A Molecular Ruler Based on Plasmon Coupling of Single Gold and Silver Nanoparticles

Carsten Sönnichsen[1,x], Björn M. Reinhard[2,x], Jan Liphardt[2,**] , A. Paul Alivisatos[1,**]

[1]Department of Chemistry, University of California, Berkeley and Materials Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

[2]Department of Physics, University of California, Berkeley and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

** Correspondence should be addressed to J.L. (liphardt@physics.berkeley.edu) or A.P.A. (alivis@berkeley.edu)

[x] These authors contributed equally to this work

Molecular rulers based on Förster Resonance Energy Transfer (FRET) that report conformational changes and intramolecular distances of single biomolecules have helped to understand important biological processes[1-4]. However, these rulers suffer from low and fluctuating signal intensities from single dyes and limited observation time due to photobleaching. The plasmon resonance in noble metal particles has been suggested as an alternative probe to overcome the limitations of organic fluorophores[5, 6] and the coupling of plasmons in nearby particles has been exploited to detect particle aggregation by a distinct color change in bulk experiments[7-9]. Here we demonstrate that plasmon coupling can be used to monitor distances between single pairs of gold and silver nanoparticles. We use this effect to follow the directed assembly of gold and silver nanoparticle dimers in real time and to study the time dynamics of single DNA hybridization events. These "plasmon rulers" allowed us to continuously monitor separations of up to 70 nm for more than 3000 seconds. Single molecule *in vitro* studies of biological processes previously inaccessible with fluorescence based molecular rulers are enabled with plasmon rulers with extended time and distance range.

The optical properties of colloidal gold have been studied for over 100 years with major contributions by Zsigmondy[10] and Mie[11]. The discovery of surface enhanced Raman scattering (SERS) of molecules near metal structures has renewed the interest in plasmon resonances of metal particles[12]. Functionalized metal particles are currently being used to detect biomolecules with up to zeptomole sensitivity in bulk experiments[7, 8, 13]. Moreover, the binding of proteins to single functionalized particles can be followed by dark-field microscopy, by exploiting the dependence of the plasmon resonance wavelength on the refractive index of the particle's surroundings[14, 15]. The plasmon resonance wavelength of a metal nanoparticle is also affected by other nanoparticles that are in its immediate environment. When two nanoparticles are brought into proximity, their plasmons couple, which shifts the resonance wavelength depending on the particle separation. Since this effect is well known theoretically[16, 17] and experimentally observed for fixed distances[18, 19], we sought to explore its use as molecular ruler. We applied this 'plasmon ruler' to study the dynamics of DNA hybridization on a single molecule level. DNA was chosen as our model system for four reasons: I. DNA has been used successfully to assemble discrete nanostructures[20, 21], II. Plasmon coupling has been demonstrated

in bulk experiments to detect specific DNA targets[7, 13] III. The length of DNA can be readily chosen, controlled, and is well understood[22], IV. Kinetics of single DNA hybridization is relevant for biotechnology and nanoscience applications [23, 24].

We employed 40 nm diameter gold and silver nanoparticles. The particle diameter was chosen to ensure a sufficiently intense light scattering signal while minimizing any effects of the particles on proximal biomolecules. However, we subsequently found that a reduction in particle size to 20 nm for silver and 30 nm for gold is possible with the current technique. Particles were illuminated with unpolarized white light and light scattered by individual particles was collected by a darkfield microscope in transmission mode (**Fig. 1a**)[25]. Upon introduction of streptavidin-functionalized particles into the BSA-biotin coated glass chamber, we immediately observed numerous scattering sources adhering to the chamber surfaces. Nanoparticles were vividly colored: individual silver nanoparticles were blue (**Fig. 1b**), gold nanoparticles were green (**Fig. 1c**), aggregates were red-shifted compared to individual particles (typically by about 50 nm for gold, 150nm for silver), and dust and scratches were white.

