Quantum Dot Probes for Tracking Nerve Growth Factor Ligand-Receptor Dynamics in Live Neurons

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ABSTRACT

The mechanisms by which cell-surface receptors interact with their specific ligands to initiate signaling cascades is a dynamic process which involves ligand-receptor binding, transport, and clearance across and within the cell plasma membrane. Nerve growth factor (NGF) is a neurotrophin that plays a significant role in neural survival, growth and regeneration. The spatial and temporal dynamics of NGF-TrkA receptor binding has been shown to be important in regulating different aspects of neurotrophin signal transduction. However, the process by which growth factor-receptor complexes are shuttled from distal axons to neuronal cell bodies is still unclear and an active area of investigation. Organic fluorophores currently used for locating receptors on neurons suffer from the drawbacks of limited spatial resolution and photobleaching. Nanometer sized, photostable quantum dots (QDs) offer the potential to track NGF-TrkA dynamics with high-resolution (molecular detection) over long periods of time. Antibody conjugated QDs have been used for labeling and tracking single receptors [1, 2]. In addition, ligand-conjugated QDs retain their activity and bind to receptors [3, 4]. In this study we employ anti-TrkA-QD antibody conjugates to visualize TrkA receptor spatio-temporal dynamics in the neuronal PC12 cell line.

Keywords: receptor, drug, neuron, quantum dot, ligand

1 INTRODUCTION

Nanoparticles may provide an effective means for delivery of ligands to live cells in order to identify specific types of receptors, assay receptor functionality, and track receptor transport. Assay of receptors often use ligand-radiolabels. While these types of probes offer high sensitivity, they do not offer any temporal-spatial information. Ligands have been adsorbed on to microbeads for delivery to cells [ref] .However, in the past few years, efforts have been made to conjugate ligands to nanoparticles and nanoparticle technology is being explored as a means for efficient drug delivery [3,4]. Typically, a ligand from the micro/nanoparticle surface is released through enzymatic dissociation [5]. In other cases, ligands tethered to microbeads remain intact and bind to receptors on the surface of a cell. Currently, few examples of both these cases are available. In addition, there is little understanding of general principles necessary for optimizing interaction of a surface tethered ligand and intact extracellular membrane receptors. It has been proposed that properties such as the length of the surface tether, site of ligand conjugation, size of the ligand, and hydrophilicity/hydrophobicity play an important role in these interactions.

Semiconductor quantum dots (QDs) may serve as good candidates for use as nanoparticle probes to identify and track receptor function. QDs are extremely uniform, possess a high surface to volume ratio, and are endowed with intrinsic fluorescent properties including very bright intensity and photostability. Moreover, the property of intermittent fluorescence emission (optical blinking) and the electron dense nature of these nanoparticles allows for easy identification of individual nanoparticles in cell preparations. Early studies using ligands coupled to QDs have shown successful interaction of ligand-QD conjugates with TrkA, erbB and serotonin receptors [3,4,6]. Consequently, a number of applications such as single receptor tracking along extracellular membranes of hippocampal neurons, stimulation of neuritogenesis in PC12 cells, and imaging trafficking of erbB1 receptors in filopodia of A431 cells have been made possible by this technique. Little attention has been directed to the feasibility of QD probes for studying the spatio-temporal dynamics of binding and transport of receptors from the extracellular surface to intracellular sites. Ligand-QD conjugates may serve as an efficient probe that would allow for characterization of receptor transport with high spatial resolution in live cell cultures over substantial lengths of time (>12 hrs).

TrkA receptors are a subset of the superfamily of evolutionarily well-conserved receptors responsible for maintenance of neuronal function, growth, regeneration, as well as involvement in the basic mechanisms underlying high cognitive function, such as synaptic plastiticity. TrkA receptors bind ligand nerve growth factor (NGF) and growing evidence suggest that availability of the receptor and its ligand may play broad and critical roles in neurodegenerative disease such as Alzheimer's, Parkinson's, as well as inflammation and pain [7]. The ability to localize and track with high resolution single or groups of TrkA receptors under different conditions and over long term time intervals of a few days would allow

novel insight into TrkA receptor function. In this initial study, we investigate the ability of anti-TrkA antibody coupled to QDs to: 1) bind to individual TrkA receptors in live cells and 2) to track the extracellular and intracellular distribution of receptors over long periods of time (secs to days).

2 MATERIALS AND METHODS

PC-12 cells were grown on collagen coated T-75 flasks in RPMI-1640 containing 10% horse serum and 5% fetal bovine serum. Upon reaching confluence, the cells were trypsinized and re-plated onto 35 mm collagen coated glass-bottom petridishes (MatTek Corp.) and allowed to incubate overnight.

