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Articles

A Fluorescence-Based Method for Determining the Surface Coverage and Hybridization Efficiency of Thiol-Capped Oligonucleotides Bound to Gold Thin Films and Nanoparticles

Linette M. Demers,[†,‡] Chad A. Mirkin,*[,†,‡] Robert C. Mucic,[†,‡] Robert A. Reynolds, III,[†,‡] Robert L. Letsinger,*[,‡] Robert Elghanian,[‡] and Garimella Viswanadham[‡]

Department of Chemistry and Center for Nanofabrication and Molecular Self-Assembly, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

Using a fluorescence-based method, we have determined the number of thiol-derivatized single-stranded oligonucleotides bound to gold nanoparticles and their extent of hybridization with complementary oligonucleotides in solution. Oligonucleotide surface coverages of hexanethiol 12-mer oligonucleotides on gold nanoparticles (34 ± 1 pmol/cm^2) were significantly higher than on planar gold thin films (18 ± 3 pmol/cm^2), while the percentage of hybridizable strands on the gold nanoparticles (1.3 ± 0.3 pmol/cm^2, 4%) was lower than for gold thin films (6 ± 2 pmol/cm^2, 33%). A gradual increase in electrolyte concentration over the course of oligonucleotide deposition significantly increases surface coverage and consequently particle stability. In addition, oligonucleotide spacer sequences improve the hybridization efficiency of oligonucleotide-modified nanoparticles from ∼4 to 44%. The surface coverage of recognition strands can be tailored using coadsorbed diluent oligonucleotides. This provides a means of indirectly controlling the average number of hybridized strands per nanoparticle. The work presented here has important implications with regard to understanding interactions between modified oligonucleotides and metal nanoparticles, as well as optimizing the sensitivity of gold nanoparticle-based oligonucleotide detection methods.

There has been a great deal of effort directed toward elucidating the surface coverage, structure, and function of immobilized oligonucleotides on electrode and thin-film metallic surfaces, primarily for use in the development of nucleic acid detection methodologies. A number of techniques, including electrochemistry, XPS, and surface plasmon resonance spectroscopy, have been utilized to characterize such systems.[1−7] In contrast, there is very little known about the loading of oligonucleotides on inorganic nanoparticles.

Recently, our group reported several new DNA detection schemes based upon the hybridization of target DNA molecules to gold nanoparticle probes with surface immobilized alkanethiol-capped oligonucleotides.[8−10] This detection method takes advantage of a colorimetric change associated with particle aggregation caused by oligonucleotide hybridization events between particles and target molecules within the aggregate. Two factors that affect the magnitude of the observed optical changes are (1) the extent of surface modification of the gold probes with oligonucleotides and (2) the ability of complementary target oligonucleotides to access surface-bound oligonucleotides. Understanding these factors not only is important for determining and controlling the inherent sensitivity of gold nanoparticle-based detection methods but also should lead to a greater understanding of the interactions between gold nanoparticles and surface-bound thiol-functionalized oligonucleotides.

Herein we report a fluorescence-based method for determining the surface coverage of alkanethiol-functionalized oligonucleotides adsorbed onto Au nanoparticles and the accessibility of these immobilized strands for hybridization with complementary oligo-

* Corresponding authors: (e-mail) camirkin@chem.nwu.edu; rletsinger@chem.nwu.edu.

† Center for Nanofabrication and Molecular Self-Assembly.

‡ Department of Chemistry.

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Table 1. Sequences of Modified Oligonucleotide Strands

oligonucleotide	sequence (5′ to 3′)
$SN_{12}f$	$HS(CH_2)_6$-5′CGCATTCAGGAT-$(CH_2)_6$-f
$SA_{20}N_{12}f$	$HS(CH_2)_6$-5′$(A)_{20}$CGCATTCAGGAT-$(CH_2)_6$-f
$S3'X_{20}N_{12}f$	f-$(CH_2)_6$-5′TCTCAACTCGTA$(X)_{20}$-$(CH_2)_3SH$, X = A or T
$S3'N_{12}f$	f-$(CH_2)_6$-5′TCTCAACTCGTA-$(CH_2)_3SH$
$N'_{12}f$	f-$(CH_2)_6$-5′ATCCTGAATGCG
$N_{12}3'f$	5′ATCCTGAATGCG-$(CH_2)_6$-f

nucleotides in solution. This method has enabled us to investigate parameters that influence the surface coverage and structure of immobilized single-stranded oligonucleotides, such as the electrolyte concentration in the adsorbate deposition solution, the use of oligonucleotide spacers that differ by sequence and length, and the role of coadsorbed diluent molecules. This is the first study aimed at gaining such information for nanoparticle-based materials, and importantly, it provides a way of predicting and controlling the hybridization efficiencies of oligonucleotide-modified nanoparticles and oligonucleotides either in solution or on thin-film surfaces.

