Expanding the Chemical Space of Biocompatible Fluorophores: Nanohoops in Cells

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Supporting Information

ABSTRACT: The design and optimization of fluorescent molecules has driven the ability to interrogate complex biological events in real time. Notably, most advances in bioimaging fluorophores are based on optimization of core structures that have been known for over a century. Recently, new synthetic methods have resulted in an explosion of nonplanar conjugated macrocyclic molecules with unique optical properties yet to be harnessed in a biological context. Herein we report the synthesis of the first aqueous-soluble carbon nanohoop (i.e., a macrocyclic slice of a carbon nanotube prepared via organic synthesis) and demonstrate its bioimaging capabilities in live cells. Moreover, we illustrate that these scaffolds can be easily modified by well-established “click” chemistry to enable targeted live cell imaging. This work establishes the nanohoops as an exciting new class of macrocyclic fluorophores poised for further development as novel bioimaging tools.

INTRODUCTION

Fluorescent molecules have fueled the now widespread use of optical imaging to observe biological processes in living systems.^{1–3} The power of such imaging methods has led to increased interest in identifying new types of dyes, optically active materials, and nanoparticles that have enhanced photophysical properties suitable for multimodal, multiplexed, and super-resolution imaging.^{4–14} Because fluorophores play such a critical role in understanding biological processes, it is somewhat surprising that most advances in small molecule dye technology today rely on structural modifications of scaffolds discovered over a century ago.¹⁵ For example, the robust Janelia Fluor and some AlexaFluor dyes are structurally modified versions of rhodamine scaffolds discovered 130 years ago. Similarly, commercially available CyDyes, which have found widespread use as probes for targeted live cell imaging, are based off the cyanine core structure synthesized first in 1924 (Figure 1).⁷ Clearly the modification of these core dye scaffolds is still yielding fruitful discoveries (e.g., Janelia Fluor 549);^{16,17} however, fundamentally new types of fluorophore scaffolds could offer advantageous photophysical properties for exploitation in biological contexts.^{18–21} Inspired by this prospect, we report here the first biological studies demonstrating carbon nanohoops, short macrocyclic slices of carbon nanotubes prepared by organic synthesis, as exciting new biocompatible fluorophore scaffolds (Figure 1).

The $[n]$ cycloparaphenylenes ($[n]$ CPPs, n = number of benzene rings) are the smallest macrocyclic slices of carbon nanotubes (CNTs). These structures, coined “carbon nanohoops” due to their structural relationship to carbon

nanotubes, were intensely pursued synthetic targets for over 70 years before finally succumbing to synthesis in 2008 (Figure 1).^{22,23} Since then, the development of synthetic methods to prepare nanohoops has unveiled several unique, size-dependent photophysical properties that are a direct result of the radially oriented π -system of this unusual architecture.^{24–27} First, the bending of the π -system increases delocalization around the hoop due to induction of a small amount of quinoidal character in these strained systems.²⁸ Second, the hoop architecture forces neighboring aromatic units to have smaller dihedral angles than in an acyclic oligomeric system due to conformational constraints of the macrocyclic geometry, again leading to increased conjugation.²⁹ These two factors together result in a size-dependent fluorescence emission (λ_{em}) where the HOMO $\rightarrow$ LUMO gap narrows as nanohoop diameter decreases.³⁰ Additionally, due to Laporte forbidden HOMO $\rightarrow$ LUMO transitions, all nanohoops share a common absorption maxima (λ_{abs} = 340 nm) with high absorption coefficients (ϵ) and large effective Stokes shifts ranging from 100 to 200 nm depending on size.^{31–34} Taken together, the nanohoop scaffold offers the possibility of multiplexed imaging using a single excitation source. Moreover, the nonplanarity of the benzene rings in the nanohoop also leads to better solubility when compared to planar aromatic systems. Lastly, despite molecular strain, nanohoops are only reactive under forcing reaction conditions.³⁵ The inherent attributes provided by the nanohoop structure highlight their potential as new

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Common Organic Dye Scaffolds:

Nanohoops:

Figure 1. Traditional organic dye scaffolds and the new nanohoop fluorophore scaffold.

Scheme 1. Synthesis of Disulfonate[8]CPP

fluorophores for biological imaging. Despite this exciting proposition, to date, there are no reported biological investigations of these small molecular slices of carbon nanotubes. Herein for the first time we report a strategy to prepare an aqueous-soluble nanohoop (1), demonstrate that the desirable optical properties of this scaffold are maintained in aqueous buffer and in live cells, and provide insights into the toxicity and permeability of the nanohoop. We also demonstrate that targeting groups can be easily appended to the nanohoop using copper catalyzed “click” chemistry. This study provides the foundation for the study of nanohoops and their derivatives as an exciting new class of biological imaging tools.

