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A Clickable Spherical Nucleic Acid Probe for Fluorescence and Synchrotron Radiation X-ray Dual-Modality Imaging

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Abstract

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Full Text

A Clickable Spherical Nucleic Acid Probe for Fluorescence and Synchrotron Radiation X-ray Dual-Modality Imaging

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Abstract

Synchrotron radiation imaging technology offers nanoscale spatial resolution, but there is a scarcity of neuron-specific probes developed using this technology. In this study, we have developed a dual-modal probe based on click chemistry spherical nucleic acids (SNAs), achieving highly specific fluorescence imaging and synchrotron X-ray microscopy of neuronal cells. Monodisperse gold nanoparticles (AuNPs) with a diameter of 5 nm were used as the core, and their surfaces were covalently functionalized with both alkynylated DNA strands and Cy5 fluorescent groups. This design enables the particles to undergo click chemistry reactions with azide groups expressed by cells, thereby facilitating the labeling of neuronal cells. We successfully labeled PC12 neuroendocrine cells and primary neuronal cells with the probe and performed dual-modal imaging using fluorescence and synchrotron X-ray microscopy. This study combines the spatiotemporal controllability of click chemistry with the synergistic effect of spherical nucleic acid gold cores, providing a novel molecular tool for the study of brain neuron-specific marker imaging and a new approach for the design of synchrotron X-ray specific probes.

Keywords

Synchrotron Radiation, Dual-Modality Imaging, Spherical Nucleic Acids, Click Chemistry

1. Introduction

The primary challenge in current bioimaging technologies is achieving molecular specificity alongside high spatial resolution using a single imaging modality [1,2]. While fluorescence microscopy can achieve single-molecule detection sensitivity through targeted molecular labeling, it is limited by poor tissue penetration

(<200 m) and vulnerability to photobleaching [3-5]. In contrast, Synchrotron Radiation Scanning Transmission X-ray Microscopy (SR-STXM) offers high penetration depth, nanoscale spatial resolution, and element specificity, enabling analysis of tissue and cellular ultrastructure while providing the sensitivity and spatial resolution required at the subcellular level [6-8]. However, SR-STXM lacks specific molecular labeling methods. Combining these two technologies to design multimodal probes—particularly by leveraging the functionalized interface of spherical nucleic acids (SNAs) and the physical properties of heavy metal nanomaterials—represents a promising approach to address this bottleneck.

Spherical nucleic acids (SNAs) are spherical structures in which nucleic acid molecules are conjugated to the surface of a nanoparticle core. This architecture endows SNA probes with unique properties, such as high stability, programmability, and multifunctionality [9-12]. As a novel nucleic acid delivery system, SNAs have been utilized for immune regulation, gene regulation, drug delivery, biosensing, and bioimaging [13-15]. By meticulously designing nucleic acid sequences, SNAs can accurately label specific organelles or biomarkers through targeted molecules such as aptamers or antibodies [16,17]. Numerous studies have investigated the self-stability [18], contractile properties [19], intracellular uptake [20], extracellular uptake, intracellular distribution [21], and tumor-targeted delivery [22] of SNAs with gold nanoparticle cores (Au-SNAs). However, these studies predominantly focus on the properties and functions of the nucleic acid shells. Leveraging the high atomic number (Z=79) of the gold core, Au-SNAs generate significant contrast in synchrotron X-ray microscopy [23], making them suitable for high-contrast synchrotron X-ray imaging applications.

Click chemistry comprises highly efficient and specific chemical reactions, including copper-catalyzed azide-alkyne cycloaddition (CuAAC), strain-promoted azide-alkyne cycloaddition (SPAAC), photo-induced thiol-ene reactions, and inverse electron-demand Diels-Alder (IEDDA) reactions [24,25]. These reactions are characterized by mild conditions, high yields, and high selectivity, and have been widely applied in bioconjugation and molecular labeling [26,27]. By integrating click reactions with metabolic engineering, imaging probes can achieve highly specific and sensitive molecular labeling of cells [28]. Additionally, modifying alkynylated DNA on SNA surfaces facilitates specific covalent binding between probes and biological targets through click reactions. This approach significantly reduces nonspecific adsorption while enabling spatial and temporal control in complex biological systems.

