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Polyvalent Oligonucleotide Iron Oxide Nanoparticle "Click" Conjugates

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ABSTRACT We have utilized the copper-catalyzed azide−alkyne reaction to form a dense monolayer of oligonucleotides on a superparamagnetic nanoparticle core. These particles exhibit the canonical properties of materials densely functionalized with DNA, which can be controlled by modulating the density of oligonucleotides on the surface of the particles. Furthermore, like their Au analogues, these particles can easily cross HeLa (cervical cancer) cell membranes without transfection agents due to their dense DNA shell. Importantly, this approach should be generalizable to other azide-functionalized particles.

KEYWORDS DNA, click, cycloaddition, bioconjugation, nanomaterials

Polyvalent nucleic acid gold nanoparticle (AuNP) conjugates are a unique class of hybrid bionanomaterials formed by functionalizing gold nanoparticles, typically 2−250 nm in diameter, with a dense oligonucleotide shell. The ability to generate such structures with high surface densities of oligonucleotides (~2×10^{13} oligos/cm^2) has led to the discovery and subsequent study of many fundamentally new properties, including cooperative melting transitions,[1] enhanced affinities for complementary oligonucleotides,[2] hybridization-dependent optical responses,[3] enhanced catalytic behavior,[4] resistance to enzymatic degradation,[5] and high cellular uptake without the need for transfection agents.[6] These conjugates have led to many important applications in several areas of research, including programmable colloidal assembly and crystallization,[7] gene regulation,[6] and high sensitivity metal ion,[8] cancer,[9] and molecular diagnostics,[10] some of which have been commercialized and recently FDA cleared.[4]

Although there have been important efforts to extend such chemistry to other particle compositions, including silver,[11] semiconductor quantum dots,[12] silica,[13] and oxides,[14] the thiol adsorption on gold chemistry still stands as one of the most versatile ways of making stable conjugates with tailorable oligonucleotide surface compositions and densities. These other approaches have primarily been limited to heterobifunctional cross-linkers (e.g., EDC); however many of these methods are subject to competing reactions and hydrolysis.[15] New chemistry is needed for broadening the scope of inorganic nanomaterial conjugates that exhibit the aforementioned properties unique to the polyvalent nucleic acid gold nanoparticle conjugates. Herein, we report an approach that utilizes superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with azides as click reagents that can be rapidly coupled to alkyne-modified oligonucleotides to create stable polyvalent conjugates with exceptionally high surface densities of oligonucleotides. Importantly, this approach leads to superparamagnetic conjugates that exhibit many of the attributes of the nonmagnetic polyvalent AuNP system.

The copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) click reaction[16] has been recognized as a facile and versatile chemistry for bioconjugation[17] and has thus garnered significant interest in the field of nanotechnology due to its ability to effectively couple nanosynthons together.[18] It is a functional group tolerant reaction that forms triazole linkages under a vast array of conditions.[16] For conjugation of oligonucleotides to nanoparticles, this reaction is a superior choice due its bioorthogonality and high heat of formation, which can promote conjugation in a high salt environment required to overcome the Coulombic repulsion of neighboring oligonucleotides. Indeed, Simon has previously shown that azide-functionalized AuNPs could be assembled in a linear fashion along the backbone of alkyne modified double helices of DNA.[19] Because of the proven compatibility of the CuAAC with DNA,[18b,20] we hypothesized that by using copper(I) click chemistry, we could synthesize nanostructures other than gold which contain a dense monolayer of oligonucleotides. To investigate this possibility, we have chosen superparamagnetic iron oxide nanoparticles (SPIONs) as a proof-of-concept example because of their potential for use in biomedicine,[21] magnetic resonance imaging,[22] assembly,[23] and environmental remediation.[24]

In a typical conjugation experiment (Scheme 1), ~20 nmol of an oligonucleotide modified with a terminal alkyne (**1**, 5′ alkyne-A_{10}-ATT-ATC-ACT 3′; **2**, 5′ alkyne-A_{10}-AGT-GAT-AAT 3′) were added to a DMSO solution of a $CuSO_4$, THPTA (tris-hydroxylpropyl triazolylamine), and ascorbic acid (AA). The THPTA ligand is a necessary addition to the reaction to

