Luminescent images of single gold nanoparticles and their labeling on silica beads

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Abstract

Luminescent Au nanoparticles were synthesized in a modified Brust method (average diameters of metal core = 1.6 nm). The fluorescence images were measured using scanning confocal microscopy and validated as compared with organic fluorophores. The metal particles were functionalized with succinimidyl ester terminated ligands and bound as fluorophores on surface-aminated silica beads to mimic labeling of biological functionalities. The labeled silica beads were shown to display bright signals and good photostability. Our results indicate that the luminescent metal nanoparticles can be employed as the probes to label the biological functionalities in developing molecule imaging agents.

Introduction

Fluorescence microscopy is a useful tool in early detection, diagnosis, and treatment of disease, as well as aid in studies of biological and biochemical mechanisms, immunology, and neuroscience on single cells. These measurements provide for specific visualization of one or two particular targets in cells while all other biomolecules remain invisible [1]. In the research, molecular fluorescence imaging agent plays a critical role [2,3]. Generally, an imaging agent consists of an organic fluorophore moiety and a targeting functionality, such as an antibody, peptide, DNA, or a special ligand [4,5]. However, the most molecular imaging agents by organic fluorophores have poor photostability so the applications of them are limited in some cases. In the past decade, the synthesis of nano-scale particles has been developed rapidly [6-8]. Some self-fluorescent semiconductor particles, known as quantum dots (QDots), are synthesized in nano-scale [9-12]. Compared with most organic fluorophores, QDots display a wide region of excitation wavelength and characteristic narrow emission band as well as high quantum yield. Moreover, because of their inorganic semiconductor structures, QDots exhibit excellent photostability. Because QDots are coated by organic layers on semiconductor cores, they can be readily decorated by versatile functionalized groups via surfaces reactions. However, QDots have their deficiencies as compared with organic fluorophores: large size. This feature of QDots can change the properties of their labeled functionalities or block the labeled functionalities being in proximate of the cell surface due to steric hindrances. It is thus of high interest to develop novel fluorophores that simultaneously have the advantages of small size as organic and good photostability as QDots for the new molecular imaging agents.

It has been reported that small metal nanoparticles can emit fluorescence when the metal cores are small and generate the energy band gap on the surfaces [13]. Because this emission mechanism is close to that of QDot, the metal particles are also called as the metal QDots.
The emissive metal particles can be excited at a wide range of wavelengths and the emission bands are narrow, although the quantum yields are not high in some cases. Their emission wavelengths are shown to depend on the metal specie, core size, and ligand specie, etc. Therefore, they can be employed as fluorescent probes in biological imaging applications. Herein, the organic monolayer-protected gold particles, known to emit at near IR region, were synthesized with small metal cores. Fluorescence images of these Au particles were monitored with scanning confocal microscopy. Their luminescence images were validated as comparing with organic fluorophores. We also bound these molecule-like metal nanoparticles on the artificial silica beads to mimic the labeling of biological functionalities (Scheme 1). The luminescence images of the labeled beads, including the brightness and photostability, were compared with organic fluorophores and semiconductor QDots.

**Experimental method**

All reagents and spectroscopic grade solvents were used as received from Fisher or Aldrich. RC dialysis membrane (MWCO 50,000) was obtained from Spectrum Laboratories, Inc. Nanopure water (>18.0 Ω cm) purified using Millipore Milli-Q gradient system, was used in all experiments. (2-mercaptopropionylamino) acetic acid 2, 5-dioxo-pyrrolidin-1-ylester was synthesized as previously reported.

Gold particles were prepared using a modified Brust reaction in methanol. In a typical reaction, HAuCl₄ and N-(2-mercaptopropionyl)glycine (abbreviated as tiopronin) were co-dissolved at mole ratio of Au salt / tiopronin = 1/6 in methanol. Excess amount of NaBH₄ in methanol was added with rapid stirring at 0 °C. The black suspension was stirred for an additional 1 h, then isolated on a Millipore porous filter, washed with excess amount of methanol and acetone, and dried in air. The residuals were dissolved in water and purified by dialysis (MWCO 8,000) against water. The tiopronin-coated gold particles were succinimidylated via ligand exchange. (2-mercapto-propionylamino) acetic acid 2, 5-dioxo-pyrrolidin-1-ylester (1 × 10⁻⁷ M) and tiopronin-coated gold particle (1 mg/mL, 1 × 10⁻⁷ M) were co-dissolved in a mixing solvent of water/methanol (v/v = 1/1) and stirred for 24 h. Solvent was removed under vacuum. The residue was washed by methanol and acetone.