EXPERIMENTAL SECTION

General Methods. $HAuCl_4 \cdot 3H_2O$ and trisodium citrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Gold wire, 99.999% pure, and Ti wire were purchased from Goldsmith Inc. (Evanston, IL). Silicon wafers (100) with a 1-μm-thick oxide layer were purchased from Silicon Quest International (Santa Clara, CA). 5′-Thiol modifier phosphoramidite reagent, 3′-propanethiol modifier CPG, fluorescein phosphoramidite, and other reagents required for oligonucleotide synthesis were purchased from Glen Research (Sterling, VA). 5-(and 6)-carboxyfluorescein, succinimidyl ester was purchased from Molecular Probes (Eugene, OR). All oligonucleotides were prepared with an automated DNA synthesizer (Expedite) using standard phosphoramidite chemistry.[11] Sequences are given in Table 1. Oligonucleotides containing only 5′-hexanethiol modifications were prepared using procedures previously reported,[9] all other oligonucleotide synthesis and purification procedures are available as Supporting Information. NAP-5 columns (Sephadex G-25 Medium, DNA grade) were purchased from Pharmacia Biotech. Nanopure H_2O (>18.0 MΩ), purified using a Barnstead NANOpure ultrapure water system, was used for all experiments. An Eppendorf 5415C or a Beckman Avanti 30 centrifuge was used for centrifugation of Au nanoparticle solutions. High-performance liquid chromatography (HPLC) was performed using a Hewlett-Packard (HP) series 1100 HPLC.

Physical Measurements. Electronic absorption spectra of the oligonucleotide and nanoparticle solutions were recorded using a HP 8452a diode array spectrophotometer. Fluorescence spectroscopy was performed using a Perkin-Elmer LS50 fluorometer. Transmission electron microscopy (TEM) was performed with a Hitachi 8100 transmission electron microscope operating at 200 kV. A Thermo Jarrell Ash AtomScan 25 atomic emission spectrometer with an inductively coupled plasma (ICP) source was used to determine the atomic concentration of gold in the nanoparticle solutions (gold emission was monitored at 242.8 nm).

Preparation and Characterization of Gold Nanoparticles. Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$.[12,13] TEM was used to determine the size distribution of the resulting nanoparticles. At least 250 particles were sized from TEM negatives via graphics software (ImageTool). The average diameter of a typical particle preparation was 15.7 ± 1.2 nm. Assuming spherical nanoparticles and density equivalent to that of bulk gold (19.30 g/cm^3), an average particle mass was calculated (2.4×10^7 g/mol). The atomic gold concentration in a solution of gold nanoparticles was determined by inductively coupled plasmon atomic emission spectroscopy (ICP-AES). A gold atomic absorption standard solution (Aldrich), was used for calibration. Comparison of atomic gold concentration in the particle solution to the average particle volume obtained by TEM analysis yielded the molar concentration of gold particles in a given preparation, typically ~10 nM. By measuring the UV−visible absorbance of nanoparticle solutions at the surface plasmon frequency (520 nm), we calculated molar extinction coefficients (ϵ at 520 nm) for the particles, typically 4.2×10^8 M^{-1} cm^{-1} for 15.7 ± 1.2 nm diameter particles.

Preparation of Gold Thin Films. Silicon wafers were cut into ~10 mm × 6 mm pieces, cleaned with piranha etch solution (4:1 concentrated H_2SO_4/30% H_2O_2) for 30 min at 50 °C, and then rinsed with copious amounts of water, followed by ethanol. (*Warning*: *piranha etch solution reacts violently with organic materials and should be handled with extreme caution*.) Metal was deposited at a rate of 0.2 nm/s using an Edwards Auto 306 evaporator (base pressure of 3×10^{-7} mbar) equipped with an Edwards FTM6 quartz crystal microbalance. The polished side of the silicon was coated with a Ti adhesion layer of 5 nm, followed by 200 nm of gold.

Preparation of Alkanethiol Oligonucleotide-Modified Gold Nanoparticles. Gold nanoparticles were modified with fluorescein-alkanethiol oligonucleotides by adding oligonucleotides to aqueous nanoparticle solution (particle concentration ~10 nM) to a final oligonucleotide concentration of 3 μM. After 24 h, the solution was buffered at pH 7 (10 mM phosphate), and NaCl solution was added (final concentration of 0.1 M). The solution was allowed to "age" under these conditions for an additional 40 h. Excess reagents were then removed by centrifugation for 30 min at 14 000 rpm. Following removal of the supernatant, the red oily precipitate was washed twice with 0.3 M NaCl, 10 mM phosphate buffer (pH 7) solution (0.3 M PBS) by successive centrifugation and redispersion and then finally redispersed in fresh 0.3 M PBS. Invariably, a small amount (~10−20% as determined by UV−visible spectroscopy) of the nanoparticles is discarded with the supernatant during the washing procedure. Therefore, final nanoparticle concentrations were determined by TEM, ICP-AES, and UV−visible spectroscopy (vide supra). Extinction coefficients and particle size distributions did not change significantly as a result of the oligonucleotide modification.

