Expanding the Chemical Space of Biocompatible fluorophores: Nanohoops in Cells

Brittany M. White,[a] Yu Zhao,[a] Taryn E. Kawashima,[a] Bruce B. Branchaud,[a] Michael D. Pluth,[a]*
Ramesh Jasti,[a]*

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 non-planar 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. This work establishes the nanohoops as an exciting new class of macrocyclic fluorophores poised for further development as novel bioimaging tools.

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 on the cyanine core structure synthesized first in 1924 (Figure 1).[17] 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 have unveiled several unique, size-dependent photophysical properties that are a direct result of the radially oriented pi-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 results in a size-dependent fluorescence emission (λem) where the

---

[a] B. M. White, Dr. Y. Zhao, T. E. Kawashima, Prof. Dr. B. B. Branchaud, Prof. Dr. M. D. Pluth, Prof. Dr. R. Jasti
Department of Chemistry and Biochemistry and Materials Science Institute
University of Oregon
Eugene, OR 97403-1253 (USA)
E-mail: pluth@uoregon.edu, rjasti@uoregon.edu

Figure 1. Traditional organic dye scaffolds and the new nanohoop fluorophore scaffold.
HOMO→LUMO gap narrows as nanohoop diameter decreases.\textsuperscript{[20]} Additionally, due to Laporte forbidden HOMO→LUMO transitions, all nanohoops share a common absorption maxima ($\lambda_{\text{abs}} \approx 340$ nm) with high absorption coefficients ($\epsilon$) and large effective Stokes shifts ranging from 100-200 nm depending on size.\textsuperscript{[21-34]} Taken together, the nanohoop scaffold offers the possibility of multiplexed imaging using a single excitation source. Moreover, the non-planarity of the benzene rings in the nanohoop also leads to better solubility when compared to planar aromatic systems. Lastly, most traditional fluorophores are electrophilic (see Figure 1), which can lead to undesired side reactivity in biological systems. Despite molecular strain, nanohoops are only reactive under forcing reaction conditions.\textsuperscript{[35]} The inherent attributes provided by the nanohoop structure highlight their potential as new 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 aqueous-soluble nanohoop (1), demonstrate that the desirable optical properties of this scaffold are maintained in live cells and provide insight into the toxicity and permeability of the nanohoop. This study provides the foundation for the study of nanohoops and their derivatives as an exciting new class of biological imaging tools.

Numerous studies have documented the promise of carbon nanotubes as biological imaging agents.\textsuperscript{[29]} 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.\textsuperscript{[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 Supplementary Information Figure 7 and Figure 8). 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 diborane 5 gave macrocycles 6 and 7 in a 28% combined yield. Global deprotection of both macrocycles followed by H$_2$SnCl$_4$-promoted reductive aromatization provided benzyl alcohol[8]CPP 8 in 35% yield.\textsuperscript{[29]} Deprotonation of the benzyl alcohols with sodium hydride and triethyl orthoformate with 1,3-propanesultone 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.\textsuperscript{[35]} This structural control is certainly a hallmark of the bottom-up organic synthesis of graphitic materials.

Characterization of the nanohoop with $^1$H and $^{13}$C($^1$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 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 x 10$^4$ M$^{-1}$ cm$^{-1}$. Upon excitation, we observe a bright green fluorescence ($\lambda_{\text{em}} = 510$ nm) with a quantum yield of 0.17 and a large effective Stokes shift of over 180 nm. The fluorescence emission is insensitive to acidic or basic environments (pH = 3-11), which is in contrast to many common fluorophores (e.g. fluorescein, Figure 2c).

![Scheme 1. The Synthesis of Disulfonate[8]CPP](image)

| Solvent | $\lambda_{\text{abs}}$ (nm) | $\epsilon$ (M$^{-1}$ cm$^{-1}$) | $\lambda_{\text{em}}$ (nm) | $\Phi_e$ | $\lambda_{\text{em}} - \lambda_{\text{abs}}$ (nm) | Brightness ($L/E_\text{p}$) |
|---------|-----------------|-----------------|-----------------|-------|-----------------|-----------------|
| [8]CPP | CHCl$_3$ | 340 | 1.0 x 10$^4$ | 533 | 0.10 | 193 | 1.0 x 10$^4$ |
| 1 | DMSO | 337 | 6.4 x 10$^4$ | 523 | 0.17 | 186 | 1.1 x 10$^4$ |
| 1 | PBS Buffer | 328 | 5.8 x 10$^4$ | 510 | 0.17 | 182 | 9.9 x 10$^3$ |

![Figure 2. Characterization of disulfonate[8]CPP (1). a) Summary of nanohoop photophysical properties. *Contains 0.1% SDS. \textsuperscript{1}Standard deviation is >5% of the measurement (n=3). b) $\lambda_{\text{em}}$ and $\lambda_{\text{em}}$ 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).](image)

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.

