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Polyvalent DNA Nanoparticle Conjugates Stabilize Nucleic Acids

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Abstract

Polyvalent oligonucleotide gold nanoparticle conjugates have unique fundamental properties including distance-dependent plasmon coupling, enhanced binding affinity, and the ability to enter cells and resist enzymatic degradation. Stability in the presence of enzymes is a key consideration for therapeutic uses; however the manner and mechanism by which such nanoparticles are able to resist enzymatic degradation is unknown. Here, we quantify the enhanced stability of polyvalent gold oligonucleotide nanoparticle conjugates with respect to enzyme-catalyzed hydrolysis of DNA and present evidence that the negatively charged surfaces of the nanoparticles and resultant high local salt concentrations are responsible for enhanced stability.

Over the past decade, researchers have developed many uses for polyvalent oligonucleotide nanoparticle conjugates (DNA-Au NPs).¹ These structures, which consist of a nanoparticle core (typically 2–250 nm in size) and many oligonucleotide strands covalently attached to their surface,^{2,3} exhibit several unusual properties that make them attractive for both diagnostic and therapeutic applications.^{4–9} These properties include cooperative binding and enhanced affinities for complementary nucleic acids,^{10,11} catalytic properties that can be used for signal amplification,¹² unusual distance dependent plasmonic properties,^{13,14} and the ability to enter cells without the use of auxiliary transfection agents.¹⁵ They also exhibit an extraordinary intracellular stability that makes them useful for antisense studies, drug delivery, and intracellular molecular diagnostics.^{16–18} Indeed, nucleic acid stability is a key property of any system that aims to use such structures for intracellular regulatory or diagnostic events. The problem is that Nature has evolved an arsenal of enzymes, known as nucleases, to degrade foreign nucleic acids that enter cells.¹⁹ Herein, we describe a study that examines the stability of the DNA on a nanoparticle surface as a function surface coverage and provide an explanation for why these structures resist enzymatic degradation.

We prepared DNA-Au NPs and studied their nuclease stability using fluorescence spectroscopy and literature methods¹⁶ (see Supporting Information). The DNA-Au NPs consists of a 13 ± 1 nm Au-NP functionalized with a dense monolayer of oligonucleotides composed of a 20-base DNA sequence, a 10-base DNA linker, and propylthiol anchor (see Supporting Information). Each probe was allowed to hybridize with fluorescein-labeled DNA complements, and the enzyme deoxyribonuclease I (DNase I), a common endonuclease,²⁰ was added to interrogate DNA-Au NP stability. Since DNase I is known to bind ssDNA (although with much lower affinity than dsDNA), we initially varied the nanoparticle surface coverage using duplex and single-stranded DNA. DNA-Au NPs were

allowed to hybridize with different molar ratios of fluorophore-labeled complements (5, 10, 20, and 30 complements per DNA-Au NP). Hybridization was achieved by heating to 70 °C and cooling for 12 h. After hybridization, the total dsDNA in each sample was adjusted to 50 nM. Next, the samples were treated with DNase I, and the rate of degradation was measured by a fluorescence-based assay (see Supporting Information). The results of these experiments reveal similar reaction rates in each sample. We conclude that dsDNA is the substrate of DNase I and the effect of ssDNA or dsDNA on the nanoparticle surface is similar. This is consistent with the ~500 times lower activity of DNase I for ssDNA.²¹

To develop a basis for comparing DNA-Au NPs with molecular DNA, we prepared molecular probes consisting of the same DNA sequence with a 3' dabcyl quencher. To compare duplex degradation using fluorescence methods, each probe was allowed to hybridize with fluorescein-labeled DNA complements. The degradation reactions were recorded (Figure 1), and the half-lives were calculated (Table 1). Under these conditions, DNA-Au NPs have a half-life that is 4.3 times longer than the molecular probe with the identical sequence. The longer half-life observed in the case of the DNA-Au NP is consistent with the stabilization of DNA with respect to enzymatic degradation.

Enzyme-catalyzed DNA degradation can be modeled as a 2-step reaction that involves (1) the association of the enzyme with the substrate, and (2) the hydrolysis of the nucleic acids.²² The enhanced stability observed for DNA-Au NPs could result from a decrease in rate of either of these two steps. We performed a double-reciprocal analysis (Lineweaver–Burk plot) of the enzyme reactions (Figure 1), which allowed us to calculate enzyme–substrate association ($1/K_m$, step 1) and maximum reaction velocity (V_{max} , step 2) for both the DNA-Au NPs and molecular DNA (Table 1). These data reveal that the enzyme association is more favorable in the case of DNA-Au NPs, while the maximum reaction velocity is slower than that of molecular DNA (Table 1). Therefore the hydrolysis step (step 2) is the step that contributes to the enhanced nuclease resistance of DNA-Au NPs, despite somewhat greater enzyme association.