Preparation of Alkanethiol Oligonucleotide-Modified Gold Thin Films. Silicon-supported Au thin films were immersed in deposition solutions of alkanethiol-modified oligonucleotides for

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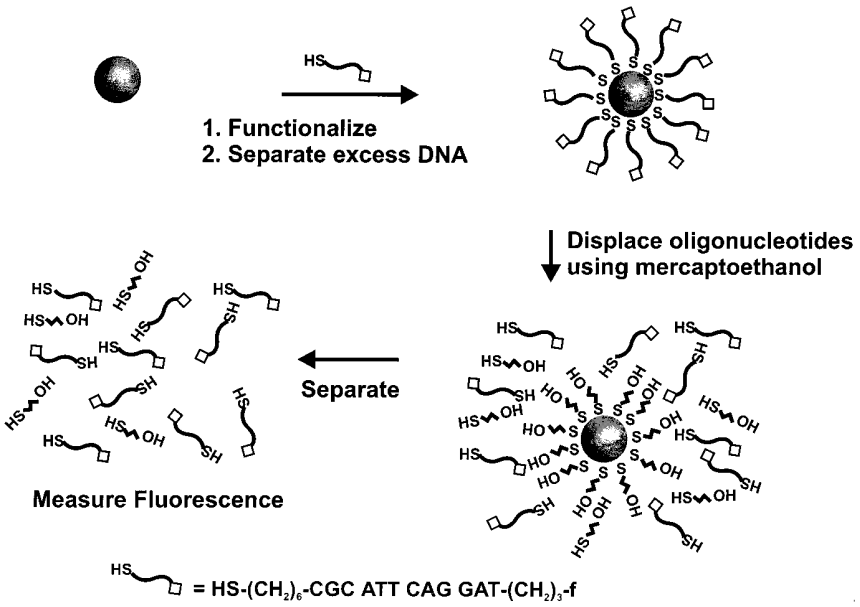
Scheme 1



times and buffer conditions equal to those for the Au nanoparticles. Following oligonucleotide deposition, the films were rinsed extensively with 0.3 M PBS and stored in buffer solution. Gold was evaporated on one side only, leaving an unpassivated silicon/silicon oxide face. Alkanethiol-modified DNA adsorbed to the bare silicon oxide side was easily removed by rinsing with PBS.

Quantitation of Alkanethiol-Oligonucleotides Loaded on Nanoparticles. Mercaptoethanol (ME) was added (final concentration 12 mM) to fluorophore-labeled oligonucleotide-modified nanoparticles or thin films in 0.3 M PBS, Scheme 1. After 18 h at room temperature with intermittent shaking, the solutions containing displaced oligonucleotides were separated from the gold by either centrifugation of the gold nanoparticle aggregates or by removal of the gold thin film. Aliquots of the supernatant were diluted 2-fold by addition of 0.3 M PBS, pH 7. Care was taken to keep the pH and ionic strength of the sample and calibration standard solutions the same for all measurements due to the sensitivity of the optical properties of fluorescein to these conditions.[14] The fluorescence maximums (measured at 520 nm) were converted to molar concentrations of the fluorescein-alkanethiol-modified oligonucleotide by interpolation from a standard linear calibration curve. Standard curves were prepared with known concentrations of fluorophore-labeled oligonucleotides using identical buffer pH, salt, and mercaptoethanol concentrations. Finally, the average number of oligonucleotides per particle was obtained by dividing the measured oligonucleotide molar concentration by the original Au nanoparticle concentration. Normalized surface coverage values were then calculated by dividing by the estimated particle surface area (assuming spherical particles) in the nanoparticle solution.[15]

Nanoparticle Dissolution with KCN. A solution of 0.2 M KCN and 2 mM $K_3Fe(CN)_6$ in water was used to dissolve the gold nanoparticles. The etching solution was added to 16.5 ± 1.2 nm Au nanoparticles derivatized with 3′-propanethiol, 5′-fluorescein 12-mer oligonucleotides ($S3'N_{12}f$) in 0.3 M PBS (final concentration 0.08 M KCN and 0.8 mM $K_3Fe(CN)_6$, ~3.9 nM nanoparticles). After several minutes, the nanoparticle surface plasmon resonance absorption band at 520 nm completely disappeared, and the resulting pale yellow solution had absorption bands at 420 (due to excess ferricyanide) and 494 nm (due to the fluorescein-labeled oligonucleotides in solution). A linear absorption versus concentration calibration curve was constructed by adding known amounts of the 3′-propanethiol, 5′ fluorescein 12-mer oligonucleotide to solutions of citrate-stabilized gold colloids in 0.3 M PBS which had been digested under the etching conditions described above. Normalized surface coverage values were then calculated as described above.

