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Probing the interior of synaptic vesicles with internalized nanoparticles

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Abstract

Synaptic vesicles are subcellular organelles that are found in the synaptic bouton and are responsible for the propagation of signals between neurons. Synaptic vesicles undergo endo- and exocytosis with the neuronal membrane to load and release neurotransmitters. Here we discuss how we utilize this property to load nanoparticles as a means of probing the interior of synaptic vesicles. To probe the intravesicular region of synaptic vesicles, we have developed a highly sensitive pH-sensing polymer dot. We feel the robust nature of the pH-sensing polymer dot will provide insight into the dynamics of proton loading into synaptic vesicles.

Keywords

synaptic vesicles; synaptosome; polymer dots; Förster resonance energy transfer (FRET); pH sensor; single molecule

1. INTRODUCTION

Synaptic vesicles are subcellular organelles within synapses that load, store, and release the neurotransmitters that are essential to the communication between neurons. Studies have shown links between intravesicular concentrations of small molecules, such as protons¹ and chloride², and the uptake of neurotransmitters into synaptic vesicles. Current methods for probing the intravesicular region include the use of dyes that can cross the synaptic vesicle membrane, such as acridine orange³ and Oregon green,⁴ but the non-specificity of the dyes and self-quenching at high dye concentrations⁵ make them unreliable for real-time measurements within the synaptic-vesicle lumen. Similarly, genetic transfections of fluorescent proteins, such as pH sensitive green-fluorescent protein, have been used as probes within synaptic vesicles,⁶ but genetic modifications potentially alter composition and function of synaptic vesicles. We have sought to develop a technique in which nanoparticles incorporating fluorescent based sensors are loaded into synaptic vesicles for real-time monitoring of the intravesicular concentration of important molecules with minimal interference to synaptic-vesicle function.

Semiconducting polymer dots (Pdots) have emerged as an ideal platform for nanoparticle fluorescent-based sensors for a variety of reasons: (1) Pdots have a broad size range (tens of

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nanometers to hundreds of nanometers) providing the ability to tune the size of the nanoparticle for various applications.^{7, 8} (2) The surface of a Pdot is easily functionalized to improve biocompatibility and to allow further bioconjugation.^{9–11} (3) Finally, Pdots show improved brightness and high photostability compared to other nanoparticles.^{12, 13} By conjugating a pH-sensitive dye with a pH-insensitive Pdot, we were able to develop a sensitive and robust pH sensing probe that can be used to measure the pH in a range of biological applications.¹⁴

We are particularly interested in using the pH sensing Pdots to monitor the fluctuation of pH within the lumen region of synaptic vesicles at a single-vesicle level. Though Pdots are small enough to fit inside a synaptic vesicle, they are too large to diffuse through the membrane or be transported into the vesicle and a technique in which synaptic vesicles uptake nanoparticles is needed. To address this, we explored loading nanoparticles into synaptic vesicles using synaptosomes, which are isolated nerve terminals that allow synaptic vesicles to still undergo endo- and exocytosis. Utilizing synaptosomes, we were able to stimulate synaptic vesicles to uptake nanoparticles through endocytosis and then further isolate the synaptic vesicles for single-vesicle studies.¹⁵ This loading capability in conjunction with our pH- sensing Pdots will enable us to develop a better understanding of the transporters that play an essential role in the loading of neurotransmitters into synaptic vesicles.

2. pH SENSING SEMICONDUCTING POLYMER DOTS

2.1 Materials

The following materials were purchased from Sigma-Aldrich: poly-(2,5-di(3',7'-dimethyloctyl)phenylene-1,4-ethynylene (PPE) and tetrahydrofuran (THF; anhydrous, 99.9%, inhibitor free). Amino-terminated polystyrene (PSNH₂) was purchased from ADS Dyes, Inc. (Quebec, Canada) and all other chemicals were purchased from Invitrogen (Carlsbad, CA). All chemicals were used as received without further purification.