Our first application of plasmon coupling was to monitor the directed assembly of functionalized particle pairs. We used the surface immobilized particles (**Figs. 1b** and **c**) as anchors for single stranded DNA (ssDNA) functionalized particles. The 33-nucleotide ssDNA molecules had a biotin at their 3' end, allowing them to bind to the streptavidin coated anchor particles (**Fig. 1a**). Shortly after introducing the ssDNA-functionalized particles into the chamber, some scattering centers suddenly changed color due to dimer formation. Silver particles turned from blue to green (**Fig. 1b**), gold particles turned from green to orange (**Fig. 1c**). The spectral shift upon dimer formation is considerably larger for the silver particles (102 nm) than for gold particles (23 nm, **Fig. 1d**). The fraction of surface immobilized particles that captured a ssDNA-functionalized particle ranged from 10% to 86 % depending on the time the samples had been stored, with fresher particles performing better. Aggregates of more than two particles were easily identified by their intensity and distinct color with multiple peaks in the spectrum (see for example the purple dot in **Fig 1b**). To avoid these aggregates of more than two particles in the following studies, we adjusted incubation time and particle concentration to get less than 10% coupling events for the surface immobilized particles. No color change was observed in a control experiment with particles lacking biotin, confirming the streptavidin-biotin directed assembly. The two-particle complexes were stable and could be continuously monitored for hours and we

routinely recorded their spectra for 3000 seconds at 0.5 Hz. The connection axis of the particle dimer is randomly oriented in space as judged from the polarization of the scattered light.

Having established that particle pairs linked by ssDNA could be assembled on surfaces, we set out to establish that the DNA linked nanoparticle pairs could be used to report the interparticle distance, *r*. As with many other rulers, macroscopic or microscopic, the observable for the plasmon ruler (in our case, the spectral shift) needs to be converted to a distance using either empirical calibration or by exploiting an established physical theory. In the absence of an experimental study, we used the spectral shift distance relationship, $\Delta\lambda(r)$ as obtained by Wei *et al.* [17] (see **Fig. 3a** therein) solving the electrodynamic Maxwell equation for 40 nm diameter gold nanoparticles in a dielectric medium. Caution has to be used in applying these theoretical values to our system because of unknown influences, e.g. of the glass surface and the undefined orientation of our dimers, but for the current discussion, the above mentioned relationship is sufficient. As a first qualitative test of the plasmon ruler, we changed the inter-particle distance by adjusting the ionic strength of the solution. The Debye screening length is larger in low salt concentrations, which should increase the electrostatic repulsion between the charged gold particles[26]. Consistent with this model, we observed a blueshift in the spectrum of individual nanoparticle pairs when we decreased the salt concentration of our buffer (normally 0.1 M NaCl) to 0.005 M NaCl (**Fig. 2a**). When we restored the ionic concentration to the original value, we observed a redshift. An effect due to a change in the refractive index was ruled out by observing nanoparticle pairs formed by precipitation (see method section). Such random particle pairs, not linked by DNA and unable to move relative to each other, showed no detectable spectral shift for salt concentrations between 0 and 0.1 M NaCl (data not shown). For DNA tethered particles, the observed shifts induced by buffer exchange were reversible, although not fully: the salt dependent shifts were superimposed on a background redshift of the spectrum, which was caused by flushing the chamber (**Fig. 2a**). The most likely explanation of this background redshift is a leaching of weakly bound ssDNA from the particle surface, which reduces their repulsive steric interactions. The gradual formation of additional DNA tethers was excluded in a control experiment by blocking all free streptavidin sites with excess free biotin after formation of the initial complex, which left the redshift unaffected. It is important to note that the gradual redshift was only observed when the chamber was flushed with buffer. As long as the DNA tethered particles were incubated in buffer without flushing, the plasmon wavelength remained constant (**Fig. 2b).** Subsequently we found that this redshift could be avoided altogether

by attaching the DNA to the particles via linker molecules (biotin – neutravidin or dioxigenin – anti-dioxigenin, see **Supplementary Fig. 1a**). These linker molecules serve as spacers to prevent particles to come into close range, where the attractive Van der Waals interaction starts to dominate.