Quantitation of the Hybridized Target Surface Density. To determine the activity of adsorbed oligonucleotides for hybridization, fluorophore-labeled oligonucleotides, which were complementary to the surface-bound oligonucleotides ($N'_{12}f$), were reacted with oligonucleotide-modified surfaces (Au nanoparticles or thin films) under hybridization conditions (3 μM complementary oligonucleotide, 0.3 M PBS pH 7, 24 h). Nonhybridized oligonucleotides were removed from the gold by rinsing twice with buffered saline as described above. Then, the fluorophore-labeled oligonucleotides were dehybridized by addition of NaOH (final concentration ~50 mM, pH 11−12, 4 h). Following separation of the solution containing the $N'_{12}f$ from the nanoparticle solutions by centrifugation, and neutralization of the solutions by addition of 1 M HCl, the concentrations of hybridized oligonucleotide and corresponding hybridized target surface density were determined by fluorescence spectroscopy, Scheme 2.

RESULTS AND DISCUSSION

Quantitation of Surface Coverage and Hybridization of 12 Base Oligonucleotides on Au Nanoparticles and Thin Films. Citrate-stabilized Au nanoparticles were functionalized with 12-

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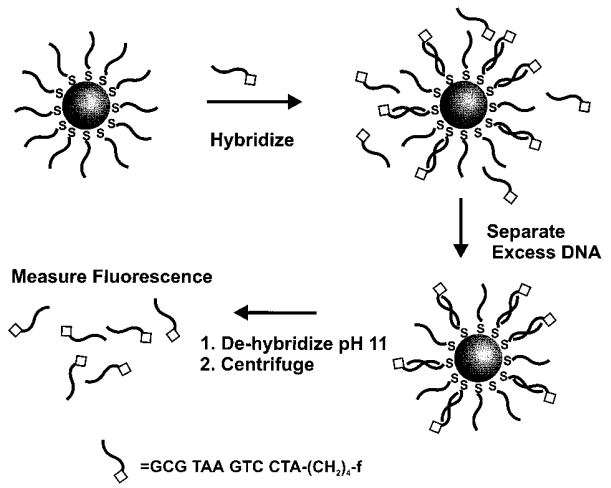
(15) The assumption of roundness is based on a measured average roundness factor of 0.93. Roundness factor is computed as: $(4\pi(\text{surface area}))/(2 \times \text{perimeter})$.

Scheme 2



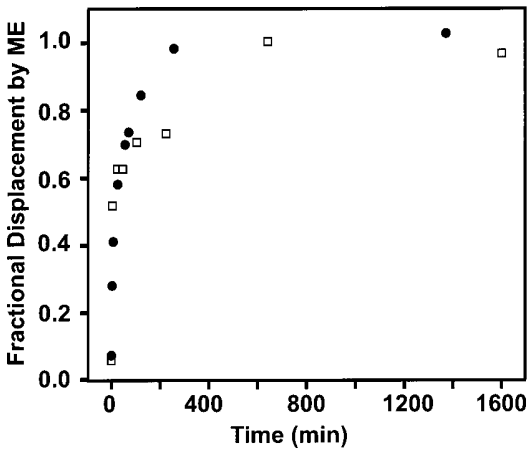


Figure 1. Fractional displacement of $SN_{12}f$ by mercaptoethanol (12 mM) in 0.3 M PBS from gold nanoparticles (closed circles) and gold thin films (open squares). Fractional displacement for each sample was plotted as the fluorescence intensity obtained at time, *t*, normalized with respect to the fluorescence intensity at the longest displacement time.

mer fluorescein-modified alkanethiol DNA (HS-$(CH_2)_6$-5′CGCATTCAGGAT-$(CH_2)_4$-f). Surface coverage studies were then performed by thoroughly rinsing away nonchemisorbed oligonucleotides, followed by removal of the fluorophore-labeled oligonucleotides from the gold surface, and quantitation of oligonucleotide concentration using fluorescence spectroscopy (as described in the Experimental Section).

Desorption of all the oligonucleotides from the Au surface and subsequent removal of Au particles from the solution are critical for obtaining accurate coverage data via fluorescence for two reasons. First, the fluorescence signal of labeled, surface-bound DNA is efficiently quenched as a result of fluorescence resonance energy transfer (FRET) to the Au particle. Indeed, there is almost no measurable signal for fluorescein-modified oligonucleotides (12−32 nucleotide strands; sequences are given in Table 1) after they are immobilized on 15.7 ± 1.2 nm Au particles and residual oligonucleotide in solution is washed away. Second, the Au nanoparticles absorb a significant amount of light between 200 and 530 nm, so their presence in solution during fluorescence measurements acts as a filter and diminishes the available excitation energy, as well as the intensity of emitted radiation. The Au surface plasmon band at 520 nm falls at the emission maximum of fluorescein.