Having determined that the hydrolysis step is slowed, we investigated the physical characteristics of the DNA-Au NPs that lead to this property. To probe the contribution of DNA surface density, we prepared a series of nanoparticles with decreasing DNA coverage by adding increasing amounts of ethylene glycol-thiol²³ diluent molecules (PEG 1 to PEG 4) during the DNA-Au NP conjugation step. Fluorescence-based quantification measurements²⁴ reveal oligonucleotide surface densities ranging from 12 to <1 pmol/cm² (Table 2). In nuclease reactions (Figure 2), the conjugates with similar DNA surface densities have similar half-lives (100 ± 16 to 97 ± 11 min, Table 3). In contrast, conjugates that had less DNA on their surfaces had shorter half-lives (67 ± 12 to 48 ± 4 min). The fact that the particles with greater surface density of DNA have the longest half-lives suggests that DNA density contributes to nuclease resistance. One hypothesis that could explain this trend is that the crowding of DNA on a nanoparticle surface results in steric inhibition of enzyme binding. It is interesting that the Lineweaver–Burk analysis reveals that enzyme binding is not hindered on DNA-Au NPs but rather somewhat enhanced (1.6 to $2.6 \mu\text{M}^{-1}$, molecular DNA to DNA-Au NP, Table 1). Thus, while increasing the DNA density increases nuclease resistance, it may be due to affects other than steric inhibition.

Two of the hallmarks of DNA-Au NPs are their elevated melting temperatures and high binding constants compared with molecular DNA.^{4,10,11} These properties result from both the ability of the conjugate to engage in multidentate interactions as well as a high local salt concentration at the nanoparticle surface.^{10,25} We hypothesized that a high local salt concentration could lead to enzyme inhibition. Indeed, previous work has demonstrated that monovalent cations, including Na⁺, inhibit of DNase I and related nucleases. Specifically,

Na⁺ displaces Ca²⁺ and Mg²⁺ ions that are bound to the enzyme and required for activity.^{20,26–28} We therefore designed experiments to investigate the local environment associated with the DNA-Au NPs. The surface potentials of the nanoparticles were calculated using zeta potential measurements. All DNA-Au NPs are negatively charged as a result of their DNA functionalized surfaces. The measurements reveal a more negative potential for more densely functionalized conjugates (Table 2). More negative charges should also lead to more charge-balancing counterions. The strong correlation observed between charge and nuclease resistance could therefore be explained by a greater amount of charge-balancing Na⁺ counterions associated with the DNA-Au NP.

To confirm that the salt associated with DNA-Au NPs leads to enhanced stability toward nucleases, we repeated degradation experiments using a nuclease engineered to be more tolerant of monovalent salts such as Na⁺ (Turbo DNase, see Supporting Information). In these experiments, we expect that the DNA-Au NPs with high salt concentrations will have an increased degradation rate when tested with the salt-tolerant enzyme. Degradation rates were normalized using the molecular system to account for inherent differences between the enzymes. Consistent with the hypothesis of low localized salt concentration, the degradation rate of molecular DNA did not increase when challenged with salt tolerant enzymes. In fact, it increases from a half-life of 23 to 32 min due to a difference in activity (Table 3). Consistent with the hypothesis of a high localized salt concentration, DNA-Au NPs have greatly reduced half-lives when challenged with salt tolerant enzymes (Figure 2). The decrease in half-life scales with both the density of the DNA on the nanoparticle and surface charge. When the differences in the two enzymes' activities are normalized to molecular DNA, DNA-Au NPs are 4.3 times more stable under salt-dependent conditions while only 1.5 times more stable under salt-tolerant conditions. The similarity in stability between DNA-Au NPs and molecular DNA under salt-tolerant conditions demonstrates that steric hindrance is not the dominant factor contributing to stability.

