Formation and Stabilization of Fluorescent Gold Nanoclusters Using Small Molecules†

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Formation and Stabilization of Fluorescent Gold Nanoclusters Using Small Molecules†

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Biological molecular imaging and sensing requires fluorophores that are not only stable and bright, but also small enough to allow the unencumbered observation of the movement of proteins. Toward this goal, we have studied the formation of fluorescent metallic gold nanoclusters stabilized by small molecule ligands. The morpholine and piperazine backbones of Good's buffers were used to template fluorescent clusters, through a process of etching of nanoparticles first formed in the reaction. The clusters are found to be subnanometer sized, with nanosecond fluorescence lifetimes and as bright, or brighter, than the commercial dye norharmane.

Development in single molecule fluorescence imaging and tracking instrumentation is tantalizingly close to allowing observation of the three-dimensional dynamics of single proteins in live cells.[1−7] Soon it will be technically feasible to image a single protein molecule within a living cell throughout the entire protein life cycle, from expression, to transport, to function, and, ultimately, to degradation. Following individual biomolecular synthesis and function is still difficult, however, due to problems with fluorescent probes used to label the biomolecules of interest. Effective labels must be photostable, bright, and small. While semiconductor quantum dots show great promise in biolabeling due to their superior photophysical properties including photostability, high quantum yield, and large Stokes shifts,[8] their large size (equal to or greater than that of most proteins) limits their use as biological labels for most dynamical studies.

Recently, fluorescent nanoclusters of gold and silver have received considerable attention as potential fluorescent labels for biological molecules.[9−11] Metallic nanoclusters (<2 nm) of several to tens of atoms have been studied for many years for their interesting catalytic and near-IR optical properties and more recently their surprising structural ligation.[12−17] Traditionally, nanoclusters have been synthesized using soft ligands, such as phosphines or thiols that form strong bonds with gold, and, with the exception of a mixed-ligand protected cluster, tend to exhibit low fluorescence quantum yields.[18] However, we and others have shown that hard ligands such as amines and carboxylates can template fluorescent clusters of gold and silver, with relatively large fluorescence quantum yields (10−70%).[19] While bright and stable, these clusters have, for the most part, been synthesized in large templates such as dendrimers and polymers and produce composites with sizes on the order of most proteins. To facilitate fluorescence imaging and sensing, nanoclusters should either be made directly within biological molecules (DNA and proteins)[11,20] or with small molecular ligands for direct conjugation to the biological molecules of interest. Toward the goal of making small and biologically attachable fluorescent probes, we have investigated the production of fluorescent gold nanoclusters using Good's buffers. Good's buffers are common pH buffers with morpholine or piperazine backbones and have previously been shown to be good Au(I) ligands[21] with reduction potentials sufficient to produce gold nanoparticles.[22] Here our goal was not only to complex gold but to stabilize the formation of fluorescent gold clusters with small molecules. Further, with a wide range of commercially available chemical moieties, this family of small molecules comprises ideal ligand targets to investigate the mechanisms of cluster formation, and their small size also enables future detailed theoretical calculations. Herein, we report a straightforward approach for the synthesis of fluorescent Au nanoclusters using small molecule templates and describe the photophysical characterization of the resultant nanoclusters.

In a typical experiment, Au nanoclusters were synthesized by mixing a gold(III) chloride aqueous solution (0.2 wt % (5.8 mM), 1 mL) with a Good's buffer (100 mM pH 7, 5 mL) at room temperature, with subsequent incubated shaking for three days (37 °C, 250 rpm). This procedure typically generated clear solutions with gold nanoparticle (NP) precipitates on the bottom of the tubes. These precipitates are nonfluorescent irregularly shaped NPs with broad absorption of 500−700 nm (Figure S5; here particles are defined as collections of hundreds of atoms supporting a surface plasmon). In contrast to the precipitates, over a matter of days the supernatant of this reaction becomes fluorescent (Figure 1). Typical fluorescence emission spectra of clear solutions after Au NP removal are shown in Figure 1. Gold nanoclusters stabilized with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) exhibit maximum excitation/emission at 395 nm/500 nm, whereas 2-(*N*-morpholino)ethanesulfonic acid (MES)-stabilized clusters yield maximum excitation/emission of 420 nm/485 nm. The fluorescent species are stable

