Published in final edited form as:

Angew Chem Int Ed Engl. 2009 ; 48(2): 318–320. doi:10.1002/anie.200804137.

Shuttle-based fluorogenic silver cluster biolabels

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Keywords

silver clusters; cell imaging; transfer; fluorogenic; live

Most molecular/cellular labeling utilizes organic dyes conjugated to bioactive molecules through general organic/inorganic chemistry. Though shortcomings of organic dyes such as poor photostability^[1] and low brightness^[2] limit observable copy numbers, lack of selectivity in labeling often more seriously limits their application in live cell imaging and single molecule studies^[3]. Specificity can be addressed through antibodies and other strong affinity pairs such as avidin-biotin^[4,5], but direct covalent technologies are crucial at sub-pM concentrations, as this is beyond the binding limits of antibody-based affinities. Genetically encoded fluorescent proteins are an excellent solution for specific fluorescent labeling in live cells, but disadvantageous organic dye photophysical instabilities (blinking and bleaching) remain, coupled with the potential perturbation due to large label size^[6]. Attempts to overcome photoinstabilities of organic labels have produced much brighter quantum dot labels^[7], but, while providing excellent signals, these emitters introduce additional problems such as large physical size, aggregation, toxicity, polyvalency, and strong fluorescence intermittency^[8–10]. Simultaneously addressing concerns of brightness, photostability, monovalency, size, and fluorescence intermittency, newly emerging silver cluster-based labels offer excellent potential as molecular labeling agents^[11–15]. Spectrally-pure emitters have been produced ranging from the blue to the near IR, with fluorescence quantum yields (Φ_F) up to 40% and hydrodynamic radii of the fully assembled ssDNA-encapsulated SCs of ~2.5 nm^[16]. Moreover, at bulk and single molecule levels, SCs also show both excellent brightness and photostability^[17].

Ag nanoclusters are typically created within ss-DNA scaffolds through direct BH_4^- reduction of Ag salts in the presence of DNA^[12]. Such methods can be employed to generate highly emissive labels on proteins of interest, but BH_4^- is a rather harsh reductant. To minimize exposure, we investigated other scaffolds in which clusters could first be synthesized, but would enable whole cluster transfer to the ss-DNA encapsulant – a fluorogenic cluster shuttle. Identified to stabilize Ag nanocluster emission^[18], poly(acrylic acid) (**PA**) was investigated as a low Φ_F silver cluster scaffold. Initial attempts at SC creation within a commercially-available, small linear **PA** by reduction with sodium borohydride yielded both very low SC concentrations and low Φ_F 's, with simultaneous generation of silver nanoparticles. Nanoparticle formation was eliminated and cluster yield greatly improved when silver ions first formed complexes with 3-(2-aminoethylamino)propyltrimethoxy silane (APTOS) before **PA** stabilization and borohydride reduction. Mass spectrometry of the complexes predominantly shows 2:1 APTOS:Ag⁺ complexes which likely limit final Ag cluster size

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(see supplementary information). Ag clusters made in this way (**PA**-SCs) exhibit a 3% Φ_F and a biexponential fluorescence decay (280 ps (27%) and 1380 ps (73%)).

