Published in final edited form as:

J Mol Cell Cardiol. 2008 December ; 45(6): 853–856. doi:10.1016/j.yjmcc.2008.08.006.

Delivery and visualization of proteins conjugated to quantum dots in cardiac myocytes

Yevgeniya E. Koshman¹, Stephen B Waters², Lori A. Walker², Tamara Los², Pieter de Tombe³, Paul H. Goldspink², and Brenda Russell³

¹Department of Bioengineering, University of Illinois at Chicago, Chicago, Illinois. USA.

²Department of Medicine, Section of Cardiology, University of Illinois at Chicago, Chicago, Illinois. USA.

³Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois. USA.

Abstract

The design of a novel transduction complex has permitted the introduction of protein-quantum dot conjugates into the cytoplasm of living cells. Appropriate subcellular localization of quantum dot conjugated cardiac troponin C to the myofibrils and a nuclear peptide to the nucleus were attained in living cardiac myocytes using this approach. This new methodology opens the possibility for live tracking of exogenous proteins and study of protein dynamics.

Introduction

Understanding the roles of specific proteins in cellular processes is a fundamental goal of cell biology. Methods to label proteins inside cells are necessary to study their localization, movement, interactions and microenvironments *in vivo*. Often, fusion proteins are created with a green fluorescent protein (GFP) tag but use is limited due to the deterioration of signal, expense of molecular constructs, and the requirement for viral delivery in some cell types [1]. Therefore, new approaches to deliver and label proteins *in vivo* are needed. The unique optical and spectroscopic properties of semiconductor quantum dots (QDs) offer an alternative to fluorescence-based applications. QDs have a broad excitation profile with narrow, symmetric emission peaks so that mixtures of different sized QDs permit simultaneous excitation with a single light source and detection of multiple targets [2]. To date a major limitation in live cell imaging with protein conjugated QDs is their inability to label cytoplasmic proteins unless laboriously injected [3]. Here, protein-conjugated to QDs are linked to TAT-HA2 by a dissociable disulfide bond that permits protein delivery to myofibrillar and nuclear locations in living cardiac myocytes.

The cellular uptake of the transactivator of transcription (TAT) protein of HIV was reported in 1988 [4,5]. This activity, termed protein transduction, is bestowed by a highly basic 11 amino acid sequence that can be fused with exogenous proteins for rapid cell entry. TAT-fusion proteins are receptor-independent and have been applied to a wide variety of cell types [6,7] including use in cardiac myocytes with a TAT conjugation via a disulfide bond with a variety of PKC regulating peptides [8]. Despite the many practical advantages of protein transduction technology, it remains functionally limited because 99% of TAT-fusion proteins remain sequestered in intracellular vesicles and subject to rapid proteolytic degradation [9].

Therefore, a dissociable cytoplasmic targeting system was designed to overcome problems of vesicle sequestration and any potential modulation of enzyme function or protein structure attributable to a fused TAT sequence. This disulphide-based dissociable system linked to TAT-HA2 contains three distinctive features: an N-terminal cysteine residue facilitates reversible linkage to the protein of interest via disulphide bonding; a TAT transduction domain responsible for cellular internalization; and a pH-sensitive, fusogenic HA2 sequence from the influenza hemagglutinin protein. Therefore, the quantum dot conjugated-protein-TAT-HA2 transduction system provides all the elements necessary for covalent linkage to a labeled protein of interest, internalization via endocytosis, escape from vesicles upon acidification, dissociation of the TAT-HA2 peptide from the protein upon exposure to the reducing environment of the cytoplasm and visualization. This new process delivers QD tagged exogenous protein to the cytosol free of protein transduction domains with appropriate subcellular destination verified by use of thin filament protein cardiac troponin C, cTnC) and a nuclear localization signal peptide.

Methods

The experiments were conducted in accordance with the Institutional Animal Care and Use Committee and NIH guidelines.

Neonatal rat ventricular myocytes and fibroblasts

Cells were isolated from 1-day neonatal rats as previously described and maintained at 37°C, 5%CO₂ [10]. Following protein transduction, myocytes were fixed in 4% paraformaldehyde and stained with phalloidin-Alexa Fluor 488 (Molecular Probes), and then counter stained with DAPI. A gallery of Z-stack images 1 µm thick were collected using confocal microscopy. Optical density profiles were obtained with a Zeiss LSM 510 META at various locations throughout several cells in each of five experiments per condition.

Protein-quantum dot conjugation

2 nmol QdotITK™Carboxyl QDs (655 nm) (in 50 nM sodium borate, pH 8.3) (Molecular Probes) were incubated with 5 mM BisSulfosuccinimidyl Suberate (BS³) (Pierce) cross linker for 30 minutes to charge dots for conjugation. Cardiac troponin C, cTnC (1 µg/µl) was reduced with TBP (tri-butyl phosphine) and incubated with charged Qdots for 1 hour. The reactions were quenched with the addition of 50 mM TRIS.