Monodispersed silica beads were prepared via Stöber method. 1.0 mL of 30% ammonia alcohol solution was added dropwise under vigorous stirring to 1.4 × 10⁻² M of tetraethyl orthosilicate in 50 mL ethanol. The solution became turbid after stirring overnight due to the formation of silica beads. The mixture was centrifuged to remove the suspension, and the residual was washed thoroughly with ethanol. The beads were re-dispersed in 50 mL ethanol. The surfaces of beads were aminated adding 10 μL of 3-aminopropyltrimethoxy silane and then kept stirring for 5 h. The silica beads were centrifuged, washed with alcohol and water, and then dispersed in 10 mL water. The silica beads were labeled by luminescent Au particles by condensation. 20 or 100 μL of 0.1 mM silica bead water solution was added in 1.0 mL of 0.1 mM metal particle water solution. The absorbance and emission spectral changes in solution with the reaction time were monitored without further purification.

Absorption spectra were monitored with a Hewlett Packard 8453 spectrophotometer. Luminescence spectra were recorded with Cary Eclipse Fluorescence Spectrophotometer. All luminescence image studies were performed using a time-resolved confocal microscope (MicroTime 200, PicoQuant) [26]. Immobilization of metal particles on glass coverslips was achieved by adding 20 μL of diluted nanoparticle suspension onto an amino-silanized coverslip following by spin drying at 4000 rpm. A single mode pulsed laser diode (470 nm, 100ps, 40 MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D467/10, Chroma) before directing into an inverted microscope (Olympus, IX 71). An oil immersion objective (Olympus, 100x, 1.3NA) was used.
both for focusing laser light onto sample and collecting fluorescence emission. The fluorescence that passed a dichroic was focused onto a 75 μm pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin Elmer Inc). Images were recorded by raster scanning (in a bidirectional fashion) the sample over the focused spot of the incident laser with a pixel integration of 0.6 ms. The excitation power into the microscope was maintained at 1 μW. In combination with a pulsed diode laser, Instrument Response Function (IRF) widths of about 300 ps FWHM is obtained, which permits the recording of sub-nanosecond fluorescence lifetimes, extendable to less than 100 ps with reconvolution. Lifetimes were estimated by fitting to a χ² value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis. All measurements were performed in a dark compartment at room temperature.

Transmission electron micrographs (TEM) were taken with a side-entry Philips electron microscope operated at 120 keV. Samples were cast from water solutions onto standard carbon-coated (200-300 Å) Formvar films on copper grids (200 mesh) by placing a droplet of a ca. 1 mg / mL aqueous solution.

Results and discussion

The Au particles were synthesized by chemical reduction of Au salt with NaBH₄. The formed Au particles were protected by the self-assembled tiopronin monolayer on the metal cores [22]. In this case, the metal particles are small because of a low molar ratio of Au salt / tiopronin (6/1) in solution [16]. This fact can be demonstrated by the absorbance spectrum that displayed a direct decay from high to low energy wavelength without a significant metal plasmon resonance (Fig. 1), consistent with the feature of small metal particles [14,22]. Their metal cores can be outlined by TEM images [Fig. 2(a)], showing a poly-dispersion of core size with an average diameter of 1.6 nm.

The small Au particles are reported to emit luminescence upon excitation at a wide wavelength range [14]. In order to match the imaging experiments, the metal particle was excited at 470 nm in solution (Fig. 1). The emission maximum was shown at 785 nm, consistent with the report from Murray [16]. The sample was cast on the glass coverslips to monitor the luminescence images of single metal particles using scanning confocal microscopy upon excitation at 470 nm (left panels in Fig. 3). Although the quantum yield was low to be 0.3% [16], the luminescent images could be recorded well in a similar operation to the organic fluorophores. The time-dependent fluorescence transients of metal particles and cyanine 5 (Cy5) were displayed in the right panels of Fig. 3, which were excited at the same power intensity. The time trace of Cy5 displayed a typical behavior of organic fluorophore with a one-step photobleaching; while the Au particles displayed decay from its initial intensity. Antibunching experiments were not done as Dickson [27] to figure out the single or aggregated metal particles in this paper. However, we have many experiences to cast the nano molar organic fluorophores solution on the glass coverslips to prepare the samples for single molecule fluorescence detection. It was shown that most spots were identified as single fluorophores and some as aggregates. Thus, we believe that like the organic fluorophore, the metal particles existed mostly as the singles in this case. In addition, the same solution was also cast on the grids for TEM measurement, showing most single particles instead of aggregates (Fig. 2).

The metal particles emit in the energy band gap mechanism on the surface similar as QDots because of their similar chemical structures. Thus, it is certain that the metal particles display better photostability than organic fluorophores. For instance, the Cy5 molecules were observed to bleach completely in 2 s, but the metal particles in 20 s. However, the metal particles displayed poor photostability than QDot, which was attributed to the photodegradation of metal particle under a strong irradiation of laser source. Contrarily, because of high quantum yield,
QDot is generally excited at a lower power laser so the decay curve of QDot is not obvious in the same time scale. In order to improve the photostability, we consider increasing the quantum yield of metal particle. According to recent publication from Murray [20], the quantum yield of small metal particles can reach to 10%, 30 fold higher than the current sample. However, the metal particles are much smaller than QDots. Thus, the metal particles are suggested using in biological and clinical assays when the small and photostable probes are required.