2.2 Generation of pH sensing Pdot

To prepare Pdot pH-sensors, initially, 13mg of NHS-fluorescein and 20mg of PS-NH₂ was dissolved in 4ml of anhydrous dimethylformamide. The solution was stirred and allowed to react overnight. Then 100 μ l of PPE at 1mg/ml in THF, 15 μ l of polystyrene polyethylene glycol carboxylic acid (PS-PEG-COOH) at 2mg/ml in THF, and 40 μ l of the PS-NH₂-Fluorescein conjugate were added to 5 ml of THF. The Pdots were formed by quickly injecting this solution into 10ml of ultrapure water under vigorous sonication. Nitrogen was bubbled through the solution of Pdots while on a hotplate set at 125°C for 30 min to purge the THF from the solution. The resulting Pdots were filtered through a 0.2 μ m cellulose acetate membrane filter to remove aggregates that may have formed during the preparation. The Pdot solution was further concentrated by adding 80 μ l of Triton X-100 at 2.5 wt% in ultrapure water and centrifuging in a centrifugal ultrafiltration tube (Amicon Ultra-4, MWCO: 100kDa) at 2,750 $\times$ g for 3 min.

2.3 Characterization of pH sensitivity

When developing the pH-sensing Pdot, it was important to maximize their sensitivity. Previously, fluorescent pH-sensitive dyes were covalently linked to quantum dots (Qdots) in which Förster resonance energy transfer (FRET) occurred to monitor the pH.^{16, 17} In this instance, the ratio between the emission intensities from the Qdot and the fluorescent dye was used to measure the pH. Ratiometric measurements are less susceptible to environmental effects providing an advantage over measuring pH based on the fluorescence from the dye alone. To take advantage of this property, PPE was chosen as the semiconducting polymer core due to the overlap of the emission of PPE and the excitation of the pH sensitive dye, fluorescein. In addition, PPE is insensitive to changes in pH serving as an internal standard when measuring the pH of the surrounding environment. Figure 2A shows how the emission of fluorescein ($\lambda = 513\text{nm}$) increases in response to an increase in pH while the emission of PPE ($\lambda = 440\text{nm}$) remains relatively unchanged.

The pH-sensing Pdots were further characterized by examining the sensitivity and robustness of the pH measurements with fluorescence spectroscopy. The correlation between pH and the emission ratio ($I_{513\text{nm}}/I_{440\text{nm}}$) was found to be linear in the range of pH = 5 to 7.5 (Figure 2B). A 0.27 per unit change of pH was found over this range. The Pdot pH sensors demonstrated excellent reversibility and reproducibility when measuring the pH of a solution that was continually toggled between 5 and 7.5 (Figure 2C).

To demonstrate the capabilities of the Pdot pH sensors in a biological sample, the Pdots were loaded into live HeLa cells to measure the intracellular pH. These live cell experiments were performed with Pdots generated as described above but with no PS-PEG-COOH. The HeLa cells were incubated in the presence of the Pdot pH sensors and the Pdots were taken up via endocytosis. After rinsing the cells with fresh phosphate buffered saline to remove excess Pdots, confocal images of the HeLa cells were acquired (Figure 3). The intracellular pH was measured by calculating the ratio of the average intensity of the fluorescein emission (507 to 518nm) to the average intensity of the PPE emission (433 to 444nm). The intracellular pH was found to be 4.8 ± 0.9 which is in agreement with previously reported pH ranges.¹⁴

3. SYNAPTOSOMES AS A LOADING PLATFORM FOR NANOPARTICLES INTO SYNAPTIC VESICLES

3.1 Materials

Mice were wild type strain C57black6 courtesy of Sandra Bajjalieh. The mice were used in accordance to University of Washington animal practices. Q605 quantum dots and FM4-64 dye were obtained from Invitrogen. All other chemicals were purchased from Sigma-Aldrich.

3.2 Loading nanoparticles into synaptic vesicles

3.2.1 Synaptosome isolation—Freshly isolated mouse brains were placed in a chilled Teflon-glass tissue grinder and homogenized in ice cold homogenization buffer (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300mM sucrose, pH 7.4) with 10 strokes. The homogenate was centrifuged at $1,000\times g$ for 10 min at 4°C to remove cellular

debris. The resulting supernatant was centrifuged again at $24,500\times g$ for 13 min at 4°C resulting in a crude mitochondrial pellet containing the synaptosomes and additional cellular organelles. The synaptosomes were further purified by re-suspending the crude mitochondrial pellet in ice cold homogenization buffer and placing on a Percoll gradient consisting of a layer of 23%, 10%, and 3% percoll in sucrose/EDTA buffer (5mM HEPES, 300mM sucrose, 1mM EDTA, pH 7.4). The gradients were centrifuged at $23,000\times g$ for 20 min at 4°C with slow acceleration and no braking during deceleration. Isolated synaptosomes were collected from the interface between the 10% and 23% percoll layers. To remove excess percoll from the synaptosomes, isolated synaptosomes were diluted into Krebs-like buffer (118.5 mM sodium chloride, 4.7mM potassium chloride, 1.2mM calcium chloride, 1.18mM magnesium chloride, 10mM glucose, 20mM HEPES, 0.1mM disodium hydrogen phosphate, pH 7.4) and pelleted by centrifuging at $18,970\times g$ for 10 min at 4°C . The final synaptosome pellet was re-suspended and the final protein concentration was adjusted to 0.3mg/ml using Krebs-like buffer.