If the nanoparticle pairs were in fact acting as molecular rulers, we reasoned that it should be possible to detect the hybridization of complementary DNA oligonucleotides to the ssDNA linkers. We chose dsDNA since it is well understood theoretically, and extensively characterized experimentally. Indeed, we found a significant blue shift upon addition of complementary DNA (**Fig. 3a**), indicative of hybridization. Since double stranded DNA (dsDNA) is significantly stiffer than ssDNA[22] the particles are pushed apart. (inset, **Fig. 3a**). To record the dynamics of the DNA hybridization, we monitored the spectrum of single gold particle pairs continuously in time. A representative time trace is shown in the bottom panel of **Figure 3b**. We extracted the spectral peak position (top panel, **Fig. 3b**) by fitting the smoothed spectral data. These time traces reveal initial discrete on/off events with a spectral amplitude of $\Delta\lambda = 0.9$ nm. The subsequent larger, irreversible annealing step leads to a total blueshift of $\Delta\lambda = 2.1$ nm. Most likely, we were observing consecutive attachment/detachment of ssDNA complements to the DNA strand on the nanoparticle, which was eventually followed by the complete zipping of the helix to yield a duplex. The sudden spectral jumps strongly indicate their origin from single molecules. To confirm the assignment of the spectral shifts as single DNA hybridization events, we converted the recorded spectral shifts to particle separations[17]. The spectral steps shown in **Figure 3b** correspond to a change in distance of $\Delta r = 0.8$ nm and $\Delta r = 2$ nm. The total change in length of 2 nm upon duplex formation agrees well with the change in DNA length of 1.9 nm upon hybridization predicted by the worm–like chain model[27] (32 nucleotide ssDNA: persistence length $p = 2.0$ nm, contour length $l = 20.8$ nm, 32 base pair dsDNA $p=53$nm, $l = 10.9$ nm.).

Hybridization reactions of surface immobilized DNA typically proceed in a series of consecutive steps. First, ssDNA strands from solution diffuse into the vicinity of the immobilized strands, where the complementary strands start to interact due to attractive van der Waals interaction betweens the bases of the nucleic acids and form hydrogen bonds. It is well known that a DNA duplex forms in a rapid zipping motion after a certain number of base pairs have spontaneously formed a stable nucleus, see for example Hagan and Chakraborty[24]. Our data indicate that for nanoparticle bound oligomers several attachments/detachments events are

required before a stable nucleus is formed and zipping can occur. This is consistent with previous observations of relatively inefficient hybridization of nanoparticle bound oligomers[28].

To verify that hybridization is indeed the reason for the observed blueshift, we performed control experiments recording the spectral positions before and after addition of complementary and non-complementary DNA. In order to record the spectra of several particles in the same chamber, we had to take the initial spectrum before flushing with DNA (This is different to the time traces discussed above, which were started after flushing the chamber with DNA). A clear difference between addition of complementary and non-complementary DNA was visible (**Fig. 3c**). On average, addition of complementary DNA led to a blueshift of $\Delta\lambda = 3.3$ nm, consistent with a hybridization-induced increase of interparticle spacing. The control shows an average red shift of $\Delta\lambda = 7.1$ nm, caused by the flushing and stripping of ssDNA from the Au as seen before in the salt exchange experiment. The average blue shift observed for DNA hybridization (3.3 nm) is larger than the shift observed in time traces on single particle dimers (~2 nm). In the latter, we used DNA conjugated nanoparticles with a low concentration of biotinylated DNA to increase the probability of a single tether whereas the particles in the control experiment carried a higher number of biotinylated DNA molecules to increase the binding efficiency. We also recorded some DNA hybridization dynamics using dimers of 40nm silver particles (**Fig. 3d**). As seen before (**Fig. 1d**), the plasmon coupling between silver particles leads to a more pronounced spectral shift. Our explanation that DNA hybridization is responsible for the observed blushift via an increase in steric repulsion between the particles is further supported by the observation that addition of DNA binding dendrimers leads to a drastic redshift consistent with DNA wrapping around the dendrimer (see **Supplementary Fig. 1b**).

The plasmon rulers we used to follow the assembly of discrete nanostructures and the detection and kinetics of single DNA hybridization events have several key advantages over FRET based rulers, which should allow a wide range of new *in vitro* single molecule experiments. In FRET the fluorescence intensities of a donor-acceptor dye pair are monitored simultaneously. Only a distance change between the donor and acceptor results in an anticorrelated intensity change, whereas most other environmental effects lead to uncorrelated intensity changes. Unfortunately, observation of single molecule fluorescence is hindered by blinking and rapid photo-bleaching, limiting the continuous observation time to a few tens of seconds. Furthermore, it is sometimes difficult to distinguish changes in relative dye orientation from changes in distance[1].