RESULTS AND DISCUSSION

Numerous studies have documented the promise of carbon nanotubes as biological imaging agents.³⁶ Inspired by some of these works, we initially investigated the use of surfactant Pluronic F108 to solubilize the unfunctionalized nanohoops in aqueous media for biological studies—a strategy that has been successful for CNTs.³⁷ Although the solubility of the nanohoop increased in the presence of surfactant, cell imaging experiments were plagued by low signal response and aggregation (see the Supporting Information, Figures S1 and S2). This complication prompted the synthesis of 1 (Figure 1), a nanohoop functionalized with sulfonate groups to promote solubility in aqueous media. The synthesis of 1 relies on the incorporation of alcohol functional groups into the nanohoop backbone for late stage manipulation (Scheme 1). The

synthesis begins with the monolithiation of 1,4-dibromobenzene and subsequent nucleophilic addition into ketone **2**, followed by protection of the resulting alcohol with triethylsilyl (TES) chloride to give **3** (96% yield, dr: >20:1). Lithiation of **3** followed by nucleophilic addition to a second equivalent of ketone **2** and TES protection provided dichloride **4** with two *tert*-butyl dimethylsilyl (TBS) protected benzyl alcohols as reactive handles. Suzuki–Miyaura cross-coupling of **4** and diboronate **5** gave macrocycles **6** and **7** in a 28% combined yield. Global deprotection of both macrocycles followed by H_2SnCl_4 -promoted reductive aromatization provided benzyl alcohol[8]CPP **8** in 35% yield.²⁶ Deprotonation of the benzyl alcohols with sodium hydride and treatment with 1,3-propane sultone delivered disulfonated[8]CPP (**1**) in 57% yield. The building block synthesis outlined here and the oligomeric nature of the nanohoop scaffold should provide access to various sizes of nanohoops, each with unique fluorescent profiles, excited state lifetimes, and Raman signatures due to the size-dependent nature of these properties.^{29,38–42} This structural control is a hallmark of the bottom-up organic synthesis of graphitic materials.

Characterization of the nanohoop with ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectroscopy revealed spectra consistent with the expected structure of **1**. Importantly, the nanohoop is completely soluble in DMSO with photophysical properties that are comparable to the parent nanohoop [8]CPP (Figure 2a). Of note, the installation of two sulfonates was sufficient to

Figure 2. Characterization of disulfonate[8]CPP (**1**). (a) Summary of nanohoop photophysical properties. (footnote a) Contains 0.1% SDS. (footnote b) Standard deviation is <5% of the measurement ($n = 3$). (footnote c) 0.01 M KOH in ethanol. (b) λ_{ex} and λ_{em} of 2 μM solutions of [8]CPP (black), **1** in DMSO (green), and **1** in PBS buffer with 0.1% SDS (yellow). (c) pH vs fluorescence (FL) intensity of **1** and fluorescein in a 1:1 MeOH:100 mM KCl, 100 mM KOH solution. Error bars represent standard deviation ($n = 3$).

render this nanohoop aqueous-soluble, a result which is consistent with our findings that these nonplanar structures are much more soluble than flat aromatics. Importantly, the photophysical properties of **1** are retained in aqueous media (PBS buffer with 0.1% SDS). Similar to [8]CPP, the absorption maximum for **1** is at 328 nm with a large molar extinction coefficient of $5.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Upon excitation, we observe a bright green fluorescence ($\lambda_{\text{em}} = 510 \text{ nm}$) with a quantum yield of 0.17 and a large effective Stokes shift of over 180 nm. This is in stark contrast to common fluorophores such as fluorescein that has a Stokes shift of 41 nm.⁴³ The

fluorescence emission is insensitive to acidic or basic environments ($\text{pH} = 3\text{--}11$), which is again in contrast to many common fluorophores (e.g., fluorescein, Figure 2c). Taken together, these findings illustrate that the desirable absorption and emission properties of the nanohoop are not perturbed when the nanohoop scaffold is manipulated to prepare aqueous-soluble versions that can be used for biological studies.

To probe the cytotoxicity of the nanohoop, we treated live HeLa cells with 5, 10, 25, 50, and 100 μM solutions of **1** for 2 h. We then monitored cell death using WST-8 formazan reduction (CKK-8 cell assay, Supporting Information, Figure S3).⁴⁴ Nanohoop **1** showed no cytotoxicity at working concentrations of $\leq 10 \mu\text{M}$. Instead, cell death was only observed at concentrations of 25 μM and above or with longer incubation times (Supporting Information, Figure S4). We note that more extensive studies of nanohoop toxicology as a function of size, composition, and even encapsulated molecules are warranted in the future. Related studies for other graphitic nanomaterials are often plagued by the inherent heterogeneity of those materials, again highlighting the advantage of the bottom-up synthetic approach for the nanohoops.³⁶

Next, using epifluorescence microscopy, we aimed to determine whether **1** is cell permeable and whether the fluorescence of the nanohoop is sufficient to generate bright images in live cells. To test this, HeLa cells were treated with a 10 μM solution of **1** in FBS free DMEM with 0.5% DMSO and the nuclear stain NucRed 647 for 1 h (Figure 3E–H). Notably, after incubation and washing, bright green fluorescence from the nanohoop is clearly observed in the cells, which does not colocalize with the nuclear dye. Interestingly, the lack of localization of **1** to specific cellular compartments is consistent with the previously reported localization of calixarenes in Chinese hamster ovary (CHO) cells.⁴⁵ Based on Pearson's correlation coefficients, we observe moderate colocalization to the cytosol (Celltracker Red CMTPX), and lower colocalization to the mitochondria (MitoTracker Red RM) and endoplasmic reticulum (ER-Tracker Red, Supporting Information, Figure S5 and Table S2).⁴⁶ In the absence of **1** (Figure 3A–D), no fluorescence was observed in the nanohoop channel confirming that the signal was not due to cellular autofluorescence. Additionally, no significant changes in cell morphology were observed through the differential interference contrast (DIC) channel after incubation with **1**, confirming a low cytotoxicity of the nanohoop at this concentration.