3.2.2 Nanoparticle loading—Isolated synaptosomes were incubated in the presence of 200nM Qdot and 20 μM of FM4–64 for 10 min while in a 37°C water bath. The synaptosomes were stimulated for 2 min with 30mM K^{+} Krebs-like buffer (63.2mM sodium chloride, 60mM potassium chloride, 1.2mM calcium chloride, 1.18mM magnesium chloride, 10mM glucose, 20mM HEPES, 0.1mM disodium hydrogen phosphate, pH 7.4). The stimulation induced the endo- and exocytosis of the synaptic vesicles causing the vesicles to take up any Qdots and FM4–64 located at the surface of the synaptosome (Figure 4). After stimulation, the synaptosomes were centrifuged at $8,400\times g$ for 3 min at 4°C and washed twice, once with Krebs-like buffer containing 1mg/ml bovine serum albumin (BSA) and once with Krebs-like buffer without BSA, to remove excess Qdots and FM4–64. Then synaptosomes were re-suspended in ice-cold homogenization buffer.

3.2.3 Synaptic vesicle isolation—Synaptic vesicles were isolated from the synaptosomes by adding nine volumes of cold ultrapure water to the re-suspended synaptosomes and then homogenized with 3 strokes in a chilled Teflon-glass tissue grinder. The synaptosomes were left on ice for 30 min to allow for full rupture. Cellular debris was removed by centrifuging at $22,460\times g$ for 9 min at 4°C . The supernatant was removed and centrifuged one final time at $217,410\times g$ for 1.5 hours at 4°C to pellet the synaptic vesicles. Finally, the pellet of synaptic vesicles was re-suspended into potassium acetate buffer (20mM HEPES, 110mM potassium acetate, 4mM magnesium sulfate, 4mM potassium chloride, pH 7.4).

3.3 Verifying loading of nanoparticles into synaptic vesicles

Previous studies have shown that Qdots can be taken up by synaptic boutons in neuronal cultures.¹⁸ Quantum dots are well-characterized nanoparticles that can be easily acquired in a size ($\sim 15\text{nm}$) that is small enough to fit within a synaptic vesicle ($\sim 50\text{nm}$). Also, Qdots can be obtained with a carboxyl functionalized surface to improve the non-specific interaction of the Qdot to neuronal membranes. Therefore, Qdots were the ideal model fluorescent sensor to use when developing the technique for loading nanoparticles into synaptic vesicles via synaptosomes. A membrane selective dye, FM4–64, was simultaneously loaded into the

synaptic vesicles as an additional label to verify the loading of Qdots. Total internal reflection fluorescence (TIRF) microscopy was used to image the synaptic vesicles (Figure 5). Overlaid images of Qdots and FM4-64 showed that $25.8 \pm 11.1\%$ of vesicles appeared to contain a nanoparticle.¹⁵

Due to the preference of Qdots to neuronal membranes, it is possible that excess Qdots may not have been removed during the washing steps in the procedure. A black hole quencher (BHQ) was utilized to distinguish Qdots encapsulated in synaptic vesicles from those that were bound to the exterior of the vesicle (Figure 5). Images of vesicles after quenching with BHQ showed that $15.6 \pm 5.1\%$ of synaptic vesicles actually had encapsulated Qdots.¹⁵

4. CONSIDERATIONS WHEN LOADING POLYMER DOTS INTO SYNAPTIC VESICLES

There were two main advantages to using Qdots as a model nanoparticle when developing the synaptic vesicle loading technique: (1) The Qdots used were small in size with a narrow size distribution (Figure 7A) which was necessary to fit within the intravesicular region of the synaptic vesicle and (2) the negatively charged surface of carboxyl functionalized Qdots improved the non-specific interaction with the synaptosome surface.¹⁸ When developing alternative options for fluorescence based nanoparticle sensors, size and surface functionalization are important properties to control. As discussed previously, Pdots are ideal fluorescent based sensors due to their sensitivity and robustness. In addition, the size and surface functional groups can be easily controlled to optimize the Pdot for use in a wide range of studies. Figure 7 shows how Pdots composed of poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-[2,1',3]-tioadiazole)] (PFBT) can be generated in various sizes through minor alterations to the procedure. In this case, PFBT Pdots with an average diameter of 16nm were generated by slowly injecting (3 min/ml) a PFBT/THF solution into ultrapure water during sonication using a probe sonicator with a high local sonicating power and an average diameter of 70nm was achieved by quickly injecting (<1 min) the PFBT/THF solution into ultrapure water under sonication with a typical bath sonicator. Using these methods, the size of Pdots can be optimized for each specific application.