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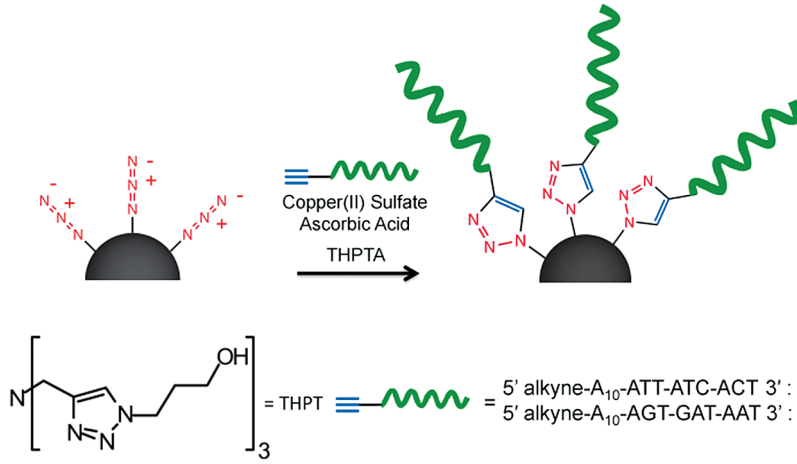
SCHEME 1. CuACC Conjugation of Oligonucleotides to Superparamagnetic Iron Oxide Nanoparticles: (Top) Schematic and (Bottom) Water-Soluble Tris-hydroxylpropyl Triazolylamine Ligand and Complementary Oligonucleotides



prevent the well-known degradation effect of Cu(I) on oligonucleotides and has been successfully used elsewhere.[18b] This mixture was prepared in a 1:4:10 ratio of the respective components, resulting in final reaction concentrations of 200 μM $CuSO_4$, 800 μM THPTA, and 2 mM AA. Then, 100 pmol of 10 nm azide-functionalized particles (experimental section) in water were added to this solution, for a final volume of 500 μL. The reaction was halted by centrifugation at multiple time points (Supporting Information), and the nanoparticles were isolated and resuspended in 0.15 M PBS. This process was repeated three times. We found that the ratio of the catalytic elements to each other were crucial to producing stable conjugates, which has been reported elsewhere,[25] as a large excess of ligand increases the stability of Cu(I) ion in solution[26] and a 10-fold excess of AA keeps copper reduced over the course of the reaction.

To study the extent of the conjugation reaction and the properties of the resulting DNA−SPION conjugates, two batches of particles were functionalized with complementary oligonucleotide sequences (**1** and **2**, Scheme 1). Unmodified 10 nm SPIONs are well-dispersed and are too small to be rapidly pulled out of solution with a conventional bar magnet. When the DNA−SPION particles are functionalized with **1** or **2**, they retain their stability and can be suspended in solution (at NaCl concentrations up to ~1 M) without evidence of aggregation. However, when particles functionalized with **1** and **2**, respectively, are combined in equal amounts, the particles aggregate within a short period of time (1−2 h). Because this process is due to DNA hybridization interactions, it is reversible, and upon heating, the aggregates disperse and the particles are released. The broad absorption of iron oxide in the visible region of the spectrum allows one to easily distinguish aggregated particles from suspended ones spectroscopically or by eye, as the aggregated particles form a heterogeneous suspension that does not efficiently absorb light.