The streptavidin-biotin system was used for targeting QDs to TrkA receptors. A rabbit anti-TrkA polyclonal antibody directed against the entire extracellular epitope of the TrkA receptor (Chemicon) was used to target the receptor sites. The antibody (38 ug/ml) was biotinylated by incubating overnight in a x500-fold excess of NHS-Biotin-PEO (Pierce) followed by dialysis to separate out the unbound biotin. 100 ul of 3.8 ug/ml antibody in DMEM was added to the cells after withdrawing the RPMI media and allowed to incubate for 15 min at 37°C. The excess antibody was washed out gently and the cells were incubated with 10nM streptavidin-QDs (Quantum Dot Corp.) in DMEM for 15 min at 37°C.The cells were again rinsed gently to remove any unattached QDs and the culture dishes were replaced with RPMI. All rinsing steps were carried out with DMEM.

The cells were fixed at various time points – within 10 secs, 10 min, 30 min, 50 min and 24 hrs after labeling. Cells were fixed by the addition of 10% neutral buffered formalin for 7 min followed by rinsing with distilled water and PBS. The cells were then mounted in a 9:1 mixture of glycerol: PBS for imaging. Images were acquired using a Zeiss Axiovert 200M microscope equipped with an Axiocam MRM camera. DIC and fluorescence images were obtained at both 40X and 100X magnifications. A series of z-stack images were acquired with an Apotome attached to the microscope and collapsed using 3D reconstruction software.

3 RESULTS

3.1 Anti-TrkA-QDs bind to TrkA receptors in live cells

QDs were found to specifically target TrkA receptors on the surface of the cells resulting in bright and punctuate staining on the membrane. Negative controls showed insignificant labeling in the absence of the antibody suggesting strong specificity of our antibody- conjugated probes (Fig. 1)

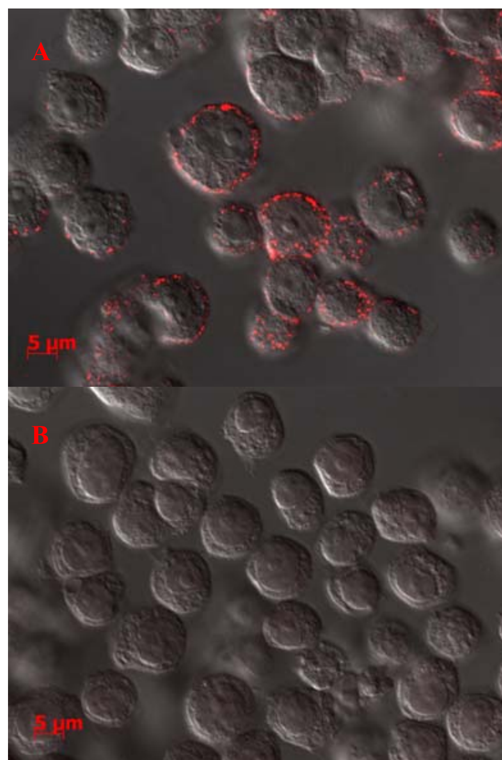

Figure 1: (A) Punctate membrane staining of PC-12 cells incubated with anti-TrkA antibody followed by SA-QDs and imaged 10 min after labeling. (B) Negative control wherein cells were exposed to 10nM SA-QDs alone. Both images were acquired with identical exposure times.

3.2 Anti-TrkA-QDs track movement and spatial distribution of TrkA receptors

Cells presented with anti-TrkA-QDs showed a clear redistribution of TrkA receptors with time. Cells were allowed to incubate at 37°C for varying time periods, subsequent to labeling and were then fixed for observation. Within 10 min of introduction of anti-TrkA-QD probes, strong punctate fluorescence was detected at the periphery of the cells. When the cells were observed 30 min after labeling, a number of QDs were found to have moved into the cytosol. By 50 min, a majority of the QDs were redistributed as polarized groups around nuclei, suggesting intracellular-directed movement of QD tagged receptor complexes. After overnight incubation, QD fluorescence was significantly reduced and only a few punctate clusters were detected. Images were subjected to 3D reconstruction and the reconstructed images clearly depict the movement of QDs from the extracellular membrane into the cell (Fig. 2).