Similarly, functionalization of Pdots is achieved with minimal variation in the method for generating Pdots. The surface of Pdots can be functionalized by including a small amount of amphiphilic polymer containing the functional group of interest with the semiconducting polymer during the formation step. Functionalization of Pdots can be verified through measurement of the ζ -potential. For example, non-functionalized poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-(1-cyanovinylene-1,4-phenylene)] (CN-PPV) Pdots were found to have a ζ -potential of -35mV while CN-PPV Pdots generated with 20% poly(styrene-co-maleic anhydride) (PSMA) were found to have a ζ -potential of -55mV . By applying techniques for controlling the size and modifying the surface functional groups, pH-sensitive Pdots can be optimized for loading into synaptic vesicles.

5. CONCLUSION

Synaptic vesicles play an important role in neuronal communication. Therefore, development of techniques and new approaches for studying these subcellular organelles is ongoing. We have successfully used synaptosomes to load nanoparticles into synaptic vesicles and then isolate the vesicles for further single-vesicle studies. By combining this loading technique with the pH-sensitive Pdots, we anticipate the ability to probe the intravesicular region of the synaptic vesicle while avoiding the drawbacks of other similar techniques. The pH sensing Pdots offer a sensitivity that will contribute to development of a better understanding of the uptake of protons into synaptic vesicles and eventually how this relates to the overall function of this organelle.

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REFERENCES

- [1]. Budzinski KL, Allen RW, Fujimoto BS, Kensel-Hammes P, Belnap DM, Bajjalieh SM and Chiu DT, "Large structural change in isolated synaptic vesicles upon loading with neurotransmitter," *Biophys. J.*, 97, 2577–2584 (2009). [PubMed: 19883601]
- [2]. Schenck S, Wojcik SM, Brose N and Takamori S, "A chloride conductance in VGLUT1 underlies maximal glutamate loading into synaptic vesicles," *Nat. Neurosci.*, 12(2), 156–162 (2009). [PubMed: 19169251]
- [3]. Zoccarato F, Cavallini L and Alexandre A, "The pH-sensitive dye acridine orange as a tool to monitor exocytosis/endocytosis in synaptosomes," *J. Neurochem.*, 72(2), 625–633 (1999). [PubMed: 9930734]
- [4]. Zeigler MB, Allen PB and Chiu DT, "Probing rotational viscosity in synaptic vesicles," *Biophys. J.*, 100, 2846–2851 (2011). [PubMed: 21641331]
- [5]. Palmgren MG, "Acridine orange as a probe for measuring pH gradients across membranes: mechanism and limitations," *Anal. Biochem.*, 192, 316–321 (1991). [PubMed: 1827963]
- [6]. Budzinski KL, Zeigler M, Fujimoto BS, Bajjalieh SM and Chiu DT, "Measurements of the acidification kinetics of single synaptophysin vesicles," *Biophys. J.*, 101, 1580–1589 (2011). [PubMed: 21961583]
- [7]. Ye F, Wu C, Jin Y, Chan Y-H, Zhang X and Chiu DT, "Ratiometric temperature sensing with semiconducting polymer dots," *J. Am. Chem. Soc.*, 133, 8146–8149 (2011). [PubMed: 21548583]
- [8]. Ye F, Wu C, Jin Y, Wang M, Chan Y-H, Yu J, Sun W, Hayden S and Chiu DT, "A compact and highly fluorescent orange-emitting polymer dot for specific subcellular imaging," *Chem. Commun.*, (In press).
- [9]. Wu C, Schneider T, Zeigler M, Yu J, Schiro PG, Burnham DR, McNeill JD and Chiu DT, "Bioconjugation of ultrabright semiconducting polymer dots for specific cellular targeting," *J. Am. Chem. Soc.*, 132, 15410–15417 (2010). [PubMed: 20929226]
- [10]. Chan Y-H, Jin Y, Wu C and Chiu DT, "Copper(II) and iron(II) ion sensing with semiconducting polymer dots," *Chem. Commun.*, 47, 2820–2822 (2011).
- [11]. Wu C, Hansen SJ, Hou Q, Yu J, Zeigler M, Jin Y, Burnham DR, McNeill JD, Olson JM and Chiu DT, "Design of highly emissive polymer dot bioconjugates for in vivo tumor targeting," *Angew. chem. Int. Ed.*, 50(15), 3430–3434 (2011).
- [12]. Jin Y, Ye F, Zeigler M, Wu C and Chiu DT, "Near-infrared fluorescent dye-doped semiconducting polymer dots," *ACS Nano*, 5(2), 1468–1475 (2011). [PubMed: 21280613]
- [13]. Wu C, Jin Y, Schneider T, Burnham DR, Smith PB and Chiu DT, "Ultrabright and bioorthogonal labeling of cellular targets using semiconducting polymer dots and click chemistry," *Angew. Chem. Int. Ed.*, 49(49), 9436–9440 (2010).

