basis), and HER2-targeting DNA–ICPS. Starred bars (**) indicate that knockdown was significant ($p < 0.05$), as determined by an unpaired Student T test.

These results suggest that DNA–ICP nanoparticles have the potential to transport large amounts of DNA to the cytosol. A dose-dependent increase in iron concentration was found after the incubation of DNA–ICPs in SKOV-3 ovarian-cancer and MCF-7 breast-cancer cells for 24 h. The color of the iron complex could be seen by the naked eye in pelleted cells treated with DNA–ICPs. Confocal microscopy experiments with SKOV-3 and C166 cells confirmed that DNA–ICPs enter such cell lines, thus demonstrating that these structures exhibit comparable uptake characteristics to AuNP-SNAs (see Figure S7 in the Supporting Information).

Having demonstrated the ability of DNA–ICP conjugates to enter cells in a manner analogous to AuNP-SNAs, we probed their ability to alter protein expression by targeting a known cancer-related mRNA transcript. SKOV-3 ovarian-cancer cells were chosen, as they overexpress human epithelial growth factor receptor 2 (HER2), which is involved in signal transduction pathways leading to malignant cell growth and differentiation.[28] We performed a series of gene knockdown experiments with anti-HER2 DNA–ICPS. SKOV-3 cells were incubated with different concentrations of antisense DNA–ICPs (**HER2-ICP**) or nontargeting DNA–ICPS (**NonTarget-ICP**), with free anti-HER2 DNA complexed with Lipofectamine (Life Technologies) as a positive control. After 3 days, cells were harvested and HER2 expression was determined by western blot analysis (Figure 3e). Treatment with anti-HER2 DNA–ICPs reduced HER2 expression by up to 81%, in a dose-dependent fashion. This result is comparable to those observed with commercial transfection kits, and no change in HER2 expression was observed with nontargeting DNA–ICPs. Finally, no toxic effects or cell death resulted from treatment with DNA–ICPs, as predicted by MTT assays (see Figure S5 in the Supporting Information).

In conclusion, we have reported a facile method to synthesize biocompatible, DNA-decorated infinite-coordination-polymer nanoparticles that are capable of cell entry and gene regulation without transfection agents. Iron(III)-based ICP nanoparticles, synthesized in water, can be conjugated directly to oligonucleotides and carry them across cell membranes. Furthermore, the core is comprised of benign building blocks that are not expected to pose significant health hazards. This study represents a major step towards the construction of clinically viable gene regulation constructs for in vivo applications in the treatment of cancer and other genetic diseases.

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