NTA-Ni$^{2+}$-Functionalized Quantum Dots for VAMP2 Labeling in Live Cells

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An efficient method for labeling individual proteins in live cells is required for investigations into biological mechanisms and cellular processes. Here we describe the preparation of small quantum dots (QDs) that target membrane surface proteins bearing a hexahistidine-tag (His$_6$-tag) via specific binding to an nitriilotriacetic acid complex of nickel(II) (NTA-Ni$^{2+}$) on the QD surfaces. We showed that the NTA-Ni$^{2+}$-QDs bound to His-tag functionalized beads as a cellular mimic with high specificity and that QDs successfully targeted His-tagged vesicle-associated membrane proteins (VAMP) on cell surfaces. This strategy provides an efficient approach to monitoring synaptic protein dynamics in spatially restricted and confined biological environments.

**Key Words:** Quantum dot conjugates, Imaging probes, Labeling, Protein tracking

**Introduction**

Single quantum dot (QD) tracking methods have been employed in a variety of biological investigations. Because QDs exhibit good photostability and bright fluorescence intensities, QD probes have been used to monitor individual biological molecules in studies on the diffusion dynamics of neurotransmitter receptors, tracking individual kinesin motors in the cytoplasm, and visualization of nerve growth factor transport in live neuron cells. Most QD probes designed for biological assays have been functionalized using the streptavidin-biotin interaction due to the high binding affinity (4.0 × 10$^{15}$ M$^{-1}$) and straightforward adaptation of this system. However, streptavidin-coated QDs are quite large (20 - 30 nm) and can impair the trafficking of labeled proteins in crowded cellular environments such as synapses. The size of QDs increases when large proteins are used to functionalize the surfaces, e.g. streptavidin, which is 53 kDa, ~4 nm, or antibodies that are essential for labeling cell surface proteins. Therefore, efforts have been made to reduce the size of quantum dots to improve the trafficking of proteins. In this report, we describe the development of small QDs that selectively target oligohistidine-tagged proteins. Nitriilotriacetic acid complexes of nickel(II) (NTA-Ni$^{2+}$) have been used to target hexahistidine (His$_6$) expressed fusion proteins.

Genetically encoded oligohistidine (His$_n$) sequences have been widely used in combination with NTA-Ni$^{2+}$ for purification applications, achieved by passing the protein mixture through a chromatography column containing NTA-Ni$^{2+}$ resins, in vitro detection of His$_n$-tagged proteins, and the surface immobilization of recombinant proteins. Because the incorporation of His$_n$ into the termini of peptides minimally disrupts the native structure or function, the manipulations required for this methodology are innocuous. Therefore, a variety of fluorochrome conjugates attached to NTA-Ni$^{2+}$ or gold nanoparticles, the surfaces of which have been derivatized using NTA-Ni$^{2+}$, have facilitated site-specific labeling of proteins for observing biological events.

Vesicle-associated membrane protein 2 (VAMP2) is a small integral membrane protein found in secretory vesicles and is a member of the VAMP family. There is great interest in imaging VAMPs because their trafficking plays an important role in Ca$^{2+}$-triggered exocytosis of neurotransmitters via various regulatory proteins that are collectively referred to as SNARE masters. Here, we demonstrate the specific labeling of VAMP2 in live cells using NTA-Ni$^{2+}$-functionalized QDs.

**Experimental**

**Chemicals.** N$_{66}$N$_{77}$-Bis-(carboxymethyl)-l-lysine hydrate (NTA) was purchased from Fluka (Buchs, Switzerland). Nickel(II) chloride hexahydrate (99.9999%), 1-ethyl-3-(3-dimethythylaminopropyl)carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), Qdot® 605 ITK® Carboxyl Quantum dots was purchased from Invitrogen (USA).

**Preparation of NTA-Ni$^{2+}$-functionalized QDs.** Carboxyl groups of the QDs (80 pmol) were activated by EDC (10 mM, 10 µL) in borate buffer (pH 7.5) for 15 min at rt. NTA (10 mM) was dissolved in borate buffer (pH 9.0) then diluted in deionized water to obtain 1 mM NTA. Subsequently, Ni$^{2+}$ (1 mM, 30 µL) was added to the NTA solution (10 µL). After 1 h incubation at rt, the resulting NTA-Ni$^{2+}$ complex was added to the activated QDs and stirred for 6 h at 4 °C. To remove unreacted NTA-Ni$^{2+}$, column purification (Sephadex G-50, Sigma-Aldrich Chemical Co., MO) was performed. The final NTA-Ni$^{2+}$-QDs were stored in deionized water at 4 °C.