In addition to probing salt concentration by changing the type of enzyme, we also adjusted the bulk salt concentration in the assay buffer. Specifically, we measured the initial velocity of DNase I catalyzed degradation reaction of molecular DNA and DNA-Au NPs for three different salt concentrations (see Supporting Information). The results of these experiments show that small increases in salt concentration (15–25 nM) significantly affect molecular DNA degradation, but not DNA-Au NP degradation. This is consistent with a higher salt concentration associated with DNA-Au NPs than molecular DNA. Additionally, at high salt concentrations (215 nM) the molecular DNA and DNA-Au NPs are degraded at a similar rate. When taken with the Lineweaver–Burk analysis and the increased nanoparticle degradation observed under salt-tolerant enzyme conditions, these results support the conclusion that salt associated with DNA-Au NPs is the dominate cause of decreased enzymatic activity, and thus the stability of polyvalent DNA-Au NP conjugates (Scheme 1).

Stability toward enzymes and nucleases is important for therapeutic and diagnostic applications. Strategies to increase the stability of nucleic acids typically rely on chemical modification²⁹ or electrostatic interaction with a surface.^{30,31} Nanoparticles have the ability to interact with enzymes in a unique and often multivalent manner.^{32–41} This work has examined the apparent stability of DNA toward nucleases that results when they are arranged in a dense monolayer on a nanoparticle surface. We have investigated various nanoparticle properties (surface density and charge) and demonstrate that charge and local salt are the main factors that correlate with the increased nuclease stability of DNA-Au NPs. Polyvalent DNA-Au NPs do not prevent enzyme binding, but rather inhibit enzyme catalyzed hydrolysis, which results in an observed half-life that is 4.3 fold greater than a molecular DNA system. When nanoparticles are challenged with a more salt tolerant enzyme, the observed rate of degradation increases, indicating that DNA-Au NP associated

salt is the main factor contributing to stability. These results show how the environment surrounding DNA-Au NPs is not only ideal for high-affinity target binding,¹¹ but also for resisting enzymatic degradation. This finding reinforces the exceptional promise and utility that these nanomaterials hold for a wide variety of diagnostic, therapeutic, imaging, and biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of the degradation rates of molecular DNA and DNA-Au NP systems. (A) Fluorescence-based progress curves of the enzyme-catalyzed reaction as a function of time. (B) Double reciprocal (Lineweaver–Burk) plot of the initial degradation velocity as a function of DNA-duplex concentration (used to calculate the kinetic parameters of the reaction, Table 1).

Figure 2.

Comparison of DNA-Au NP conjugates with different DNA coverage. (A) Fluorescence-based progress curves of the wild-type DNase I catalyzed reaction over time. (B) Fluorescence-based progress curves of the Turbo DNase catalyzed reaction over time. The activity of wild-type DNase I is more affected by salt than Turbo DNase.

^a Enzyme catalyzed DNA hydrolysis is modeled in two steps: enzyme association, and DNA hydrolysis. The high surface-charge resulting in increased associated salts is postulated to be the origin of DNA stability and the slower enzymatic hydrolysis.

Table 1

Half-Life, Maximum Reaction Velocity ($V_{\max}$), and Enzyme Association Constant ($1/K_m$) for Molecular DNA and DNA-Au NP Systems

system	half-life (min)	$V_{\max}$ (nM sec ⁻¹)	$1/K_m$ (μM^{-1})
Molecular DNA	23 $\pm$ 4	0.27 $\pm$ 0.04	1.6 $\pm$ 0.3
DNA-Au NP	100 $\pm$ 16	0.10 $\pm$ 0.03	2.6 $\pm$ 0.6

Table 2Physical Properties of DNA-Au NP Conjugates^a

system	surface coverage (pmol/cm ²)	surface potential (mV)
DNA-Au NP	12 ± 1	-32 ± 1
PEG 1	12 ± 1	-31 ± 1
PEG 2	10 ± 1	-31 ± 3
PEG 3	6 ± 1	-22 ± 2
PEG 4	<1	-17 ± 2

^a Surface coverage was determined by fluorescence measurements and surface potential was determined by ζ -potential measurements.

Table 3

Half-Life of Molecular and DNA-Au NP Systems for More Salt Inhibited (Wild-Type) and More Salt Tolerant (Turbo) Nucleases

system	wild-type DNase I (more salt dependent)		turbo DNase (less salt dependent)	
	half-life (minutes)	relative increase ^a	half-life (minutes)	relative increase ^a
Molecular DNA	23 ± 4	1	32 ± 1	1
DNA-Au NP	100 ± 16	4.3	48 ± 2	1.5
PEG 1	97 ± 11	4.2	48 ± 3	1.5
PEG 2	67 ± 12	2.9	44 ± 5	1.4
PEG 3	48 ± 4	2.1	38 ± 3	1.2

^aThe relative increase in half-life normalized to molecular DNA.