Transduction complex formation

Protein-QD samples were incubated with either unlabelled 100 µM C-TAT (CRRRQRRKKR) (Invitrogen) and C-TAT-HA2 (CRRRQRRKKRGDIMGEGWNEIFGAIAGFLG) (Invitrogen) and oxidized with ambient oxygen overnight to yield the desired complex: QD-cTnC-S-S-TAT and QD-cTnC-S-S-TAT-HA2. Covalent linkage of cTnC and peptide was confirmed via non-reducing polyacrylamide gel electrophoresis (QD)-nuclear localization signal peptide (CRKYGPK) (Invitrogen) were also incubated with 100 µM C-TAT and C-TAT-HA2 peptide. Conjugated complexes were filtered through 0.2 µm syringe filter and then added to the cell culture media for 2 hours.

Results and Discussion

Protein-QD conjugates and QDs alone did not enter the cells. (QD)TAT and (QD)TAT-HA2 conjugates entered all neonatal rat cardiac myocytes and fibroblasts very rapidly in an efficient manner. Observations of QD construct localizations were made after 2 hours in rat neonatal fibroblasts and myocytes with appropriate subcellular localization of quantum dot conjugated with troponin or a nuclear localization peptide being attained using this approach.

To validate the protein-QD transduction method specific proteins with known intracellular locations were used. The thin filament protein cardiac troponin C (cTnC) is located in the myofibrils of cardiac myocytes where it forms part of the troponin complex bound to actin via tropomyosin. Cardiac myocytes were incubated for 2 hours with protein alone, (QD)cTnC, with an additional bisulfide bond to TAT, (QD)cTnC-S-S-TAT, or with TAT and HA2, (QD)cTnC-S-S-TAT-HA2 (Fig 1). No emission signal was seen indicating that (QD)cTnC complexes were not able to enter fibroblasts or myocytes without the protein transduction peptides (TAT or TAT-HA2), data not shown. In myocytes, (QD)cTnC-S-S-TAT complexes were internalized but were not able to escape the vesicles and resulted in a perinuclear distribution (Fig 1B). Analysis of the optical density profile showed QD constructs (red signal) were located on either side of the nucleus (blue signal) but not associated with the actin filaments (green signal) (Fig 1B). The optical density profile further supports the idea that TAT constructs are encapsulated in vesicles once they cross the cell membrane and accumulate around the nucleus. The most striking results were obtained in myocytes transduced with the (QD)cTnC-S-S-TAT-HA2 where the QD signal, but found widely distributed throughout the cell associated with the myofibrils but absent in the perinuclear region (Fig 1C). The QDs appear to be sitting on top of the myofibrils suggesting that aggregates are too large for incorporation (Z stack panels, Fig 1C). The optical density profile analysis across the myocyte shows the QD distribution colocalizes with the myofibrils suggesting overlap between cTnC and the cytoskeleton. Analysis of the longitudinal profile shows colocalization occurring between the (QD)cTnC and the actin filaments (Fig 1C). While not all I-bands are equally well-labeled, these data confirm the cytosolic release of the (QD)cTnC from the TAT-HA2 complex and provide proof of the concept for targeting to the correct subcellular destination.

The specificity of the differential distribution of the (QD)cTnC with the dissociable TAT-HA2 construct is further reinforced by notable difference between a myocyte and a fibroblast. The (QD)cTnC-TAT-HA2 constructs enter all fibroblasts but, unlike in myocytes, QDs localize to the perinuclear region (Fig 1A)). Fibroblasts lack the myofibrillar destination for cTnC, so although the fusion protein escapes from the vesicles because of the HA2, unanchored proteins might be recaptured.

This methodology was further validated by transducing a nuclear localization sequence (NLS) peptide with both TAT and TAT-HA2 peptide into cardiac myocytes. The rationale being that NLS sequestered in vesicles would be unable to localize in the nucleus. (QD)NLS transduced via a TAT peptide complex once again accumulated in the perinuclear region (Fig 2A). However, a high density signal was detected in the nucleus of myocytes when transduced with (QD)NLS-S-S-TAT-HA2 construct (Fig 2B). Again, this is indicative of transduction, escape from the vesicles and appropriate targeting of the NLS peptide to the nucleus.