**Measurements.** The hydrodynamic radius (particle size) and core size of the NTA-Ni$^{2+}$-QDs were measured, respectively, using an ELS 8000 electrophoretic light scattering apparatus (Otsuka Electronics Co., Japan) and a transmission electron microscope (TEM) using a TECNAI F20 electron microscope (Philips Electronic Instruments Corp., Mahwah, NJ) operated at 200 kV. The fluorescence intensities of the QDs and the
NTA-Ni\textsuperscript{2+}-QDs were monitored at 488 nm excitation, and the emission spectrum was recorded over the range 580 - 630 nm (1.5 mm slit) on a RF-5301PC spectrophotofluorometer (Shimadzu, Kyoto, Japan). FT-IR spectroscopy (SPECTRUM 2000 of PERKIN ELMER) was used to confirm the presence of NTA-Ni\textsuperscript{2+} on the QDs.

**Hexahistidine immobilization.** The hexahistidine (His\textsubscript{6})-tagged Glutathione S-transferase (GST) and GST-virgin vector plasmids (pGEX-4T-1, GE Healthcare) were transformed into *Escherichia coli* BL-21, and the transformants were cultured in 2XYT medium supplemented with ampicillin. After overnight induction with 0.5 mm isopropyl 1-thio-β-D-galactopyranoside at 25 °C, the cultures were sonicated in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mm Tris, pH 8.0, 150 mm NaCl, 1 mm MgCl\textsubscript{2}, 1 mm EGTA, 0.1 mm PMSF) and centrifuged for 15 min at 12,000 rpm. The supernatants were incubated with glutathione-agarose-4B beads (Amersham Biosciences) at 4 °C for 30 min. After washing three times with lysis buffer, the beads were analyzed by SDS-PAGE with Coomassie staining for quantification.

**In vitro binding assay using native PAGE.** Purified GST and GST-his proteins were separated using 12% native PAGE. Briefly, the indicated quantities of purified proteins were dissolved in 2x native sample buffer (BioRad) and separated by application of 30 mA/250 V. After separation, proteins were transferred to a PVDF membrane for the *in vitro* binding efficiency assay or for quantification with Coomassie staining. The transferred membrane was incubated with 1 mM NTA-Ni\textsuperscript{2+}-QDs for 2 h at room temperature and washed three times with Tris-Buffered Saline Tween-20 (TBST). After labeling, the labeling efficiency was quantified using a phosphorimager using the optimized QD605 excitation and emission wavelengths.

**Cell culture and transfection.** COS-7 cells were maintained at 37 °C, 5% CO\textsubscript{2}; in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin. One day prior to transfection, cells were plated on glass coverslips to achieve a confluency of 50%. Cells were transfected with the desired plasmid using Lipofectamine 2000 (Roche Applied Science) reagent according to the manufacturer’s protocol.

**Cell labeling and imaging of His\textsubscript{6}-tagged GFP-VAMP.** Cos-7 cells were transfected with His\textsubscript{6}-tagged GFP-VAMP 24 h prior to labeling. Cell labeling was performed by placing the glass coverslips on a heating block at 37 °C. Cells were labeled with 1 nM NTA-Ni\textsuperscript{2+}-QD by incubation in Dulbecco’s PBS for 30 min followed by repeated washing with PBS. Cells were imaged at room temperature in PBS. Cell imaging was performed on an inverted widefield epifluorescence microscope (Olympus, IX71) equipped with a 100X oil objective (NA = 1.45, Olympus). Fluorophores were excited using a broad spectrum mercury lamp source. Images were acquired using a CoolSNAP-Hq CCD camera driven by MetaMorph Imaging software (Universal Imaging) with the QD605 optimized filter set (Omega).