ME was used to rapidly displace the surface-bound oligonucleotides via an exchange reaction, Scheme 1. To examine the displacement kinetics, oligonucleotide-modified nanoparticles were exposed to ME (12 mM) for increasing periods of time prior to centrifugation and fluorescence measurements. The intensity of fluorescence associated with the solution free of nanoparticles can be used to determine how much oligonucleotide was released from the nanoparticles. The amount of oligonucleotide freed in exchange with ME increased until ∼10 h of exposure (Figure 1), which indicates that the oligonucleotide displacement process is complete. The displacement reaction is rapid, which is presumably due to the inability of the oligonucleotide film to block access of the ME to the gold surface.

An independent set of experiments confirmed that the mercaptoethanol completely displaces the DNA under the stated conditions. In these experiments, a cyanide solution was used to dissolve 16.5 ± 1.2 nm gold particles functionalized with fluorophore-labeled oligonucleotides (see Experimental Section). This procedure has been used by others to dissolve the gold cores of polymer-coated nanoparticles.[16] After dissolution of the particles, the absorbance associated with the fluorescein at 494 nm was measured by UV−visible spectroscopy and used to determine the concentration of the released oligonucleotides. Note that a control experiment involving the CN^--induced dissolution of unmodified citrate-stabilized 16.5 ± 1.2 nm gold nanoparticles shows that there is zero absorbance at 490 nm after the particles are completely dissolved. Using this methodology, the surface coverage for a heavily loaded set of nanoparticles was determined to be 42 ± 2 pmol/cm^2. The surface coverage for this set of nanoparticles was independently determined to be 40 ± 3 pmol/cm^2 by our ME displacement procedure. The two values are identical within experimental error, confirming that ME displaces all of the oligonucleotides from the nanoparticle surface. Note that although this CN^- procedure works well for particles fully loaded with oligonucleotides, the low sensitivity of the absorbance-based methodology precludes one from using the methodology to study particles with low oligonucleotide surface coverages.

The average oligonucleotide surface coverage of alkanethiol-modified 12-mer oligonucleotide ($SN_{12}f$) on Au nanoparticles was 34 ± 1 pmol/cm^2 (average of 10 independent measurements of the sample.) For 15.7 ± 1.2 nm diameter particles, this corresponds to roughly 159 thiol-bound 12-mer strands per gold particle. Despite slight particle diameter variation from batch to batch, the area-normalized surface coverages were similar for different nanoparticle preparations.

To verify that our new method is useful for obtaining accurate oligonucleotide surface coverages, we used it to displace fluorophore-labeled oligonucleotides from gold thin films and compared our surface coverage data with experiments aimed at getting similar information but with different techniques. In these experiments, gold thin films were subjected to an oligonucleotide

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modification and ME displacement procedure similar to that used for the citrate-stabilized Au nanoparticles (see Experimental Section). The oligonucleotide displacement versus time curves for the Au thin films are very similar to those measured for Au nanoparticles. This suggests a similar rate of displacement for the thin films, even though the typical surface coverage values measured for these films were somewhat lower than the oligonucleotide coverages on Au nanoparticles. Importantly, the oligonucleotide surface coverages on gold thin films measured by our technique (18 ± 3 pmol/cm^2) fall near the reported coverages of oligonucleotides on thin films (10 pmol/cm^2 for a 25-base oligonucleotide on Au electrodes) determined using electrochemistry and surface plasmon resonance spectroscopy (SPRS).[3] Differences in surface coverages are expected due to different oligonucleotide sequences and lengths, as well as film preparation methods. Finally, we cannot completely rule out the possibility that the mercaptoethanol has not displaced all of the oligonucleotide from the gold surface. However, we think it is unlikely based upon the analogous displacement chemistry with the nanoparticles.

The extent of hybridization of complementary fluorophore-labeled oligonucleotides ($N'_{12}f$) to nanoparticles with surface-bound 12-mer oligonucleotides was measured by the method described in Scheme 2. Briefly, $SN_{12}f$-modified nanoparticles were exposed to $N'_{12}f$ at a concentration of 3 μM for 24 h under hybridization conditions (0.3 M PBS, pH 7) and then rinsed extensively with buffer solution. Again, it was necessary to remove the hybridized strands from the gold before measuring fluorescence. This was accomplished by denaturing the duplex DNA in a high-pH solution (NaOH, pH 11) followed by centrifugation. Hybridized $N'_{12}f$ amounted to 1.3 ± 0.2 pmol/cm^2 ($\sim$6 duplexes/15.7 nm particle[17]). To measure the extent of nonspecific adsorption, $SN_{12}f$-modified Au nanoparticles were exposed to fluorophore-labeled noncomplementary 12-base oligonucleotides ($N_{12}3'f$) in 0.3 M PBS. After extensive rinsing (successive centrifugation/redispersion steps) and subsequent high-pH treatment, the release of nonspecifically adsorbed oligonucleotides on the nanoparticles was determined to be on the order of 0.1 pmol/cm^2. An analogous procedure was used to measure hybridization to $SN_{12}f$-modified Au thin films in order to compare the hybridization results to reported values on Au thin-film electrodes. The degree of hybridization, 6 ± 2 pmol/cm^2, was consistent with hybridization reported for mixed base 25-mer on an Au electrode (2−6 pmol/cm^2).[3]