The plasmon ruler neither blinks nor bleaches and does not depend on the relative probe orientation. Moreover, metal nanoparticles are good labels for electron and x-ray microscopy, which permits the development of novel multimodal imaging techniques. In general, gold and silver particles are more stable under physiological conditions than organic dyes. The plasmon resonance, however, does also depend on the refractive index of the surrounding, and it can be difficult to distinguish resulting redshifts from distance changes, so refractive index needs to be carefully controlled. As in the case of FRET, deducing *absolute* distances is complex. Experimental data on the distance dependence of plasmon coupling is limited and the plasmon wavelength varies between particles of the same batch due to a variation in shape and size. It is, however, conceivable that these limitations will be overcome. For instance, the lifetime of gold particles is nearly unlimited, which permits its characterization before or after use in a plasmon ruler. The range of distances accessible with plasmon coupling in a pair of nanoparticles is dependent on the size and the coating of the particles. In general, the accessible distance range is larger than with FRET (2 to 8 nm)[1]. With 40 nm particles and a 0.1 nm spectral resolution for determining the plasmon resonance position, particle separations of up to 70 nm should be accessible with better than 1 nm resolution[17]. Larger particle separations, up to 2.5x the particle diameter, still lead to some small shift in the plasmon resonance[18]. Since light scattering efficiency scales with the square of their volume, it is difficult to employ particles with diameters much smaller than 20–30 nm. They are therefore larger than dyes used in conventional FRET based molecular rulers. This is a disadvantage, especially for *in vivo* applications. However, most single molecule studies have been performed *in vitro*, where the large particle size is not a fundamental limitation. Even micron sized magnetic and polystyrene beads have been successfully used in single molecule applications[23, 29]. Another potential problem of plasmon based rulers is the potential denaturing of proteins near metal surfaces. The routine use of antibody coated gold nanoparticles as specific staining reagents in electron microscopy make us confident that this problem can be avoided in most cases by using an appropriate spacer molecule. Therefore plasmon rulers have the potential to become an alternative to FRET for *in vitro* single molecule experiments, especially for applications demanding long observation times or large distances. In addition, analytical bulk assays based on particle aggregation can now be extended to the single molecule level, enhancing their sensitivity and allowing parallel processing. For example, extremely small numbers of DNA molecules such as the unamplified

genomic DNA of a single cell should be detectable by incorporating plasmon rulers into a microfluidic system.

METHODS

General Methods: Aqueous solutions of 40 nm gold and silver nanoparticles (British Biocell International, BBI, size deviation <10%) with a concentration of 9.0 x 10^{10} particles / mL ≈ 1.5 x 10^{-10} M were purchased from Ted Pella. Deionized water (> 18.0 MΩ) from a Barnstead D4641 water purification system was used for all experiments. All buffers were filtered with a 0.45 μm pore-size Millex-HA microfilter (Millipore) prior to use. Immediately after conjugation, the particles were immersed in storage buffer and stored at 4 degree Celsius for up to one month.

Preparation of nanoparticles conjugates of streptavidin: Streptavidin (Molecular Probes, S-888) was dissolved in T50 buffer (10 mM Tris, pH 8.0 and 50 mM NaCl) at a concentration of 1 mg/mL. 250 μL of this streptavidin solution was added to10 mL of aqueous nanoparticle solution. After mixing thoroughly, 100 μL of 1.0 M $NaHCO_3$ was added to the reaction mix, which was then allowed to react for 10 minutes. Subsequently 50 μL of a 2 % Polyethylene Glycol 6000 (Merck, 807491) solution was added and the reaction mixture was centrifuged at 800 G for 35 minutes. The pellets were redissolved in Superblock (Pierce, 37515) and washed with Superblock using Microcon YM-100 centrifugal filter devices.