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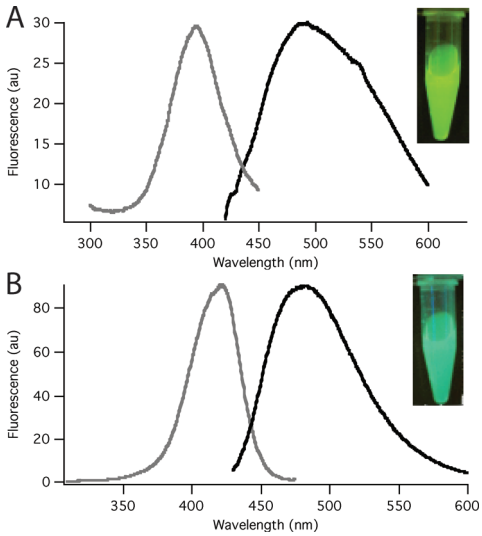


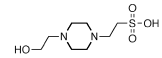
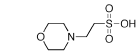
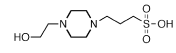
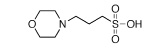
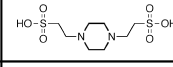
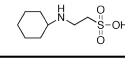
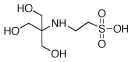
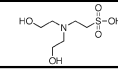
Figure 1. Fluorescence excitation (gray) and emission (black) spectra of small molecule stabilized Au nanoclusters: (a) HEPES and (b) MES. Insert photographs were taken by illuminating the solution with a mercury arc lamp through a bandpass filter centered at 405 ± 20 nm.

for at least six months at room temperature. No fluorescence was observed for the Good's buffers without addition of gold. The oxidation of MES by gold is known to be rapid and concurrent with nanoparticle formation, yet no fluorescence was observed when Good's buffer-stabilized nanoparticles are formed.[22,23] To further rule out the possibility that gold is oxidizing the Good's buffers to yield a fluorescent product, we ran reactions where $HAuCl_4$ was replaced with H_2O_2. No fluorescence from these reactions was observed even though hydrogen peroxide is known to oxidize MES (Figure S1) through the tertiary amines on the ring structure and could have potentially led to the production of luminescent products of amine oxidation.[23]

We tested a variety of Good's buffers and other similar small molecules for their ability to template gold nanoparticles and clusters (Table 1). Some small molecules produced single fluorescent species (i.e., MES and MOPS; Figure S7) and others produced mixtures of fluorescent species (i.e., HEPES, EPPS, and PIPES; Figure S8). Single species exhibit a single emission peak when excited at different wavelengths, whereas multiple species exhibit different emission peaks when excited at different wavelengths. The chart indicates the maximum excitation and emission wavelengths for the single and multiple emissive syntheses. We found that only those small molecules able to reduce gold to form nanoparticles also produced nanoclusters. However, not all particle forming small molecules could stabilize cluster formation. As we have reported here, in the process of metal cluster synthesis, gold nanoparticles are formed first, and only after many days, as these nanoparticles partially dissolve in the reaction medium, are the fluorescent clusters formed. We also find that when citrate-stabilized gold nanoparticles are exposed to MES, at the concentration used in our reactions, fluorescent species are formed identical to the original cluster synthesis (Figure S6). The appearance of fluorescence emission and the disappearance of Au nanoparticles with time suggest that the MES clusters are produced from an etching process of MES-protected nanoparticles, as has been reported previously with alternative stabilizing ligands.[24]

Ultimately, our objective is to compare experimentally observed and theoretically predicted photophysical transitions

TABLE 1: Small Molecule Production of Nanoparticles and Nanoclusters (Indicated with Maximum Excitation/Emission)

Name	Structure	pKa	Au nanoclusters excitation/emission	particles
HEPES		7.5	yes (395/500 nm)	yes
MES		6.1	yes (420/485nm)	yes
EPPS		8.0	yes (385/490nm)	yes
MOPS		7.14	yes (420/480nm)	yes
PIPES		6.8	yes (365/465nm)	yes
CHES		9.3	no	yes
HP			no	no
TES		7.5	no	no
BES		7.1	no	yes