Surface coverages and hybridization values of the $SN_{12}f/N'_{12}f$ system for both nanoparticles and thin films are summarized in Table 2. The most striking result is the low hybridization efficiency for the nanoparticle system (4%) as compared with the thin films (33%). Previous studies have shown similarly low hybridization for sufficiently densely packed oligonucleotide monolayers.[7] This may reflect a low accessibility to incoming hybridizing strands on the highly loaded nanoparticles, due to a combination of steric crowding of the bases, especially those near the Au surface, and electrostatic repulsive interactions.

Effect of Oligonucleotide Spacer on Surface Coverage and Hybridization. Although the high coverage of the $SN_{12}f$ oligonucleotide is advantageous in terms of nanoparticle stabilization, the low hybridization efficiency prompted us to devise a means of decreasing steric congestion around the hybridizing sequence. We synthesized 32-mer oligonucleotides having a 20-dA spacer sequence inserted between the alkanethiol group and the original 12-base recognition sequence. This strategy was chosen based on the following assumptions: (1) bases near the nanoparticle surface are sterically inaccessible because of interactions between the bases and the Au surface, as well as interstrand steric crowding, and (2) on a 15.7 nm diameter roughly spherical particle, 12-mer sequences attached to the end of 20-mer spacer units roughly perpendicular to the surface[7] will lead to a film with a greater free volume as compared with a film formed from the same 12-mer directly bound to the surface.

Table 2. Single-Strand Surface Coverages and Corresponding Surface Coverages for Hybridized Strands on Au Thin Films and Au Nanoparticles[a]

oligonucleotide	surface coverage (pmol/cm^2)	hybridized coverage (pmol/cm^2)	% hybridized
	Au Nanoparticles		
$SN_{12}f$	34 ± 1	1.3 ± 0.2	$\sim$4
$SA_{20}N_{12}f$	15 ± 4	6.6 ± 0.2	$\sim$44
	Au Thin Films		
$SN_{12}f$	18 ± 3	6 ± 2	$\sim$33

[a] Thiol-modified oligonucleotides were deposited onto the gold from 3 μM aqueous solutions of DNA and aged in 0.1 M NaCl. All hybridization studies were performed in 0.3 M PBS, pH 7 using 3 μM $N'_{12}f$.

While the surface density of single-stranded $SA_{20}N_{12}f$ strands (15 ± 4 pmol/cm^2) was lower than that of $SN_{12}f$ (34 ± 1 pmol/cm^2), the particles modified with a 32-mer using the identical surface modification conditions showed comparable stability compared to those modified with the 12-mer. As anticipated, the hybridization efficiency of the $SA_{20}N_{12}f/N'_{12}f$ system (6.6 ± 0.2 pmol/cm^2, 44%) was increased to $\sim$10 times that of the original $SN_{12}f/N'_{12}f$ system, Table 2.

Effect of Electrolyte Concentration during Oligonucleotide Deposition. In working with the $SN_{12}f$ sequence, we found that a salt aging step was crucial in obtaining stable oligonucleotide-modified nanoparticles.[8,9] The Au nanoparticles modified with $SN_{12}f$ in pure water fused together irreversibly to form a black precipitate upon centrifugation, while those aged in salt resisted aggregation when centrifuged, even in high ionic strength solutions (up to 1 M NaCl). We propose that the increased stability is due to higher oligonucleotide surface coverages, which lead to greater steric and electrostatic protection. Using the $SA_{20}N_{12}f$-modified particles, we examined the effect of electrolyte conditions on oligonucleotide surface loading. As shown in Table 3, final surface coverages for Au nanoparticles that were exposed to oligonucleotides in water for 48 h are much lower (7.9 ± 0.2 pmol/cm^2) as compared with those that were "aged" in salt or prepared by increasing the salt concentration gradually over the course of the final 24 h of the deposition experiment (see Experimental Section)

It is important to note that citrate-stabilized Au nanoparticles as synthesized irreversibly agglomerate even in very low ionic strength media. Indeed, in many respects, they are naturally

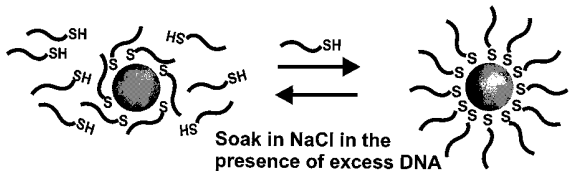
(17) The average number of duplexes per particle was computed by multiplying the normalized hybridized surface coverage (in pmol/cm^2) by the average particle surface area as found from size distributions measured by TEM.