Inspired by click chemistry, we developed click spherical nucleic acid probes (Click-SNAs) with gold nanoparticle cores. This design facilitates effective control over particle size and provides strong signals for X-ray imaging. The DNA sequence was modified with alkyne, fluorescent, and thiol groups to ensure targeting capability and fluorescence imaging signals. These probes enabled dual-mode fluorescence and STXM imaging of PC12 cells and primary neuronal cells, validating the probe's practicality.

2.1 Instruments and Materials

5 nm AuNPs (SKU EM.GC5/7) were purchased from BBI Solutions. DNA strands (Alkyne-TTTTTTTT-iCy5-TTTTTTTTTT-SH) were acquired from Autobio Engineering (Shanghai) Co., Ltd. The 5× TBE buffer was sourced from China National Pharmaceutical Group Chemical Reagent Co., Ltd., and 1× PBS (B310KJ) was obtained from Shanghai Yuanpei Biotechnology Co., Ltd.

NaCl (ST1840-500 g), BSA (ST023-50 g), penicillin-streptomycin solution (C0222), and poly-L-lysine (ST509) were purchased from Biyun Tian. Click-iT™ AHA (C10102), EZ-Link™ Biotin-PEG12-DBCO (C20042), Alexa Fluor™ 488 streptavidin conjugate (S32354), RPMI 1640 medium (11875119), fetal bovine serum (FBS, A5670801), CTS™ Neurobasal™ culture medium (A1371201), B-27™ supplements (A3695201), basic Eagle culture medium (BME, 21010046), Hanks' balanced salt solution (HBSS, 14175145), sodium pyruvate (11360070), trypsin EDTA (25200114), and Thermo Scientific DNase I (EN0521) were bought from Thermo Fisher Scientific. N-Azidoacetylmannosamine tetraacylated ($Ac_4ManNAz$, 361154-30-5) was obtained from Delta Biotech, and uranyl acetate was sourced from Henan Ruixin Experimental Supplies Co., Ltd. Carbon support film copper grids were ordered online from Zhongjing Scientific Instrument, and X-ray application silicon nitride films were purchased from Mingna (Shanghai) Information Technology Development Co., Ltd.

Transmission electron microscopy (TEM) characterization was conducted using a JEM-F200 field emission electron microscope (JEOL) operating at an acceleration voltage of 200 kV. Dynamic light scattering (DLS) particle size analysis was performed using a DynaPro NanoStar system (Wyatt). Cell fluorescence imaging was carried out using a Leica TCS SP8 confocal microscope (63× oil immersion objective, NA=1.4), with excitation/emission wavelengths of 488/519 nm for Alexa Fluor 488 and 633/670 nm for Cy5. Synchrotron Radiation Scanning Transmission X-ray Microscopy (SR-STXM) was performed at the BL08U1A Soft X-ray Spectroscopy Fiber Line Station of the Shanghai Synchrotron Radiation Facility, using a photon energy of 520 eV. Additionally, synchrotron radiation absorption edge imaging was conducted at the BL07W Soft X-ray Imaging Beamline Station of the Hefei Light Source, also with a photon energy of 520 eV.

2.2 Preparation of Click-SNAs Probes

We designed a DNA sequence carrying fluorescent (Cy5), alkyne, and thiol groups. Click-SNAs probes were then synthesized using the salt aging method [29]. Briefly, 300 l of 83 nmol/L AuNPs (5 nm) were mixed with 30 l of 100 mol/L thiol-modified DNA and 30 l of 5× TBE buffer. After shaking at room temperature for four hours, 10.5 l of 3 mol/L NaCl solution was added and mixed thoroughly. The mixture was allowed to stand for 30 minutes, and this

process was repeated four times to achieve a final NaCl concentration of 0.3 mol/L. Following overnight shaking, the solution was centrifuged (14,000 rpm, 30 min) to remove the supernatant, and the pellet was resuspended in 0.5× TBE solution. This washing process was repeated three times. Finally, the Click-SNAs probes were resuspended in 0.5× TBE buffer and stored at 4°C for further use.