Encouraged by the robust imaging capabilities of **1** in live cells we next sought to demonstrate the flexibility of this new fluorophore scaffold through the preparation of a “clickable” version of the nanohoop. We prepared azide[8]CPP **9** using a scalable synthetic strategy similar to the methods described in Scheme 1 (see the Supporting Information). In this case, we assumed the “clicked” moiety could provide the water solubility. To demonstrate the utility of azide **9**, folate[8]CPP **11** was synthesized using copper catalyzed azide–alkyne cycloaddition (Figure 4a). Folate receptors are known to be highly overexpressed on the surface of many cancer cells. Folic acid ($K_{\text{D}} = 0.1 \text{ nM}$) therefore can be an effective targeting group for imaging of cancer cells and even selective drug delivery.^{47,48}

HeLa cells were incubated with a 10 μM solution of **11** in FBS free DMEM with 0.1% DMSO for 2 h (Figure 4bA,B). The cells were then washed and incubated for 18 h with FBS-

Figure 3. DIC and fluorescent images of live HeLa cells in the absence (A–D) or presence (E–H) of disulfonate[8]CPP (**1**). (A, E) DIC; (B, F) NucRed live 647 imaged in CY5 channel; (C, G) **1** imaged in DAPI-long-pass channel; and (D, H) merge of the CY5/DAPI-long-pass channel showing no significant colocalization. Scale bar = 100 μm .

Figure 4. (a) Synthesis of folate-[8]CPP conjugate using copper catalyzed azide–alkyne click chemistry. (b) DIC and fluorescent images of live HeLa cells in the presence of **11** (A, B, E, F) and absence of **11** (C, D, G, H). As controls cells were treated with folic acid (E, F) and **9** (G, H). (A, C, E, G) DIC channel; (B, D, F, H) DAPI-long-pass channel. Scale bar = 50 μm .

free DMEM. After the second incubation period and washing, a bright fluorescent emission was observed from the nanohoop. In the absence of **11**, no fluorescence is observed confirming that the signal was a result of the emission of **11** and not cell autofluorescence (Figure 4bC,D). To further support the role of folic acid receptors on the cell uptake of **11**, we preincubated cells with free folic acid for 30 min to saturate the folate receptors. Then, we incubated the cells with a solution containing free folic acid and nanohoop **11**. Figure 4bE,F shows a marked decrease of cell fluorescence through the nanohoop channel consistent with the folic acid receptor mediated uptake of **11**. Furthermore, when cells were treated with **9** nonlocalized fluorescence was observed, which we attribute to aggregation of the azido nanohoop. These results demonstrate that azide[8]CPP **9** can be functionalized with targeting groups and imaged in live cells.

CONCLUSIONS

These initial studies establish several important points regarding the nanohoop architecture, a growing class of conjugated molecules with radially oriented π -systems, as a new macrocyclic scaffold for fluorescent dye design. First, sulfonation is a viable strategy to render the nanohoos aqueous-soluble and retain their advantageous photophysical properties. Second, these aqueous-soluble nanohoos can penetrate live cells with minimal cytotoxicity and produce bright fluorescent images. Additionally, our solution measurements show that these materials are pH insensitive, an important consideration as we begin to develop the wide applicability of this unique molecular structure for intracellular probes where pH varies dramatically in each cellular compartment. Finally, we established that the nanohoop can be derivatized with targeting groups using “click” chemistry and imaged in live cells. An exciting next step that we are currently pursuing is to establish nanohoos as multiplexed imaging tools utilizing the λ_{abs} shared by all nanohoos and their well resolved and size-dependent fluorescence, singlet lifetimes, and even Raman signatures. For example, based on modern imaging techniques and the synthetic methods available to prepare nanohoos, simultaneous imaging of 20 nanohoos in one experiment is feasible.^{19,49} As a more long-term prospect, we anticipate that the oligomeric nature and unique electron rich cavity of the nanohoop structure can be further engineered to allow for more complex function in biological settings. In conclusion, we have taken an important first step to demonstrate nanohoos as an untapped class of fluorescent dyes that are viable for fluorescent probe development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00346.

Experimental procedures and spectral data including NMR spectra, fluorescent images, cell viability, DIC images, quantum yield measurements, molar absorptivity measurements, fluorescence, and absorption and emission spectra (PDF)

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Notes

The authors declare no competing financial interest.

No unexpected or unusually high safety hazards were encountered.

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