Table 3. Effect of Salt Aging on Surface Coverage of $SA_{20}N_{12}f$ Oligonucleotides on Au Nanoparticles and Hybridization of the Nanoparticles to $N'_{12}f$[a]

buffer conditions during adsorption of alkanethiol DNA	surface coverage (pmol/cm^2)	hybridized coverage (pmol/cm^2)	% hybridized
H_2O	7.9 ± 0.2	*b*	
0.1 M NaCl, 10 mM phosphate	15 ± 4	6.6 ± 0.2	~44
1.0 M NaCl, 10 mM phosphate	20 ± 2	6.5 ± 0.2	~33

[a] All hybridization experiments were performed in 0.3 M PBS, pH 7, 3 μM $N'_{12}f$. [b] Reliable values for these experiments could not be obtained due to particle aggregation, which occurred as a result of centrifugation.

Scheme 3



incompatible with salts and especially polyanions such as oligonucleotides. This aging treatment that was initially reported by us,[9] and now understood through these quantitative measurements, is essential for preparing oligonucleotide particles stable in solutions of moderate to high ionic strength. Therefore, the particles must be initially modified with alkanethiol oligonucleotides in water prior to gradually increasing the ionic strength. It is likely that oligonucleotides initially lie flat, bound through interactions of the bases with Au. A similar mode of interaction has been proposed for oligonucleotides on thin films.[4] However, the interaction between oligonucleotides and the positively charged nanoparticle (after citrate displacement.)[18] is expected to be even stronger. In the aging step, the high ionic strength medium effectively screens charge repulsion between neighboring oligonucleotides, as well as attraction between the polyanionic oligonucleotide and the positively charged gold surface. The reduced interstrand repulsion allows more oligonucleotides to bind to the nanoparticle surface, thereby increasing oligonucleotide surface coverage, Scheme 3.

Effect of Oligonucleotide Spacer Sequence on Surface Coverage. To examine how the sequence of the spacer affects oligonucleotide coverage on Au nanoparticles, we prepared fluorescein-modified 32-mer strands, with 20-dA and 20-dT spacers inserted between a 3′ propanethiol and the fluorescein-labeled 12-mer sequence. The most notable result of surface coverage and hybridization studies of nanoparticles modified with $S3'T_{20}N_{12}f$ and $S3'A_{20}N_{12}f$ is the greater surface coverage achieved with the 20-dT spacer (35 ± 1 pmol/cm^2), in comparison to the 20-dA spacer (24 ± 1 pmol/cm^2). The percentage of surface-bound strands that hybridized to $N_{12}3'f$ was similar for $S3'T_{20}N_{12}f$ nanoparticles (~34%) and $S3'A_{20}N_{12}f$-modified nanoparticles (~38%), Table 4. These results suggest that dT-rich oligonucleotide strands interact with the nanoparticle surface to a lesser degree than dA-rich oligonucleotide strands. Consequently, 20-dT spacer segments may extend perpendicular from the Au surface, promoting higher surface coverages, while 20-dA spacer segments partially block Au sites by lying flat on the particle surface.

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Table 4. Effect of Oligonucleotide Spacer Sequence on Surface Coverage and Hybridization Efficiency[a]

oligonucleotide	surface coverage (pmol/cm^2)	hybridized coverage (pmol/cm^2)	% hybridized
$S3'A_{20}N_{12}f$	24 ± 1	9 ± 2	~38
$S3'T_{20}N_{12}f$	35 ± 1	12 ± 1	~34

[a] Hybridization experiments were performed in 0.3 M PBS, pH 7, 3 μm, $N_{12}3'f$.

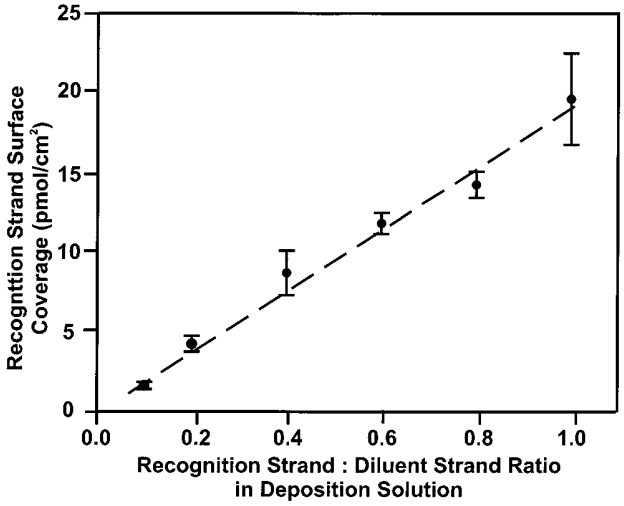

Figure 2. Surface coverages of $SA_{20}N_{12}f$ on gold nanoparticles modified using different molar ratios of $SA_{20}N_{12}f$ to diluent SA_{20} in the deposition solution. Error bars for each point were calculated from the standard deviation of multiple surface coverage measurements (see text) of the same nanoparticle.