**Results and Discussion**

The NTA-Ni\textsuperscript{2+}-QDs were synthesized in two steps (Scheme 1). First, the NTA-Ni\textsuperscript{2+} complex was prepared by coordination of Ni\textsuperscript{2+} to the deprotonated (pH 9.0, borate buffer) carboxyl groups of commercially available NTA through exposure of NTA to NiCl\textsubscript{2} solution for 1 h. The activated carboxyl groups of CdSe/ZnS core-shell polymer- capped QDs with EDC as the coupling agent were subsequently incubated with the terminal amino groups of the prepared NTA-Ni\textsuperscript{2+} complex and the final product was purified by Sephadex column. The core size and hydrodynamic radius of the NTA-Ni\textsuperscript{2+}-QDs was examined by TEM and ELS, respectively. As shown in Figure 1(a-b), the mean hydrodynamic diameter of NTA-Ni\textsuperscript{2+}-QDs was 14.2 ± 3.2 nm, which was slightly larger than the diameter of monovalent streptavidin-conjugated QDs (12.3 ± 0.2 nm).\textsuperscript{17} TEM images indicated that the diameters of the rod-shaped CdSe/ZnS core-shells ranged from 7 to 12 nm and were well-dispersed in the aqueous solution. The average size of the NTA-Ni\textsuperscript{2+}-QDs was smaller:

![Scheme 1](image1.png)

**Figure 1.** (a) Hydrodynamic size distribution of NTA-Ni\textsuperscript{2+}-QDs. (b) TEM image of NTA-Ni\textsuperscript{2+}-QDs. The scale bar indicates 20 nm. (c) Gel electrophoresis analysis of NTA-Ni\textsuperscript{2+}-QDs. (d) FT-IR spectra of NTA, NTA-Ni\textsuperscript{2+}, non-functionalized carboxyl-QDs, and NTA-Ni\textsuperscript{2+}-QDs.
than that of the three-layered streptavidin-QD conjugates (~50 nm) or the acceptor peptide-linked streptavidin-QDs (~20 nm), indicating that these QDs were less likely to disturb folding, secretion, or protein-protein interactions during trafficking to the cell surface than were fluorescent proteins fused with O6-alkylguanine-DNA alkyltransferase or acyl carrier protein. The reduced size of QD conjugates reduces the steric effects in local environments that have restricted access to cell surfaces.

We next examined the composition of the QD conjugates by analyzing surface charge and infrared spectroscopic characteristics. Gel electrophoresis separation of NTA-Ni\(^{2+}\)-QDs revealed a band that migrated toward the negative electrode, in contrast with the QDs containing anionic carboxyl functionalities, indicating more neutralized surface charges due to the presence of positively charged Ni\(^{2+}\) ions on the nanoparticle surface (Figure 1(c)). FT-IR spectroscopy of lyophilized NTA-Ni\(^{2+}\)-QDs and QDs were further scanned. As shown in Figure 1(d), deprotonated -COO\(^-\) from NTA indicated a strong absorbance at 1630 cm\(^{-1}\) and a band corresponding to COOH was also observed at 1720 cm\(^{-1}\). Incubation of NTA with NiCl\(_2\) converted the -COO\(^-\) absorption band at 1630 cm\(^{-1}\) to a band corresponding to Ni\(^{2+}\)-coordinated -COO\(^-\) at 1590 cm\(^{-1}\). NTA-functionalized QDs showed a peak at 1567 cm\(^{-1}\) that probably included contributions from both the NTA-Ni\(^{2+}\) complex and the amide groups (amide II band) formed by attachment of the primary amino groups of NTA to the carboxyl QDs. Unfortunately, the amide I band (~1650 cm\(^{-1}\)) of the NTA-Ni\(^{2+}\)-QDs was not apparent because it was masked by the strong absorbance from the QDs. These observations provided evidence for the successful conjugation of the NTA-Ni\(^{2+}\) complex to the surface of the QDs.

The photoluminescence intensity of the NTA-Ni\(^{2+}\)-QDs was 74% that of the QDs (Figure 2). This decrease in luminous intensity was smaller than the significant fluorophore emission quenching reported previously in systems that used Ni\(^{2+}\) to bridge modified NTA and His\(_6\) components. The paramagnetic Ni\(^{2+}\) moiety was found to strongly quench the photoluminescence of some organic dyes, limiting their application in biological systems.

