In vitro and Intracellular Production of Peptide-Encapsulated Fluorescent Silver Nanoclusters

Junhua Yu, Sandeep A. Patel, & Robert M. Dickson*

School of Chemistry and Biochemistry and Parker H. Petit Institute for Bioengineering and Bioscience
Georgia Institute of Technology
901 Atlantic Drive, Atlanta, GA 30332-0400 (USA)
Fax: (+)404-894-7452
E-mail: dickson@chemistry.gatech.edu

1. Experimental details.

**Chemicals.** Silver nitrate (99.9999%), phosphate buffer saline (PBS), Trypsin, Dulbecco's Modified Eagle's Medium (DMEM), Sephadex G50, and Penicillin-Streptomycin solution were purchased from Sigma-Aldrich and used as received. SYTO® RNASelect green fluorescent cell stain was obtained from Invitrogen. A gift from Prof. Y.-L. Tzeng, peptides were synthesized by the Emory University microchemical facility.

**Cell culture and metal ion staining.** NIH 3T3 cells were a gift from Dr. D. F. Doyle (Georgia Institute of Technology). Cells were incubated under 5% carbon dioxide/air at 37 °C, in DMEM with 4.5 g/L glucose, L-glutamine and pyruvate, supplemented with 10% fetal bovine serum and a 1% penicillin/streptomycin mixture. The cells were fixed by formaldehyde (4%) for 15 min, washed with PBS twice for 5 min, and then two quick-washes with deionized water. The cells were then loaded with aqueous metal ions, washed with deionized water and mounted onto slides for measurements. For colocalization experiments, silver nitrate (100 mM) was incubated with fixed NIH 3T3 cells seeded on cover-slips in deionized water for 15 hrs, and then the water was replaced with fresh water in the presence of SYTO dye (50 nM) for 30 min. The cover-slips were then washed with PBS at least 8 times and mounted onto slides for microscopy. For live-cell loading, peptide-encapsulated silver clusters (0.5 mg) were dissolved into DMEM (1 mL) and loaded into live cells at 37 °C and 5% CO₂ for 1 hr. Cells were subsequently washed with PBS before mounting onto slides for microscopy. For fixed-cell-loading, cells were fixed with pre-cooled methanol (-20 °C) for 15 min, washed with PBS for 5 min twice, and then incubated with peptide-encapsulated silver clusters (0.5 mg) in DMEM (1 mL).

**Emission from formaldehyde-fixed NIH 3T3 cells loaded with 100 mM silver nitrate for 20 hrs.** Fluorescence image; The image was taken by a CCD camera (MicroMax, Princeton Instruments) on an Olympus IX70 epifluorescence microscope (objective, UPlan-Apochromat, 60×/1.45 oil; excitation filter: BP 460-490; emission filter: LP 525). Scale bar: 15 µm.

**Colocalization of silver and SYTO® RNASelect staining.** Left: image of RNASelect fluorescence with an excitation filter of BP 460-490 nm, and 515 - 525 nm band pass emission (green channel); middle: same as blue excitation but with 665 nm long pass emission (red channel); right: merge of green and red channels. The loading concentration of RNASelect exhibiting strong emission near 520 nm and very weak emission beyond 650 nm, was set to be high enough to make the emission through BP 515-525 filter dominant from RNASelect. However, silver nanoclusters show strong emission beyond 650 nm, which enables the orthogonal imaging of colocalization when observed through a LP 665 filter to collect the silver emission.

**Photophysics of P3-Ag and its cell loading.** (A) Emission and excitation spectra of aqueous P3-Ag solution. Emission (solid) was excited at 400 nm, and excitation (dotted) was detected at 630 nm. (B) MALDI mass spectrum of P3-Ag. (C) Fluorescence images of NIH 3T3 cells loaded with P3-Ag (0.5 mg) (a) in DMEM at 37 °C and 5% CO₂, in DMEM at 4 °C (b), and methanol-fixed NIH 3T3 cells loaded with P3-Ag in DMEM at r.t. (c). The images were taken on Olympus X1-70 microscope (UPlan-Apochromat objective, 60×/1.45 oil; excitation filter: BP 460-490; emission filter: LP 525) with iXon CCD camera; incubation time: 1 hr. Scale bar, 30 µM.
Synthesis of peptide-protected silver clusters. An aqueous mixture of peptide (0.22 mM) and silver nitrate (0.37 mM) was reduced with sodium borohydride (18 mM) at room temperature. After a further 24 hrs stirring, the crude products were purified by Sephadex G50 column chromatography with water as eluant. Fluorescence and absorption spectra were taken on a QM-4/2005SE fluorometer (Photon Technology International) and Shimadzu UV-Vis spectrometer, respectively. Fluorescence lifetimes were measured on LifeSpec-ps spectrometer (Edinburgh Instruments). MALDI-mass spectra were obtained on an Applied Biosystems 4700 Proteomics Analyzer.

Emission spectra of cells. The emission spectra of the cells stained with silver nitrate were recorded with a CCD camera on an Olympus IX71 microscope with an UPlanApo objective (60×/1.45 oil) after passing through a monochromator (SpectraPro 300i, Acton).