We utilized this ability to distinguish aggregated particles from freely suspended ones to further analyze the binding properties of the conjugates via DNA melting experiments. The reversible hybridization process was monitored at 260 nm as a function of temperature (Figure 1). The DNA−SPION conjugates exhibit sharp cooperative melting transitions (fwhm ~2 °C), which are characteristic of particles functionalized with a dense monolayer of oligonucleotides.[27] The melting temperature of the aggregates increases as a function of increasing salt concentration (0.15−0.7 M), a reflection of increased charge screening of the oligonucleotides involved in hybridization.[11]

One can control oligonucleotide loading with this system by quenching the Cu(I) reaction at different time points (Supporting Information). Particles with densities of 3.18×10^{12} to 2.29×10^{13} oligos/cm^2 (10−70 strands per 10 nm

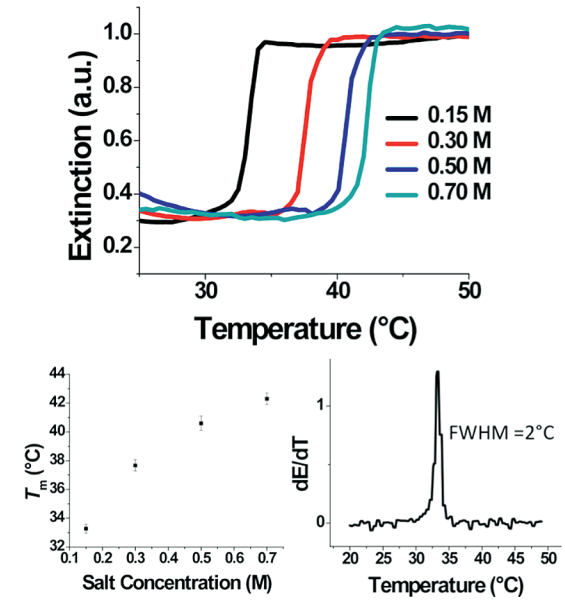


FIGURE 1. (Top) Melting transitions for DNA−SPION aggregates (10 nm in diameter) at various salt concentrations: 0.15, 0.30, 0.50, and 0.70 M. (Bottom) A plot of T_m as a function of salt concentration. An example of the derivative of a melting curve for DNA−SPION conjugates with a fwhm of 2 °C.

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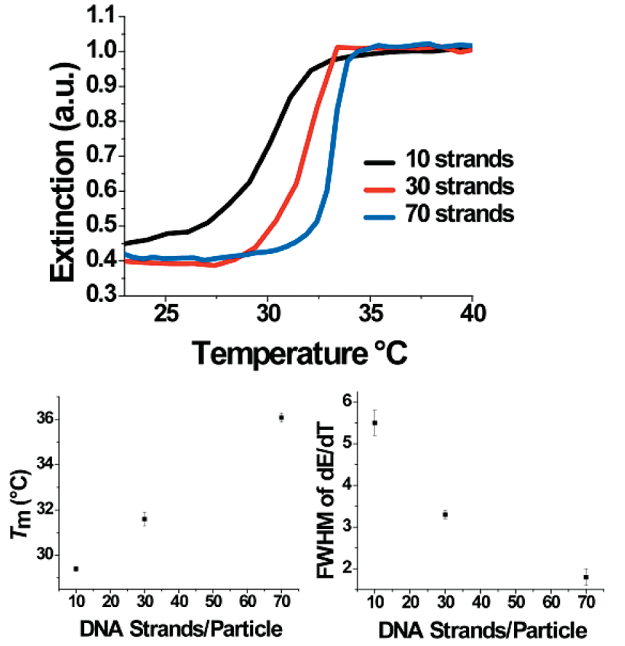

FIGURE 2. (Top) Melting transitions for DNA−SPION aggregates (10 nm in diameter) with various loadings. (Bottom) A plot of T_m as a function of DNA strands/particles. A plot of fwhm of melting transitions as a function of DNA strands/particle.

particle) have been prepared and their hybridization and subsequent melting properties studied (Figure 2). In general, higher loading results in a higher T_m and a more narrow melting transition.[27]

Our group has recently discovered that polyvalent DNA−AuNP conjugates, despite their high negative surface charge, exhibit cellular uptake as a result of dense DNA loading.[28] Having established that this novel click strategy for preparing SPION particles is effective at creating particles with high surface densities of nucleic acids, we next investigated their ability to enter HeLa cells (human cervical cancer). In a typical experiment, cells were cultured on slide chambers, incubated with 200 μL of media at a final concentration of 50 pM Cy5 labeled DNA−nanoparticle (Supporting Information) solution for 12 h, and then imaged using confocal microscopy. The resulting Cy5−DNA−SPION treated-HeLa cells were highly fluorescent, with fluorescence primarily seen in the cytoplasm, consistent with observations made from DNA−AuNP experiments.[6] Significantly, these results show that like the analogous DNA−AuNP conjugates, the polyvalent DNA-conjugated SPIONs readily enter cells without the need for transfection agents.