2.3 TEM Characterization

For TEM characterization, 10 l of probe solution was placed on a deionized carbon support membrane on a copper grid and allowed to adsorb for 10 minutes before removing excess liquid with filter paper. The membrane was rinsed twice with distilled water. Subsequently, 10 l of 1% uranyl acetate was added dropwise for negative staining and left for 1 minute. After removing the remaining liquid with filter paper, the membrane was washed three times with distilled water and dried completely before TEM imaging at 200 kV.

2.4 DLS Measurement

The size distribution of AuNPs and Click-SNAs probes was characterized using a DynaPro NanoStar DLS instrument (Wyatt) following standard operating procedures.

2.5 PC12 Cells Culture

Rat adrenal pheochromocytoma (PC12) cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 g/mL streptomycin at 37°C in a humidified 5% CO_2 incubator. PC12 cells were obtained from the cell bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences.

For experiments, silicon nitride (Si_3N_4) films were placed in a 24-well plate and sterilized by UV radiation. Logarithmic-phase cells were harvested and seeded at 1×10^5 cells/mL onto confocal dishes or 24-well plates containing Si_3N_4 membranes. Cells were incubated overnight to allow adhesion to the confocal dish or Si_3N_4 membrane.

2.6 Isolation and Culturing of Primary Neuronal Cells

Cortical neurons were isolated from embryonic day 18 Sprague-Dawley (SD) rats. Prior to plating, silicon nitride (Si_3N_4) films were placed in a 24-well plate and sterilized by UV radiation. The day before brain extraction, Si_3N_4 windows were treated with 0.5 mg/mL poly-L-lysine solution in confocal dishes or 24-well plates to enhance cell adhesion. The poly-L-lysine solution was removed before brain extraction, and fresh BME culture medium was added.

Pregnant SD rats at embryonic day 18 were euthanized by cervical dislocation, and the carcasses were disinfected by immersion in 75% ethanol. Fetal rat

brains were promptly removed, and cortical tissue blocks were separated and placed in HBSS solution. Meninges were gently removed by suction, repeated twice. The tissue was then resuspended in 4.5 mL HBSS medium, and 0.5 mL trypsin was added for enzymatic digestion in a 37°C water bath for 12-15 minutes. Subsequently, 0.5 mL DNase was added for digestion at room temperature for 2-5 minutes until the tissue was fully dissociated. The tissue was washed twice with HBSS solution and twice with fresh BME medium. Cells were then resuspended, counted, and cultured at 0.8×10^5 cells/mL in confocal dishes or at 1.2×10^5 cells/well in 24-well plates containing Si_3N_4 membranes. Four hours after seeding, once cells had adhered, the BME medium was replaced with Neurobasal medium supplemented with B-27, and the medium was changed every 3 days. Subsequent experiments were performed after 14 days in culture.

2.7 Cell Azide Modification

For PC12 cells, once they reached normal growth status, the culture medium was removed and cells were incubated with 500 M AHA in RPMI 1640 medium for 2 hours per dish or well. For primary neuronal cells, after 14 days in culture, the original medium was removed and cells were incubated with 250 M Ac_4ManNAz in Neurobasal medium for 12 hours. This procedure modified azide groups on the cell membranes of both PC12 and primary neuronal cells. Cells were then washed with 1× PBS for 2-5 minutes before fluorescence or probe labeling.

2.8 Fluorescence Labeling of Cells

The strain-promoted azide-alkyne cycloaddition (SPAAC) reaction was used to couple DBCO-biotin with surface azide groups, followed by fluorescent labeling via streptavidin-biotin binding. Azide-modified cells were washed with 1× PBS for 2-5 minutes, then incubated with 50 M DBCO-biotin in the appropriate culture medium at 37°C for 30 minutes. After replacing the medium and incubating for an additional 15 minutes, cells were washed with 1× PBS for 2-5 minutes and fixed with 4% paraformaldehyde for 15 minutes. The fixative was removed, and cells were washed three times with 1× PBS for 5 minutes each. Cells were then treated with 0.1% (w/v) sodium borohydride for 7 minutes, followed by three washes with 100 mM glycine (in 1× PBS) and three final washes with 1× PBS for 5 minutes each.