Preparation of DNA-functionalized nanoparticles: All DNA oligomers were purchased from Fidelity Systems Inc. We used 3' biotinylated ssDNA oligomers with a 5'thiol modification ($OPO_3(CH_2)_6$-S-S-$(CH_2)_6OH$) and controls which were not biotinylated. The following sequences (5' to 3') were used in our experiments:

A: (SS C6 linker)-ACATTAAAATTCCACACACGCTAACATACACA(Biotin)T

B: (SS C6 linker)-ACATTAAAATTCCACACACGCTAACATACACAT

Our strategy to functionalize nanoparticles with thiol modified oligonucleotides followed the procedure described by Kanaras *et al.* [30]. The gold nanoparticles were derivatized with modified ssDNA oligomers by incubating 600 μL of gold nanoparticle sol overnight with 10 μL of a 100 μM solution of the disulfide protected, biotinylated oligonucleotide in 10 mM Tris (pH 7.0). Then 5 μL of 5M NaCl and 50 μL of 50 mM sodium phosphate buffer (pH 7.0) were added to the reaction mix. After 12 h another 5 μL of 5M NaCl and 50 μL of 50 mM sodium phosphate buffer (pH 7.0) was added to a final concentration of 0.07 M NaCl and 7 mM sodium phosphate

buffer. The reaction mix was then allowed to react for 48 hours after which the volume was slowly reduced in vacuum to 250 μL within 48 hours. Unbound oligonucleotides were subsequently removed by centrifugation and resuspension of the pellet (7000 G, 2x). In case of silver nanoparticles 600 μL sol was incubated overnight with 10 μL of 100 μM ssDNA solution. Then the NaCl and sodium phosphate buffer concentration was slowly adjusted to 0.07 M NaCl and 7 mM sodium phosphate buffer by adding 2.5 μL 5M NaCl and 25 μL 50 mM sodium phosphate buffer (pH 7.0) every 24 hours for a total of 96 hours. After further incubation of 24 hours the volume was slowly reduced to 250 μL within 48 hours. Unbound DNA was removed by centrifugation (7000 G, 1x) and resuspension of the pellet. DNA coated gold and silver nanoparticles were stored in a storage buffer (100 μL) containing NaCl (0.1 M) and sodium phosphate buffer (10 mM, pH 7.0). The zeta potential of the functionalized particles was determined to be –40 meV.

Surface immobilization of ssDNA linked nanoparticle dimers: All attachment chemistry was conducted within a hollow rectangular glass capillary (Vitrocom) that served as flowchamber. The chamber had the dimensions (pathlength x width x length) 0.1 mm x 1.00 mm x 100 mm and a wall thickness of 0.1 mm. Biotin-BSA (Sigma, A8549) was dissolved in T50 buffer to a total concentration of 1 mg/mL, and 100 μL was flowed into the chamber and allowed to incubate for 15 minutes after which the chamber was washed with 200 μL of deionized water. Then 50 μL of a 1:500 dilution of nanoparticle conjugate of streptavidin in storage buffer was added to the chamber and incubated for two minutes. Next, the chamber was washed with 300 μL storage buffer. Eventually 50 μL of a 1:10 dilution of DNA derivatized nanoparticles in storage buffer was flowed into the chamber and incubated for 20 minutes. Then the chamber was washed again with storage buffer.

Solid Phase Hybridization Experiments: 25 μL of a 100 μM solution of target ssDNA – oligonucleotides complementary to the probe ssDNA linking the nanoparticles – in 10 mM Tris (pH 7.0) was mixed with 25 μL of hybridization buffer, which contained 20 % formamide (Aldrich, F7508), 16 % dextran sulfate (Aldrich, D6924) and 1 mM $MgCl_2$ (Ambion, #9530G) and incubated for up to an hour. Afterwards the chamber was washed with storage buffer. In control experiments the target ssDNA was replaced by non-complementary ssDNA. The following sequences (5' to 3') were used in the hybridization and control experiments:

C: TGTGTATGTTAGCGTGTGTGGAATTTTAATGT (target ssDNA, complementary to sequence A)

D: AGGTCGCCGCCCGCACAGTAGCGATTCAACGC (control)

Experimental Setup: The darkfield optical setup consists of a 100W tungsten lamp, an oil immersion dark-field condenser and a 40x objective. The scattered light was analyzed by a liquid nitrogen cooled CCD camera attached to an imaging spectrometer[25].