Next, we performed in vitro assays to evaluate the specific binding of NTA-Ni\(^{2+}\)-QDs with His\(_6\)-tagged proteins. For this, we constructed a cell mimic system using agarose beads (45 - 180 \(\mu\)m in diameter). The His\(_6\) peptide-functionalized (GST-His) beads mimicked cells that express His\(_6\)-tagged proteins on the surface membrane. Non-functionalized (GST) beads were used as a negative control. Both types of agarose beads were incubated with non-functionalized QDs or NTA-Ni\(^{2+}\)-QDs and were imaged by fluorescence microscopy. Figures 3(a-d) show the specific interactions between NTA-Ni\(^{2+}\)-QDs and the His\(_6\)-functionalized bead surface. No fluorescence was observed on GST beads treated with either non-functionalized QDs or NTA-Ni\(^{2+}\)-QDs, and no fluorescence was observed for GST-His beads treated with non-functionalized QDs. Only GST-His beads treated with NTA-Ni\(^{2+}\)-QDs showed strong fluorescence, confirming the specific binding between the His\(_6\)-tagged proteins and the NTA-Ni\(^{2+}\)-QDs. These results suggested that decoration of the surface of QDs with NTA-Ni\(^{2+}\) complexes was a good strategy for achieving noncovalent site-specific fluorescence labeling of proteins.

We next tested the binding sensitivity between NTA-Ni\(^{2+}\)-QDs and His\(_6\)-tagged GST proteins. Recently, magnetic nanoparticles have been shown to selectively trap and separate His-tagged proteins with high sensitivity from cell lysates. Figure 4 demonstrates the sensitive binding capabilities of NTA-Ni\(^{2+}\)-QDs. After incubation of NTA-Ni\(^{2+}\)-QDs with different quantities of GST and GST-His proteins, a band appeared in the lane containing 50 ng GST-His. No band was observed in the lane corresponding to the same quantity of GST, suggesting efficient specific labeling of NTA-Ni\(^{2+}\)-QDs to the His\(_6\)-tagged protein.

We extended the site-specific binding of NTA-Ni\(^{2+}\)-QDs to His\(_6\)-tagged proteins displayed on living cell membranes. CoS-7
cells that expressed GFP tagged VAMP2 on their cell surface was chosen as a model system. Here, a hexahistidine tag was genetically incorporated into the free N-terminus of GFP (His₆-tagged GFP-VAMP2). Following transfection with His₆-GFP-VAMP2, green fluorescence was detected in ~60% of the cell population (Figure 5). After incubation with 1 nM NTA-Ni²⁺-QDs for 30 min, selective labeling was obtained in cells expressing GFP, indicating the presence of the hexahistidine target and the site-specific binding to the cell surface. Although non-specific binding in the non-targeted cells was also observed because of remaining free carboxyl acid groups on the QD surface,¹⁴,²⁵ distinguished selectivity was shown according to the time. These results clearly showed that NTA-Ni²⁺-QDs could be used to determine the localization and diffusion dynamics of specific proteins in living cells without the need for fixation and/or permeabilization of the cells.

Over the past few years, an alternate strategy for the in vitro targeting of His₆-tagged proteins using QDs has exploited QD surface coatings that allow direct coordination between histidine tags and the free Zn²⁺ ions present in the ZnS shell of QDs.²⁶,²⁷ This method was simple and efficient for in vitro labeling; however, it was intrinsically limited in that it yielded poor control over binding stoichiometry and engaged in extensive non-specific binding interactions. In this report, we describe the development of a small QD complex with exposed surface NTA-Ni²⁺ moieties that are capable of specifically recognizing histidine-tagged target neuronal proteins in living cells, and we expect that this system will allow monitoring of synaptic proteins located in very narrow and confined volumes.

Conclusion

In conclusion, we fabricated small QD conjugates (~14 nm in size) for specific labeling of tagged cellular proteins. NTA-Ni²⁺ complexes were covalently bound to the QD surface through amide bond formation that could selectively recognize His₂-tagged VAMP2 in live cells as well as His₂-tagged GST proteins as a cellular mimic. The obvious answer for ‘ideal’ probes for future single molecule/particle tracking is to develop probes which are as small as possible, photo-resistant, and with very limited blinking.²⁴ We anticipated that NTA-Ni²⁺-QDs developed herein may be utilized to monitor the membrane proteins in the synaptic cleft or secretory vesicles in neuron cells.

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References