Effect of Coadsorbed Diluent Molecules. In addition to efficient hybridization, another important property of oligonucleotide-modified nanoparticles is the possibility of adjusting the total number of hybridization events. This is most readily accomplished by adjusting the surface density of recognition strands. Other researchers have used coadsorbed diluent alkanethiols such as mercaptohexanol with modified oligonucleotides on Au electrodes to control hybridization.[3,4] However, the inherent low stability of unprotected Au nanoparticles poses serious constraints on the choice of diluent molecule. A thiol-modified 20-dA sequence (SA_{20}) proved to be suitable in terms of maintaining particle stability in the high ionic strength buffers which are needed for hybridization and protecting the surface from nonspecific adsorption.

Nanoparticles were modified using deposition solutions containing different $SA_{20}N_{12}f$ to SA_{20} diluent strand molar ratios. The

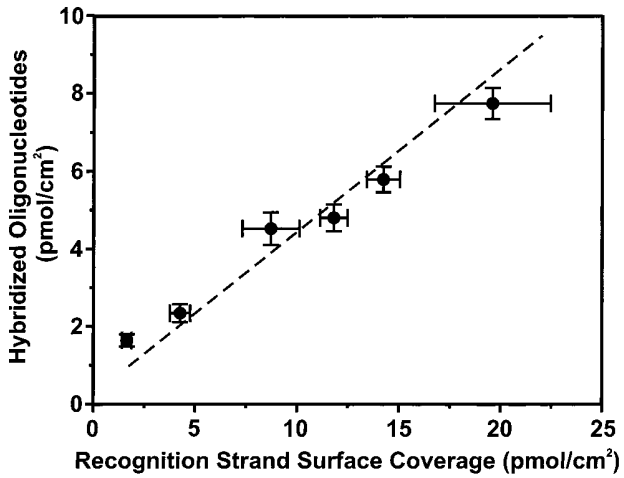

Figure 3. Surface coverages of hybridized $N'_{12}f$ strands on gold nanoparticles plotted as a function of increasing recognition strand ($SA_{20}N_{12}f$) surface coverages. Surface coverages of the fluorescein-modified oligonucleotides were determined using the fluorescence-based method described in the text. Error bars for each point were calculated from the standard deviation of multiple surface coverage and hybridization measurements of the same nanoparticle preparation.

resulting particles were analyzed by the fluorescence method described above to determine the $SA_{20}N_{12}f$ surface density and then tested for hybridization efficiency with $N'_{12}f$.

The $SA_{20}N_{12}f$ surface density increased linearly with respect to the proportion of $SA_{20}N_{12}f$ to SA_{20} in the deposition solution, Figure 2. This is an interesting result because it suggests that the ratio of $SA_{20}N_{12}f$ to SA_{20} reflects that of the deposition solution. This result is in contrast to what is normally seen for mixtures of short-chain alkyl or ω-functionalized thiols, where solubility and chain length play a crucial role in adsorption kinetics.[19,20]

The amount of complementary $N'_{12}f$ oligonucleotide that hybridized to each different sample also increased linearly with increasing $SA_{20}N_{12}f$ surface coverage, Figure 3. The fact that this relationship is well defined indicates that it is possible to predict and control the maximum extent of hybridization of the nanoparticle probes.

(19) Bain, C. D.; Evall, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 7155−7169.
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CONCLUSIONS

Although there are some similarities between the properties of oligonucleotide-modified Au thin films and nanoparticles, there are some striking differences with respect to the factors that lead to stable oligonucleotide-modified structures. This study has shown that with gold nanoparticles it is important to achieve a balance between oligonucleotide surface coverage, particle stability, and hybridization efficiency. Coverage must be high enough to stabilize particles, yet low enough so that a high percentage of the strands are accessible for hybridization with oligonucleotides in solution. This can be accomplished by the following: (1) adjusting salt conditions during oligonucleotide deposition to gain high oligonucleotide surface coverages, (2) introducing oligonucleotide spacer segments to reduce electrostatic and steric interactions between surface-bound oligonucleotides and incoming oligonucleotides involved in hybridization, and (3) coadsorbed diluent strands to control the number of hybridization events that can occur at each particle. We also have shown that the nature of tether sequence influences the number of oligonucleotide strands loaded onto gold nanoparticles. A detailed study of this sequence-dependent interaction of oligonucleotides with gold nanoparticle surfaces will be the subject of a forthcoming paper.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures for synthesis and purification of modified oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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