- [14]. Chan Y-H, Wu C, Ye F, Jin Y, Smith PB and Chiu DT, "Development of ultrabright semiconducting polymer dots for ratiometric pH sensing," *Anal. Chem.*, 83, 1448–1455 (2011). [PubMed: 21244093]
- [15]. Budzinski KL, Sgro AE, Fujimoto BS, Gadd JC, Shuart NG, Gonen T, Bajjalieh SM and Chiu DT, "Synaptosomes as a platform for loading nanoparticles into synaptic vesicles," *ACS Chem. Neurosci.*, 2, 236–241 (2011). [PubMed: 21666849]
- [16]. Jin T, Sasaki A, Kinjo M and Miyazaki J, "A quantum dot-based ratiometric pH sensor," *Chem. Commun.*, 46, 2408–2410 (2010).
- [17]. Suzuki M, Husimi Y, Komatsu H, Suzuki K and Douglas KT, "Quantum dot FRET biosensors that respond to pH, to proteolytic or nucleolytic cleavage, to DNA synthesis, or to a multiplexing combination," *J. Am. Chem. Soc.*, 130, 5720–5725 (2008). [PubMed: 18393422]
- [18]. Zhang Q, Cao Y-Q and Tsien RW, "Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles," *Proc. Natl. Acad. Sci. U.S.A.*, 104(45), 17843–17848 (2007).

Figure 1.
Schematic showing route for the preparation of pH-sensitive Pdot.

Figure 2.

(A) Fluorescence spectra of pH-sensing Pdot at different pH (red line: pH = 7.5, blue line: pH = 7, green line: pH = 6.5, black line: pH = 6, brown line: pH = 5.5, and pink line: pH = 5). 390nm excitation wavelength. (B) Ratiometric pH calibration plot of the emission ratio (I_{513nm}/I_{440nm}). (C) Emission ratio (I_{513nm}/I_{440nm}) when pH is toggled between 5.0 and 7.5 to demonstrate the reversibility and reproducibility of pH-sensing Pdots.

Figure 3. Confocal microscopy images of pH-sensitive Pdts inside live HeLa cells at $\lambda_{\text{exc}} = 405\text{nm}$. (A) Emission from PPE (integrated from 433 to 444nm). (B) Emission of fluorescein (integrated from 507 to 518nm). (C) Overlay of the emissions from PPE and fluorescein. (D) Brightfield image of the HeLa cells. The insets show a magnified version of a single HeLa cell and the scale bar is $20\mu\text{m}$.

Figure 4.
Schematic of the uptake of FM4-64 and Qdots into synaptic vesicles via synaptosomes.

Figure 5.

Images of isolated synaptic vesicles loaded with Qdot and FM4–64. (A) Qdots in synaptic vesicles. (B) Synaptic vesicles labeled with FM4–64. (C) Overlay showing successful loading of Qdot into synaptic vesicles.

Figure 6. Overlay images of synaptic vesicles loaded with Qdot (green) and FM4–64 (red). (A) Synaptic vesicles before treatment with BHQ-2. (B) Synaptic vesicle after treatment with BHQ-2.

Figure 7. Dynamic light scattering measurements of the size distributions of (A) Q605 Qdots, (B) PFBT polymer dots generated with a slow injection and high powered sonication technique, and (C) PFBT polymer dots generated with a fast injection and low powered sonication technique.