Though easily scaled up to large amounts (more than 100 mL solution, Fig 1a inset) and quite stable for weeks, **PA**-SCs, readily transfer SCs to high affinity ss-DNA sequences resulting in loss of **PA**-SC fluorescence and generation of characteristic DNA-encapsulated SC emission. Concomitant with the spectral shifts, cluster transfer from low Φ_F **PA**-SC to high Φ_F ss-DNA-SC increases brightness >10-fold, with excellent specificity. Immediately upon mixing **PA**-SC with 12-mer oligocytosine (**C12**), emission intensity increases significantly (about 10-fold), and the excitation spectra shift correspondingly from that of **PA**-SC ($\lambda_{\max} = 515$ nm) to that of 12mer polycytosine (**C12**-SCs, $\lambda_{\max} = 570$ nm) (Fig. 1a). The transfer efficiency depends on composition of **PA**, Ag^+ , and APTMOS (supporting information), with each Ag^+/PA ratio having an optimal ratio of $\text{Ag}^+/\text{APT MOS}$. At lower Ag^+/PA ratios, lower $\text{Ag}^+/\text{APT MOS}$ yields more efficient SC transfer, and *vice versa*. **C12**-SCs exhibit reasonably high initial count rates after SC transfer with Ag^+/PA and $\text{Ag}^+/\text{APT MOS}$ ratios of 3, as used in this work. The emission intensity continues to increase, reaching the emission of **C12**-SCs prepared by direct synthesis within half an hour. Solution pH and buffer conditions also influence SC transfer, with higher pH further facilitating cluster transfer, presumably through destabilization of the **PA**-SC complex. While working well for multiple 12-mer sequences, precise transfer conditions may need to be optimized for specific sequences exhibiting secondary structures or intermolecular interactions. For example, cluster transfer to 5'-AATTCCCCCCCCCCCCAATT-3' (**ATC12**) does not proceed at room temperature, but transfer readily occurs when warmed to 50 °C. This moderate selectivity allows SC transfer to ssDNA-tagged proteins with little interference from other cellular components. Generally, once protein fluorescence is desired, SCs can be transferred to the ssDNA-protein conjugate. For an ordinary antibody, the DNA tag is much more easily handled than are light-sensitive dyes. Most importantly, the brightness and photostability advantages of SCs can be utilized for higher sensitivity imaging.

Using our fluorogenic SC shuttle, we fluorescently labeled cellular components by first conjugating **C12** to antibodies (Abs), staining with these non-fluorescent DNA-conjugated antibodies, and transferring SCs as the final step. Actin, one of the three major cytoskeleton components^[19,20], was stained by anti-actin Ab **C12** conjugate (**actinC12**) prepared from anti-actin Ab FITC conjugate (Sigma, Saint Louis, Missouri, USA) and **C12** linked with sulfo-SMCC (4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt). Efficiency was examined on methanol fixed, acetone permeated BPAECs (Bovine pulmonary artery endothelial cells), which were first stained with **actinC12** (1:50 dilution) overnight at 4 °C or 1 hr at r.t., and subsequently incubated with **PA**-SCs for 20 min. The emission image from SCs (Fig. 1b) colocalized well with that of FITC (Fig. 1c) which was tagged to the anti-actin Ab, revealing antibody location and indicating actin staining with SCs via SC transfer (Fig. 1d). Controls showed that long-time staining with **PA**-SCs alone (without antibody staining) yielded no such emission images and there was no silver cluster transfer between **PA**-SCs and antibody unless the ss-DNA label was also present. The images show typical actin morphology, implying that conjugation to DNA does not interfere with antibody function. The conjugate, however, produces some non-specific nuclear staining that is readily reduced by blocking with BSA (Fig. 1g).

In identical labeling and imaging conditions, brightness of BPAECs was compared stained with either anti-actin Ab Cy3 conjugate or **actinC12** with SC transfer as the last step. The images from SCs are about seven times brighter than those of Cy3 (Fig. 1f and g). Since the Cy3/Ab ratio is 8, and the **C12**/Ab ratio is 3, each SC is ~20-fold brighter than is each Cy3 under these conditions. In addition to excellent brightness, SCs also exhibit outstanding photostability. Over 2 hrs of slow scanning on a Zeiss confocal microscope, the SC-stained BPAECs showed only 10% decrease in emission intensity, significantly better than that of Cy3

(Fig. 1f), while simultaneously being much brighter overall. This silver cluster transfer protocol also works well on other cell lines, such as NIH 3T3 (see supporting information). The availability of SC transfer, together with the outstanding brightness and photostability of SCs further illustrates their promise as bulk and single molecule labels.

Anti- α -tubulin **C12** conjugate (**tubulinC12**, Invitrogen) was synthesized similarly to stain microtubules^[21]. In this case, SCs were transferred to **tubulinC12** (primary Ab) after an Alexa-488-tagged secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG, Invitrogen) had been immunologically targeted to **tubulinC12**. Good colocalization of the emission image of BPAECs from SCs and Alexa 488 (supporting information) indicates that, again, transfer is quite efficient even though the secondary Ab may partially block the access of **PA-SCs** to **tubulinC12**. The small size of **PA-SCs** may contribute to this significant silver cluster permeability.