Lifetime measurements of cells. Fluorescence lifetime measurements were made using a Becker and Hickl SPC-630 time correlated single photon counting board using a frequency-doubled modelocked, 84 MHz, 100 fs Ti-Sapphire oscillator operating at 850 nm with average power ranging from 100 mW/cm² to 5 W/cm² as the excitation source on an epifluorescent inverted microscope with a 100×, 1.40 NA oil objective. A Hamamatsu HP4722-40 PMT with a 300 ps instrument-response function was used as the detector.

Picosecond-gated images of cells. Lifetime images were taken using a LaVision Picostar time-gated intensifier with an Andor front-illuminated iXon CCD camera. The gate window was 212ps and images were taken in steps of 20ps. Images had an integration time of 1s. The same modelocked Ti-sapphire laser used in lifetime measurements was used for excitation images. Fluorescence image of formaldehyde-fixed NIH 3T3 cells loaded with 100 mM silver nitrate for 20 hrs. (A) Fluorescence image; the inset is the intensity profile along the line drawn across the cell. The image was taken by a CCD camera (MicroMax, Princeton Instruments) on an Olympus IX70 epifluorescence microscope (objective, UPlan-Apochromat, 60×/1.45 oil; excitation filter: BP 460-490; emission filter: LP 525). Scale bar: 15 μm. (B) Colocalization of silver and SYTO RNASelect staining. Left: image of RNASelect fluorescence with an excitation filter of BP 460-490 nm, and 515 - 525 nm band pass emission (green channel); middle: same as blue excitation but with 665 nm long pass emission (red channel); right: merge of green and red channels. The loading concentration of RNASelect exhibiting strong emission near 520 nm and very weak emission beyond 650 nm, was set to be high enough to make the emission through BP 515-525 filter dominant from RNASelect. However, silver nanoclusters show strong emission beyond 650 nm, which enables the orthogonal imaging of colocalization when observed through a LP 665 filter to collect the silver emission.

2. Time and concentration dependence of fluorescent silver staining
Figure 1S. Time and concentration dependence of staining efficiency by silver nitrate. Upper panels: formaldehyde-fixed cells were loaded with silver nitrate at various concentrations. The emissive cells can be observed within 20 hrs at a low loading concentration of 20 mM (upper left). With higher silver nitrate concentration, the time to obtain bright cells becomes shorter, i.e., 50 mM for 5 hrs (upper middle) and 100 mM (upper right) for 2 hrs. The images were adjusted to the same contrast and background brightness with WinView/32. Lower panels: formaldehyde-fixed cells were loaded with silver nitrate at a concentration of 100 mM. from lower left to right, the longer the loading time, the brighter the image.

3. Silver cluster emission spectra and photostability in cells

Figure 2S. Emission spectra, recorded with a CCD camera after passing through a monochromator (SpectraPro 300i, Acton); the spectrum of a single bright region in the nucleus was obtained by narrowing the aperture of the monochromator to let just the light of the spot through. Excited with a 476-nm Ar⁺ laser.
Figure 3S. The time-intensity curve of the silver-stained nucleolus. The emission is quite stable. Within the first seven minutes of irradiation, the total intensity of the emission decreases about 30%, but then levels off with much greater stability (Spectra recorded as in Fig. 2S).

4. Control experiments loading with other metal ions.

Control experiments of loading copper and gold ions under similar conditions, failed to produce significant intracellular luminescence. When silver stained cells were treated with potassium hexacyanoferrate(III) and sodium thiosulfate to remove the fluorescent silver nanoclusters in the cell, the image intensity became very weak and lost nucleolar specification (Figure 4S, c), further indicating that the emission results from silver.

Figure 4S. Comparison of luminescence images of cells. (a) autofluorescence; (b) stained with silver nitrate; (c) stained with silver nitrate, irradiated with blue light for 15 minutes, and then incubated with potassium hexacyanoferrate(III) (36 µM) and sodium thiosulfate (3600 µM) for a hour to remove reduced silver in cells; The exposure time for all the images was 10 s except for (b) (0.5 s).

5. Emission lifetime measurement
Figure 5S. The lifetime measurement of cells stained with silver nitrate, and fit to 220 ps (33%) and 1769 ps (67%).

6. The emission and excitation spectra of P1-Ag.

Figure 6S. Emission (solid, 460-nm excitation) and excitation (dashed, 580-nm emission) spectra of aqueous P1-Ag solution in 10 mm cuvette.
**Figure 7S.** Emission spectra of aqueous solution of P1-Ag. Legend: excitation wavelength. Besides the major emissive species at 610 nm, there are also minor species which have bathochromic emissions: the emission peaks red shift along with the red-shift of excitation wavelength and the emissions become very weak beyond the excitation at 605 nm, suggesting the presence of several species with slightly different excitation and emission spectra.

7. Mass spectrum of **P1-Ag**

**Figure 8S.** The MALDI mass spectrum of **P1-Ag**

8. Strong interaction between silver and ligand is necessary. Other simple polypeptides, such as, poly-L-Lys (MW 2.5k-40k Da), poly(Glu,Ala (6:4)) (MW 20k-50k Da), and poly(D-Glu, D-Lys (6:4)) (MW 20k-50k Da), can not generate any emissive silver clusters, but yield large silver nanoparticles. Strong interaction requires a combination of several amino acids to form a strongly chelating ligand.
References