In this case, we simply labeled the silica beads using the luminescent metal particles in order to mimic the labeling of biological functionalities. The metal particles were succinimidylated by a single functionalized ligand via ligand exchange (Scheme 1) [23]. Because the multiple succinimidyl moieties were expected to cause the aggregation of silica beads, the metal particles were controlled with one succinimidyl ligand / metal particle. It was noted that the absorbance and emission spectra were not altered significantly with the surface reaction indicating that only few ligand was exchanged. TEM images showed insignificant change from the originally synthesized metal particles further confirming the point.

The silica beads were synthesized using Stöber method [24] and the sizes were identified to be 50 nm from the TEM images [Fig. 2(b)]. In order to label them by the luminescent metal particles, the surfaces of beads were aminated using 3-aminopropyltrimethoxy silane in solution. The aminated silica beads were covalently bound with the succinimidylated metal particles through condensation. The number of bound metal particle / per silica bead was controlled with the molar ratio of metal particle / silica bead in solution that was 10 or 50, respectively, in this case. It is difficult to accurately estimate the binding number, but the binding can be simply proven to occur by the existence of small metal particles near the silica beads on the TEM images [Fig. 2(c)] as well as an absorbance rising at 510 nm and an emission decrease at 765 nm due to the aggregation of small Au particles on the silica beads [22].

Although the ensemble emission displayed a decrease intensity after conjugation between the metal particles and silica beads (Fig. 1), the images of conjugates displayed an increase of 8-fold in brightness (left panel in Fig. 3) as compared with the unbound metal particles when the molar ratio of metal particle / silica bead was 10. The brightness of images was further increased to 12-fold when the molar ratio was 50. Because the bare silica beads show relatively dim emission from their scattering (right panel in Fig. 3), the increase of brightness on the labeled silica beads was suggested to be due to the overlapping of emission signals by the bound Au particles on the same beads. The fluorescence spots of single labeled silica beads were shown to be larger than those of the free metal particles furthermore demonstrating the spectral overlapping effect. We also noticed the presence of some faint spots near the bright spots, which had almost the same brightness as the free Au particle, suggesting from the unbound metal particles. We also simply mixed the non-functionalized Au particles and silica beads in solution as control. It was shown from the TEM images that the metal particles could not be bound on the silica beads. The ensemble absorbance and emission spectra were altered insignificantly with the mixing, and the emission images of Au particles remained almost unchanged from those of free Au particles. This fact indicates that the simple mixture of Au particles and silica beads cannot alter the luminescence and the enhanced emission for the Au-silica complex comes from the emission overlapping. The time trace of labeled silica bead displayed a similar decay to the free metal particle (right panel in Fig. 3), though the photodegradation time was extended to 50 s, indicating that the overlapping effect from the bound metal particles resulted in bright emission and long observation time.

The lifetime is an important parameter to investigate the luminescence mechanism. The decay curves of metal particles were analyzed by multi-exponential kinetics. The lifetime values are observed to range in 3-7 ns, which are due to polydisperse size-distribution of the Au particles.
This value is not altered significantly with binding to the silica beads, indicating that the emission of metal particles is influenced insignificantly with the labeling.

Conclusively, we synthesized the luminescent Au nanoparticles in molecule size. The images of single metal particles were monitored using scanning confocal microscopy showing relatively stronger signal and longer detective time. These metal particles were also bound on the surface-aminated silica beads. The labeled beads displayed stronger signal and better photostability. Hence, we suggest these small metal particles can work as the fluorescent probes in developing the molecule imaging agents. For instance, the luminescent metal particles can be used to label antibody molecules and then transport through the cell membrane to track an in-vivo biological activity in single cell. These labeled complexes are expected to be lack of the bulky size from the QDots and poor photostability from the organic fluorophores in the experiments.

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Fig. 1.
Absorbance and luminescence spectral changes of free and bound Au particles on the silica beads in water. Luminescence spectra were measured upon excitation at 470 nm.
Fig. 2.
TEM images of (a) free Au particles, (b) free silica beads, and (c) Au particle labeled-silica beads when the molar ratio of metal particle/silica bead was 50.
Fig. 3.
Emission images of free Au particles, Au particle labeled-silica beads when the molar ratio of metal particle/silica bead was 10, Au particle labeled-silica beads when the molar ratio of metal particle/silica bead was 50, and free silica beads without the bound metal particles. The right panels are time traces of free Au particles and Au particle labeled silica beads when the molar ratio was 10 as well as free Cy5 as comparison.
Scheme 1.
Labeling the aminated silica beads with the luminescent succinimidylated Au particles by condensation.