for each complex, so that we may tailor the chemical synthesis to produce highly stable fluorescent nanoclusters with predicted photophysical properties. Toward this goal and, in particular, toward determining the molecular sizes of the gold nanoclusters, we have characterized the nanoclusters by bulk fluorescence measurements, fluorescence correlation spectroscopy (FCS), and time correlated single photon counting (TCSPC), the details of which are discussed in the Supporting Information. FCS was used to measure the emission rate per cluster, hydrodynamic radius, and to estimate the concentration of the fluorescent clusters synthesized. In the FCS experiments, the average count rate per fluorescent cluster was obtained by dividing the total count rate on both single-photon counting detectors by the average occupancy in the detection volume (N, derived from autocorrelation analysis) at various excitation intensities. This calculation yielded maximum detection rates of 5700 photoelectrons detected per second, per cluster, for MES-stabilized clusters and 1700 photoelectrons detected per second, per cluster, for HEPES-stabilized clusters. These photon detection rates for MES (5.7 kHz) and HEPES (1.7 kHz) are comparable to the maximum photon detection rate of norharmane (~2 kHz), a UV dye with similar excitation spectra and a large quantum yield (Q.Y. = 58%).[25] We note that conventional methods to determine the bulk quantum yields of the MES- and HEPES-stabilized clusters returned unreasonably low values (<3%) for the quantum yields, most likely due to a large population of species in the reaction mixture that absorb light at the excitation wavelengths, but are nonemissive.

The fluorescence emission rates were maximal at slightly different excitation intensities for these two clusters (Figure 2). Further increases in the excitation intensity resulted in an apparent reduction of fluorescence emission per cluster (data not shown), due to optical saturation of fluorescence and increased background emission at elevated excitation intensity.[26] From the average occupancy of fluorescent clusters in the probe volume, determined by FCS, we estimated a fluorescent cluster concentration of 1.6 μM in the MES reaction solution and 0.7 μM in the HEPES reaction solution. The fluorescence lifetime was ~ 4.34 ns for the MES clusters and was fit relatively well with a single exponential, suggesting a single fluorescent species

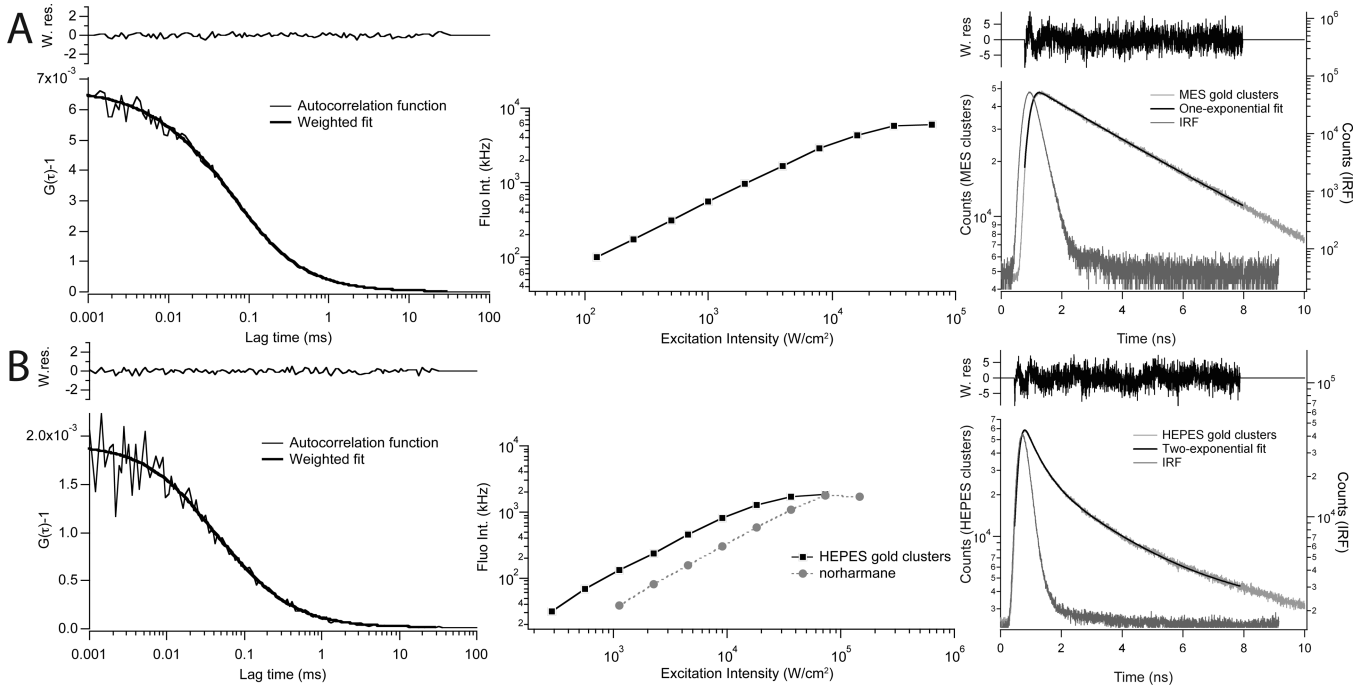


Figure 2. Representative FCS spectra, brightness per MES (A) and HEPES (B) clusters, and fluorescence lifetime decay, and instrument response function (IRF), with reduced fit residuals.