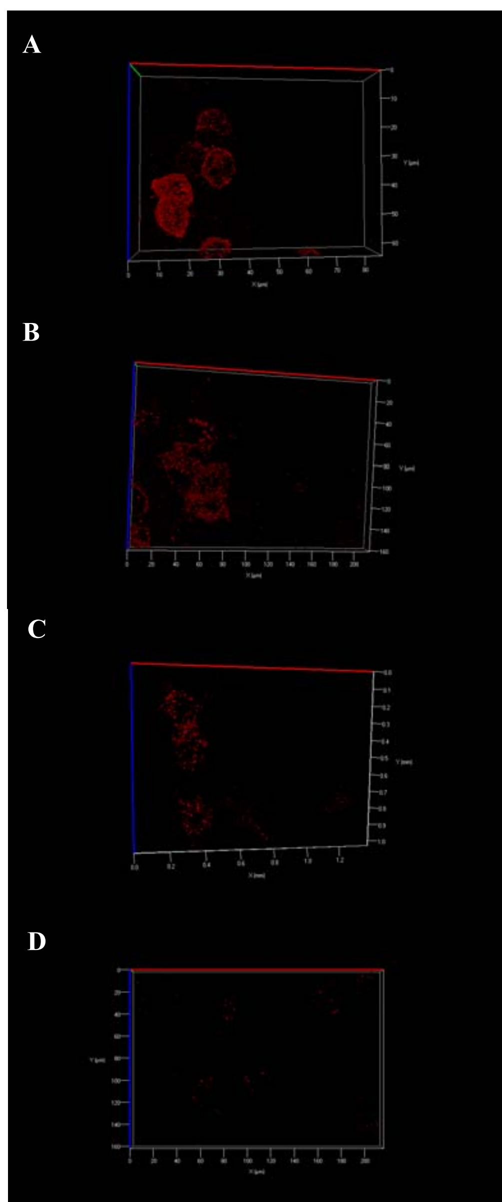

Figure 2: PC- 12 cells labeled with anti-TrkA and SA-QDs fixed (A) within 10 min showing a rich distribution of TrkA receptors on the membrane (B) 30 mins depicting movement into the cytosol (C) 50 min showing punctate fluorescence around the nucleus (D) 24 hr showing sparser distribution of punctate clusters.

3.3 Evidence for receptor clustering in intracellular compartments

Time analysis of punctate QD fluorescence revealed the characteristic blinking property representative of single QDs [8]. We found blinking of QDs bound to the membrane of cells suggesting that single QDs were detected and might have attached to individual TrkA receptors. At t=30 min, most punctuate clusters within the cells did not exhibit the characteristic blinking observed from QDs bound to the membrane. Comparison of the time profiles and size of the intracellular clusters with those that are membrane bound indicate that they may be QD aggregates bound to more than one TrkA receptor. This suggests the possibility of clustering of individual receptors once the receptor-QD complexes get endocytosed into the cell.

4 DISCUSSION

Current interest in understanding the action of NGF and its signaling pathway has created a need for high resolution techniques to detect NGF receptors and track their response to the ligand. A number of studies using the PC-12 model system have been conducted to understand the distribution of TrkA receptors and their response to neurotrophic factors. *Grimes et al.* have used antibody-based immunolabeling techniques to locate TrkA receptors on the surface of PC-12 cells and their redistribution on treatment with NGF [9]. They showed that NGF induced rapid endocytosis of TrkA and the ligand-receptor complex was localized in vesicles near the plasma membrane, some of which were found to contain clathrin and α-adaptin. NGF was bound to TrkA in these vesicles and the TrkA receptors were activated as determined by tyrosine phosphorylation and association with PLCγ-1. This gives rise to the possibility that NGF is transported retrogradely via signaling endosomes that contain activated TrkA. Moreover, *Howe et al.* found that NGF treatment induces the formation of clathrin coated vesicles and activates proteins of the Ras-MAPK pathway, which further substantiates the “signaling endosomes” theory [10].

Our results indicate that QDs conjugated to antibodies can be used as effective high resolution probes to label and track individual and groups of receptors in live cells. Antibody-QD probes bind with high specificity to TrkA receptors and these receptors, once bound to QDs, are capable of being transported from extracellular to perinuclear sites in PC12 neural cells. Spatial and temporal analysis of imaged TrkA receptors suggested that individual and groups of TrkA receptors can be resolved., demonstrating the high-resolution capability of these probes in live cells. These results hold promise that studies to characterize dynamic tracking of receptors bound to NGF ligand-QDs will be useful to evaluate receptor functionality in intact membranes. In addition, antibody and ligand conjugated QDs, once bound to receptors would be useful in studies involving treatment of cells with chemical modulators and provide further insight into neurotrophin signaling mechanisms.

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