Calculation of refractive index effect: On random dimer pairs formed by precipitation of gold and silver particles on the glass surface, we measured the plasmon peak in water (n = 1.333), dimethylsulfoxide (DMSO, n = 1.4782), and mixtures of water and DMSO. The plasmon shift $\Delta\lambda$ increased linearly with refractive index change Δn. For $\Delta n = 0.1$ we obtained spectral shift $\Delta\lambda = 12.5$ nm for silver and $\Delta\lambda = 6.2$ nm for gold. The refractive index of salt solutions was proportional to the molar salt concentration with $\Delta n = 0.0091 * [NaCl]$. The range of buffers used in our experiments (0.005 M – 0.1 M) change therefore the refractive index less than 0.001. The resulting spectral shift for gold dimers would amount to less than $\Delta\lambda = 0.06$ nm. To account for the observed shift upon buffer exchange (**Fig. 2**) of 12 nm, the refractive index would have to change by $\Delta n = 0.2$, i.e. from 1.33 to 1.53, well above that of most organic substances. This proves that the change in interparticle distance due to repulsion between the particles in low ionic strength environments indeed causes the observed shift.

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COMPETING INTEREST STATEMENT

Authors declare they have no competing financial interests.

Figure Captions

Figure 1 Color effect on directed assembly of DNA functionalized gold and silver nanoparticles**. (a)** First, nanoparticles functionalized with streptavidin are attached to the glass surface coated with BSA-biotin (left). Then, a second particle is attached to the first particle (center), again via biotin-streptavidin binding (right). The biotin on the second particle is covalently linked to the 3' end of a 33 base pair long single stranded DNA strand bound to the particle via a thiol group at the 5' end. Inset: principle of transmission dark-field microscopy. **(b)** Single silver particles appear blue (left) and particle pairs blue-green (right). The orange dot in the bottom comes from an aggregate of more than two particles. **(c)** Single gold particles appear green (left), gold particle pairs orange (right). Inset: representative TEM image of a particle pair to show that each colored dot comes from light scatted from two closely lying particles, which cannot be separated optically. **(d)** Representative scattering spectra of single particles and particle pairs for silver (top) and gold (bottom). Silver particles show a larger spectral shift (102 nm) than gold particles (23nm), stronger light scattering, and a smaller plasmon line width. Gold, however, is chemically more stable and is more easily conjugated to biomolecules via –SH, –NH2 or -CN functional groups.

Figure 2 Effect of buffer exchange. (**a**) Peak wavelength of the plasmon resonance of a particle dimer when flushed with different salt concentrations (0.1 M NaCl and 0.005 M NaCl. The plasmon resonance shifts red with higher salt concentration, indicative of a decreased interparticle distance. This is due to the reduced electrostatic repulsion of the particles in high ionic strength environment. The Debye screening length is reduced from 4 nm to 1 nm. A gradual overall redshift is also observed. **(b)** Without flushing, the peak wavelength of the plasmon resonance remains stable within 0.4 nm over 700 s.

Figure 3 Spectral shift upon DNA hybridization. **(a)** Example of a spectral shift between a gold particle pair connected with single stranded DNA (ssDNA) (red) and double stranded DNA (dsDNA) (blue). The shift is clearly visible. **(b)** Spectral position as a function of time after addition of complementary DNA: The scattered intensity (Isca) is shown color-coded on the bottom; the peak position obtained by fitting each spectrum is traced on the top. Discrete states are observed, indicated by horizontal dashed lines. **(c)** Cumulative probability distribution of the spectral shift for approx. 80 particles induced by adding complementary DNA (blue) and non-complementary DNA (red). Inset: histogram of the observed spectral shift. A Student's T-test on this data proves the difference between experiment and control to be statistically significant (probability of this result obtainted randomly is <0.0001). **(d)** Time dynamics for a silver particle dimer after addition of complementary DNA. The random fluctuations in the timetraces due to solution effects and Brownian motion could be used to gain insight into the interparticle potential.

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