For fluorescent labeling, 5 g/mL Alexa Fluor 488-conjugated streptavidin (in 1% w/v BSA) was added and incubated for 1 hour. Cells were washed three times with 1× PBS for 5 minutes each, with unbound fluorescent label retained for subsequent imaging.

2.9 Labeling of Cells with Probes

The copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction was used to label cells with biocompatible, nanoscale gold spherical nucleic acid probes

(Click-SNAs). After azide modification, cells were washed with 1× PBS for 2-5 minutes and fixed with 4% paraformaldehyde for 15 minutes. The fixative was removed, and cells were washed three times with 1× PBS for 5 minutes each. A labeling mixture containing 20-50 nM Click-SNAs probes, BTTAA-$CuSO_4$ complex (50 M $CuSO_4$, BTTAA/$CuSO_4$ molar ratio 6:1), and 2.5 mM sodium ascorbate was prepared in 1× PBS and incubated on the cell surface for 1.5 hours to label the Click-SNAs probes onto the cell membrane via click chemistry. Finally, cells were washed three times with 1× PBS for 5 minutes each, with unbound probes retained for imaging.

2.10 Cell Fluorescence Imaging

After washing probe-labeled cells with 1× PBS, fluorescence imaging was performed using confocal fluorescence microscopy (Leica SP8). Imaging was conducted using AF488 and Cy5 channels as appropriate.

2.11 Cell-Based Synchrotron Radiation Scanning Transmission X-ray Microscopy and Absorption Edge Imaging

X-ray images were acquired through Scanning Transmission X-ray Microscopy (STXM) and absorption edge imaging. STXM measurements were performed at the BL08U1A Soft X-ray Spectroscopy Fiber Line Station of the Shanghai Synchrotron Radiation Facility (SSRF). Absorption edge imaging measurements were conducted at the BL07W Soft X-ray Imaging Beamline Station of the National Synchrotron Radiation Laboratory (NSRL), China. The STXM imaging system employs 520 eV photons to scan samples in a vacuum environment. Samples on transparent Si_3N_4 films are manipulated by a piezoelectric stage and scanned point-by-point by a focused beam, with transmitted X-ray intensity captured by a CCD detector to generate complete images. The absorption edge imaging system produces contrast images by measuring the X-ray absorption edge of the sample, also using 520 eV photons in a vacuum environment.

3.1 The Preparation and Working Principle of Click-SNAs Probes

We designed a DNA sequence (Alkyne-TTTTTTTT-iCy5-TTTTTTTTTT-SH) modified with click reaction functional groups (alkyne), a fluorescent group (Cy5), and a thiol group (SH). Click-SNAs probes were then synthesized using the salt aging method [29]. The NaCl concentration in solution plays a crucial role in the composition of Click-SNAs probes (Fig. 1A [Figure 1: see original paper]). Once successfully prepared, the probes were used for cell membrane labeling and imaging through click reactions (Fig. 1B).

L-Azidoalanine (AHA) is a non-canonical amino acid analog of methionine containing an azide group that can be randomly incorporated into nascent proteins during translation, resulting in full-length azide-labeled proteins [34]. N-Azidoacetylmannosamine tetraacylated (Ac_4ManNAz) is a metabolic glycopro-

tein labeling reagent containing an azide group. It can be incorporated into the cellular sialic acid biosynthesis pathway, promoting expression of azide-modified sialic acid in cells [35]. This study utilized these biosynthetic pathways to incorporate AHA or $Ac_4ManNAz$ into newly synthesized membrane proteins or sialic acid conjugates, achieving cell membrane azide modification. Probe labeling and detection were accomplished by connecting probes through click reactions.

3.2 Characterization of Click-SNAs Probes

We selected 5 nm AuNPs as the core for spherical nucleic acid probes and characterized the structure and size of Click-SNAs probes using TEM and dynamic light scattering (DLS). TEM imaging clearly revealed a distinct nucleic acid coupling structure on the periphery of Click-SNAs probes (Fig. 1B [Figure 1: see original paper]) compared to bare AuNPs (Fig. 1A [Figure 1: see original paper]). Statistical analysis of particle sizes from TEM images yielded average diameters of approximately 5.57 nm for AuNPs and 6.25 nm for Click-SNAs probes (Figure 1C [Figure 1: see original paper]). Furthermore, DLS measurements showed a hydrodynamic size of approximately 10 nm for Click-SNAs probes, larger than that of bare AuNPs (~7 nm) (Fig. 1D [Figure 1: see original paper]). These results demonstrate that the synthesized Click-SNAs probes have uniform particle size without aggregation, indicating successful synthesis.