The HA2 domain has previously been used to disrupt vesicles containing TAT-fusion protein. Cotransduction of a TAT-HA2 peptide resulted in greater release of Cre-fusion protein from macropinosomes as measured biochemically, however only approximately 1% of total transduced protein escaped to the cytosol. In contrast, when both a protein transduction domain and the HA2 domain are present as a fusion protein, the transduced protein is located throughout the cytoplasm [9,11]. The covalent linkage might explain the difference in cellular distribution in these two experiments. The covalent linkage with the disulphide bond described here is consistent with this explanation. Inclusion of 10mM glutathione in the reaction competes for thiol groups and prevents formation of the transduction complex. Cells transduced with reactions containing glutathione exhibit greatly reduced red emission signal in their cytoplasm (data not shown). These experiments verify the requirement for a disulphide bond for introduction of the conjugates into the cytoplasmic compartment.

QDs with the dissociable transduction system offers many advantages and the potential applications of this methodology are extensive [8]. The technique of introducing dissociable QD-protein constructs into the cytoplasm of the cell provides a powerful new method of studying specific proteins at subcellular resolution in real time. The simple addition of the peptide transduction complexes permit studies on the effect of increased intracellular concentration of the protein of interest, something difficult to accomplish with current transfection technologies or from adenoviral systems. This is beneficial not only in saving time and resources involved with the requisite cloning required to create TAT-fusion proteins, but also time and effort spent to determine the effect of such a tag on enzyme activity or protein structure. The only requirement is that the peptide of interest contains at least one cysteine. Multiple cysteines would serve to improve the TAT-HA2:protein ratio and therefore the efficiency of transduction and escape from the vesicular apparatus. This makes proteins such as arsenic(III) methyltransferase, which contains 14 cysteinyl residues excellent candidates for dissociable transduction [12]. Additionally, this method will allow studies on the effect of rapid changes protein concentration, developmentally lethal proteins and mutant proteins without the need for transfection or transgenic animals. This is very important for cells that are difficult to culture for long periods or when cell lines are not available, such as cardiac myocytes. For example, the dissociable (QD)protein might be an ideal way to study cardiac myocyte remodeling in response to mechanical stimuli from the sensing of mechanical changes at the Z-disks or focal adhesion complexes, to the incorporation of new proteins into the contractile myofibrils.

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Acknowledgments

Y.E.K. was supported American Heart Association fellowship. S.B.W was supported by T32-HL-07692 and P01-HL-62426 (P.deT., P.H.G and B.R). The authors thank Dr. G. Farman for the cTnC protein.

Figure 1. Sarcomeric and myofibrillar localization of QDs conjugated to cTNC

A. Fibroblast transduced with the (QD)cTnC-S-S-TAT-HA2 complex shows accumulation of QDs (red) in the perinuclear region. **B.** Myocyte transduced with the (QD)cTnC-S-S-TAT complex shows QD (red) accumulation in the perinuclear region. Confocal Z stack (panels above and to right of 1B) show lack of QD (red) associated with the myofibrils and an empty nucleus. Optical density profile from the white line (1-2) through the cell confirms lack of signal from (QD)cTnC-S-S-TAT constructs (red profile) from the nucleus, large vesicular clumps in the perinuclear regions and no periodic colocalization with actin (green profile). **C.** Myocyte transduced with the (QD)cTnC-S-S-TAT-HA2 complex shows widespread distribution throughout the cell. The confocal Z stack (panels above and to left) show QDs are

above the I-bands or between myofibrils (green). There is no nuclear or perinuclear vesicular clumping. These observations are confirmed by optical density profiles across the myofibrils (white line, 1–2) and along the sarcomeres (white line, 3–4). QD construct peaks (red signal) coincide with the actin filaments (green signal) for the myofibrils (line 1–2) and show sarcomeric periodicity (line 3–4). The experiments were repeated on five primary cultures. QD diameter is 655 nm. Red-QD, green-phalloidin, blue-DAPI. Cells were observed with X63 objective, scale bar 10 μ m.

Figure 2. Nuclear translocation of QDs conjugated to a NLS peptide

A. Myocyte incubated with **QD-NLS-S-S-TAT** shows an empty nucleus and no myofibrillar localization but a few QDs in the perinuclear region. This is confirmed with optical density line scan (white line, 1–2). **B.** Myocyte incubated with QDs conjugated with a nuclear localization signal QD-NLS-S-S-TAT-HA2 peptide. QD localization is clearly seen in the nucleus and in perinuclear vesicles. This is confirmed in the confocal Z stack (panels above and to right) and with the optical density profile through the nucleus (white line, 3–4) where QD (red signal) coincides with the nucleus (blue signal) confirming nuclear translocation of NLS-QD construct (red profile) but not to the myofibrils (green profile). QD emission only (red) shows QDs in the nucleus. The experiment was repeated on five primary cultures. QD diameter is 655 nm. Red-QD, green-phalloidin, blue-DAPI. Cells were observed with X63 objective, scale bar 10 μ m.