In order to quantify the uptake efficiency of the SPION−DNA conjugates, we examined the iron content of the cells using inductively coupled plasma mass spectrometry (ICP-MS). HeLa cells were cultured in 24-well plates, incubated with 200 μL solutions at final concentrations of 50 pM and 500 pM DNA−SPIONs for 24 h, and collected for the iron content. Carboxylic acid modified SPIONs (COOH-SPIONs) of the same concentrations were used as a control. Due to the dense functionalization of oligonucleotides on the surface of the nanoparticles, the DNA−SPIONs readily enter cells (50000−150000 number per cell), while negatively charged COOH-SPIONS in solution exhibit a consistently lower uptake (~10000 per cell) when compared to the DNA−SPIONS (Figure 3). This is further evidence that a dense layer of oligonucleotides on a nanoparticle surface, regardless of core, can mediate cellular uptake without transfection agents. This is significant because most methods for delivery of genetic material, utilizing SPIONs, require the use of potentially toxic transfection agents[29] or targeting epitopes.[30]

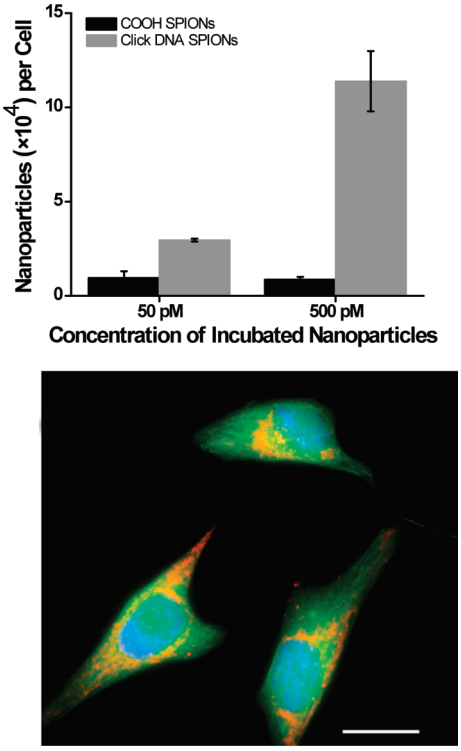

FIGURE 3. (Top) ICP analysis of SPIONs in HeLa cells shows that the monolayer of DNA on the surface of the nanoparticles greatly promotes cellular uptake. (Bottom) Fluorescence microscopy image of HeLa cells incubated with DNA−SPIONs for 24 h. The red channel is fluorescence associated with DNA−SPIONs labeled with Cy5, the green channel is fluorescence associated taxol−Alexa 488 specific for tubulin, and the blue channel is fluorescence associated with Hoechst 3342 specific for the nucleus. Scale bar is 20 μm.

In conclusion, we have developed a strategy to immobilize oligonucleotides to the surface of SPIONs using click chemistry. This method has afforded particles that exhibit properties such as sharp melting transitions and high cellular uptake, indicative of their dense functionalization. The ability to densely functionalize SPIONS with DNA will allow a myriad of applications such as magnetic resonance imaging (MRI), magnetic hyperthermia therapy strategies, and assembly of magnetic structures for electronic memory applications.[22] In addition, we expect that click chemistry will be a used as a general strategy for the conjugation of oligonucleotides to nanoparticles regardless of core material.

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Supporting Information Available. A description of the materials and methods used. This material is available free of charge via the Internet at http://pubs.acs.org.

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