3.3 Click-SNAs Probes for Labeling and Imaging of Cells

We first performed fluorescence and probe labeling of PC12 cells and primary neuronal cells (NC) for fluorescence imaging (Fig. 3 [Figure 3: see original paper]). After incorporating azide groups into cell membrane components, we utilized the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction to couple biotin, followed by fluorescent labeling of the cell membrane using Alexa Fluor™ 488 streptavidin conjugate (SA-AF488) through streptavidin-biotin binding (Fig. 3A, B [Figure 3: see original paper]). This approach enabled clear visualization of the basic morphology of PC12 cells and the fine fibrous structures of primary neurons. The results indicated that AHA and $Ac_4ManNAz$ were evenly distributed on the cell membrane surface, demonstrating excellent membrane azide modification.

Subsequently, we used Click-SNAs probes to label and image cells via click reactions (Fig. 3C, D [Figure 3: see original paper]). The imaging results allowed observation of the basic morphology of both cell types. However, compared to fluorescence imaging achieved through biotin-streptavidin coupling, the fluorescence signal intensity after probe labeling was lower. This discrepancy may be attributed to the larger size and lower concentration of the probes, resulting in fewer probes binding to the cell membrane and suboptimal imaging. In PC12 cells labeled with probes, the nucleus exhibited strong fluorescence signal (Fig. 3C [Figure 3: see original paper]), suggesting that some AHA-incorporated proteins are components of the nuclear membrane in PC12 cells, leading to substan-

tial nuclear labeling. In contrast, Ac_4ManNAz only participates in sialic acid synthesis on neuronal cell membranes, and Click-SNAs probes did not label the nucleus (Fig. 3D [Figure 3: see original paper]).

Finally, we utilized Click-SNAs probes to label two types of cells for synchrotron radiation scanning transmission X-ray microscopy (SR-STXM) imaging (Figure 4 [Figure 4: see original paper]). We used STXM to scan labeled cells with 520 eV photons, observing cell morphology through imaging contrast (Figure 4A [Figure 4: see original paper]). STXM imaging of PC12 cells showed that nuclei labeled through AHA metabolic incorporation also exhibited strong X-ray signals (Figure 4B [Figure 4: see original paper]), corroborating the fluorescence imaging results. We then performed neuronal absorption edge imaging at the NSRL BL07W soft X-ray Imaging Beamline Station and STXM imaging at the SSRF BL08U1A soft X-ray Spectroscopy Fiber Line Station (Figure 4C, D [Figure 4: see original paper]), enabling detailed visualization of neuronal cell fiber structures.

In this study, Click-SNAs probe-labeled cells exhibited specific fluorescence signals, and well-defined specialized structures of neuronal fibers were observed using synchrotron X-ray microscopy. This indicates that the probes can effectively label PC12 cells and neuronal cells, enabling dual-mode imaging through fluorescence and synchrotron radiation scanning transmission X-ray microscopy.

4. Conclusion

In conclusion, this study innovatively developed a multimodal molecular probe, Click-SNAs, based on the synergistic effect of click chemistry and spherical nucleic acids. It successfully achieved highly specific fluorescence and synchrotron X-ray dual-modality imaging of PC12 neural cells and primary neuronal cells. For the first time, this probe combines the gold core characteristics of spherical nucleic acids with efficient click chemistry coupling, significantly enhancing labeling specificity for synchrotron X-ray imaging while enabling fluorescence multimodal imaging. This research provides new insights for designing synchrotron X-ray imaging probes and opens new avenues for studying brain neuron-specific labeling imaging. Looking forward, the click chemistry-based labeling strategy is anticipated to find broader application in synchrotron radiation imaging. The modular design concept of Click-SNAs probes will likely provide robust technical support for three-dimensional analysis research at the mesoscale of the whole brain.

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