(Figure 2A), consistent with our observation of a single fluorescence peak in the MES reaction. In contrast, for the HEPES clusters, two-exponentials were needed to fit the observed fluorescence decay (2.46 and 0.49 ns, accounting for 74.2% and 25.8% of the integrated fluorescence emission; Figure 2B), consistent with our observation of multiple fluorescence peaks in the HEPES reaction.

With appropriate characterization of the optical probe volume, FCS can be used to estimate the size of the fluorescent species. The half radii of the FCS detection volumes (ω_0) were estimated to be 0.37 μm at 390 nm and 0.40 μm for 425 nm excitation, as determined by measurements on commercial quantum dots we have previously characterized.[4] For MES and HEPES templated clusters the diffusion times, τ_d, from the autocorrelation analysis were 68 and 73 μs (Figure 2) and corresponded to diffusion coefficients of 582 and 477 $\mu m^2/s$, respectively. These diffusion coefficients are substantially slower than the diffusion coefficient for benzene in water (1020 $\mu m^2/s$)[21] or ethyl benzene in water (810 $\mu m^2/s$).[27] From the measured diffusion coefficients above we infer that the fluorescent species is substantially larger than a complex between a single gold atom and a single MES or HEPES molecule.

Further analysis can be performed using the Stokes−Einstein relation, where the measured diffusion coefficients for the MES and HEPES-templated clusters were found equal to the calculated diffusion coefficients of solid spheres of hydrodynamic diameters of 7.4 ± 0.7 (for MES-templated clusters) and 9.0 ± 4.4 Å (for HEPES-templated clusters). Considering the MES-templated cluster in particular, a sphere of diameter 7.4 Å has a volume of ∼210 $Å^3$. Dividing this volume by the volume of a single gold atom (17 $Å^3$, obtained from a density of gold of 19.3 g/cm^3) suggests that up to 12 gold atoms could fit into a sphere of 7.4 Å in diameter, providing an approximate upper limit to the number of Au atoms present. Another simple analysis that takes ligands into account as well is to consider a trimer of gold atoms, with each Au ligated to a MES molecule. Using 2.75 Å as the gold−gold bond distance and 2.28 Å as the Au−N bond distance, one can calculate a radius of gyration for such a fully ligated MES−gold triad of 4.4 Å, corresponding to a diameter of gyration of 8.8 Å (note that a diameter of gyration may differ slightly from a hydrodynamic diameter). Interestingly, increasing the number of core gold atoms from three to eight, each with an associated MES, only increases the calculated radius of gyration of the overall cluster by a factor of 1.2, if one assumes an hcp-like geometry for the gold cluster. While these calculations yield results slightly larger than our estimated hydrodynamic diameter, both experimentally and theoretically our results are more consistent with a structure consisting of a core of a few atoms of gold (here cluster is defined as a group of two or more metal atoms with close metal−metal interactions) surrounded by a ligand shell than they are with a molecular gold−ligand complex. In addition to cluster size, the observed ns fluorescence lifetimes for these species are also inconsistent with the lifetimes (μs) of gold-ligand molecular complexes.[28]

In summary, we have reported a new method for preparing fluorescent and water-soluble Au nanoclusters using small molecules as reducing agents and stabilizing ligands. The reaction proceeds by first forming irregularly shaped nanoparticles, which are subsequently etched by excess ligands to produce fluorescent nanoclusters. These reaction mixtures yield fluorescent species with sizes consistent with gold cores of a few atoms and have large effective quantum yields with nanosecond fluorescence lifetimes. Although the detailed mechanisms of Au-ligand nanocluster coordination warrant further studies, the findings of the current work hold promise for designing capping ligands for the synthesis of fluorescent Au nanoclusters that can be directly linked to biological molecules. This study also provides a starting point toward understanding the formation and stabilization of small size nanoclusters and may represent an inexpensive method to produce fluorescent species from bulk colloidal gold.

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Supporting Information Available: Experimental details of the synthetic procedures and photophysical analysis for the products and the controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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