The transfer fidelity, coupled with the excellent brightness and photostability of SCs encouraged us to further apply SC transfer to live cell labeling. We utilized anti-fibroblast surface protein antibodies (anti-FSP) to recognize an epitope on live fibroblast cells^[22]. The synthesis of anti-FSP 24mer polycytosine conjugate (**FSPC24**) is also straightforward, with sulfo-SMCC as a linker between **C24** and anti-FSP (Sigma). The conjugate was then loaded into the growth medium of NIH 3T3 cells at 4 °C for 15 min, followed by SC transfer from **PA-SCs** at 4 °C for 10 min. At recommended 1:500 dilution of **FSPC24**, the cell surface showed uneven staining after SC transfer (data not shown) because of short incubation time, low temperature, and low antibody concentration. To improve staining, Ab concentration was increased 30-fold and incubation time was decreased to avoid the characteristic cellular detachment resulting from the propensity of anti-FSP to eliminate fibroblast cells from a mixture of varied cell lines^[23]. Under these conditions, much brighter and more uniform staining resulted (Fig. 2a). Reconstruction of z-stack confocal images also shows clear cell surface staining (Fig. 2b). Cells incubated with **C24**-encapsulated silver clusters alone, either at 4 °C or 37 °C, showed no staining from silver clusters, suggesting antibody-mediated staining. While quite promising as a fluorogenic cluster shuttle, only surface proteins can currently be labeled for live cells due to the poor cell-permeability of **PA-SCs**. However, SC transfer can be useful in the study of receptor-mediated target delivery. As shown in Fig. 2c, when target **FSPC24** staining cell surface protein was marked with silver clusters by SC transfer at 4 °C, and then incubated at 37 °C for 30 min, **FSPC24** silver clusters were internalized, quite likely via endocytosis, resulting in punctuate staining inside cells. Nevertheless, coupled with both DNA sequence directed SC emission colors^[16] and specific peptide scaffolds for Ag-cluster binding^[24] the design of new shuttles may enable direct multi-color intracellular labeling in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge financial support from Invitrogen Corp., NIH R01GM68732, and NIH P20GM072021. We also thank Prof. D. F. Doyle and Prof. C. J. Fahrni (Georgia Tech) for use of their cell culture facility, and Prof. Jens Karlsson (Drexel University) for the gift of BPAECs.

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Figure 1.

BPAECs stained with anti-actinC12 (**actinC12**) and visualized by SC transfer. (a) SCs transferred from **PA** to **C12**. Left panel, normalized excitation spectra of reaction mixture detected at 640 nm before (red) and after (white) the addition of **C12**, which corresponds to individual excitation spectra of **PA**-SCs and **C12**-SCs, respectively. Right panel, the fluorescence intensity increases 10-fold after the addition of **C12** (white); excited at 545 nm. Inset: **PA**-SCs (100 mL) prepared in a Pyrex conical flask, under 354 nm excitation. (b) fluorescence images of BPAEC from SCs and (c) FITC tagged to **actinC12** to indicate the location of actin, with colocalization in (d). (e–g) Photostability (e) and brightness (f, g) comparison between Cy3- (f) and SC- (g) labeled cells under identical staining and imaging conditions. Insets show the intensity profiles of the selected images. SCs and Cy3 were excited at 543 nm (20 W/cm²); FITC was excited at 488 nm. Scale bar, 25 μm.

Figure 2.

Live NIH 3T3 cells stained with **FSPC24** with SC transfer as the last step. **a, c**, merge brightfield and SC fluorescence (pseudo color red, excited at 543 nm). Cells were incubated with **FSPC24** at 4 °C (**a**) or 37 °C (**c**). **b**, an orthoaxis slice of the long cell in the lower right region of (**a**), reconstructed from z-stacks of confocal images, showing cell surface staining at 4°C. Scale bar, 30 μ m.