We next sought to investigate the photostability of the nanohoop 1 in cells, which is important for applications in bioimaging. Due to the highly strained nanohoop scaffold ([8]CPP has 73 kcal/mol of strain energy compared to its linear analogue), it is tempting to conclude that these structures will be less photostable than more classical unstrained fluorophore structures. To test this, we incubated fixed and permeabilized cells with nanohoop 1 or fluorescein. We then irradiated these cells at the \( \lambda_{\text{abs}} \) for 1 and fluorescein, respectively, and monitored the fluorescence intensity over time. Gratifyingly, our experiments show that 1 is more photostable than fluorescein (Figure 3a). Further synthetic modification of the nanohoop scaffold, as has been done with dyes such as fluorescein\(\text{in}^{[2,15]}\) could lead to enhanced stability in the future.

In order to probe the cytotoxicity of the nanohoop, we treated live HeLa cells with 5, 10, 25, 50 and 100 \( \mu \text{M} \) solutions of 1 for two hours. We then monitored cell death using the CCK-8 cell assay (Figure 3b). Nanohoop 1 showed no cytotoxicity at working concentrations of \( \leq 10 \mu \text{M} \). Instead, cell death was only observed at concentrations of 25 \( \mu \text{M} \) and above or with longer incubation times (Supplementary Information Figure 9). 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 graphic nanomaterials are often plagued by the inherent heterogeneity of those materials, again highlighting the advantage of the bottom-up synthetic approach for the nanohoops.\(\text{in}^{[38]}\)

Finally, using epi-fluorescence microscopy, we next aimed to determine whether 1 is cell permeable and whether the fluorescence of the nanohoop is sufficient to generate bright images in live cells. HeLa cells were treated with a 10 \( \mu \text{M} \) solution of 1 in FBS free DMEM with 0.5% DMSO and the nuclear stain NucRed\(\text{in}^{[2,15]}\) 647 for 1 hour (Figure 3c, E-H). Notably, after incubation and washing, bright green fluorescence from the nanohoop is clearly observed in the cells, which does not co-localize with the nuclear dye. Interestingly, apparent localization of 1 in the cytosol is consistent with the previously reported localization of calixarenes in Chinese Hamster Ovary (CHO) cells.\(\text{in}^{[43]}\) In the absence of 1 (Figure 3c, A-D), no fluorescence was observed in the nano-hoop channel confirming that the signal was not due to cellular auto fluorescence. 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.

These initial studies establish several important points regarding the nanohoop architecture, a growing class of conjugated molecules with radially oriented \( \pi \)-systems, as a new macrocyclic scaffold for fluorescent dye design. First, sulfonation is a viable strategy to render the nanohoops aqueous-soluble and retain their advantageous photophysical properties. Second, these aqueous-soluble nanohoops 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. The nanohoop architecture is also more photostable under constant irradiation with high energy light when compared to fluorescein without any further synthetic modification of the nanohoop. An exciting next step that we are currently pursuing is to establish nanohoops as multiplexed imaging tools utilizing the \( \lambda_{\text{abs}} \) shared by all nanohoops 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 nanohoops, simultaneous imaging of 20 nanohoops in one experiment is feasible.\(\text{in}^{[19,44]}\) 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 nanohoops as an untapped class of fluorescent dyes that are viable for fluorescent probe development.

![Figure 3](image-url)  
**Figure 3.** a) Time vs. FL intensity of 1 and fluorescein. Error bars represent standard deviation (n=3). b) Cytotoxicity of 1 in HeLa cells measured with the CCK-8 cell assay. Error bars represent standard error in measurement (n=6). c) 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\(\text{in}^{[2,15]}\) live 647 imaged in CY5 channel; (C, G) 1 imaged in DAPI-LP channel; and (D, H) merge of the CY5/DAPI-LP channel showing no significant co-localization. Scale bar = 10 \( \mu \text{m} \).

**Acknowledgements**

Financial support was provided by the National Science Foundation (RJ, CHE-1255219), the National Institute of Health (MDP, R01GM113030), the Sloan Foundation, the Camille and Henry Dreyfus foundation and generous startup funds from the University of Oregon. NMR support was provided by NSF-MRI (CHE-1427987), Oregon BEST, and ONAMI and microscopy support in the UO CAMCOR facility was provided by the NSF (CHE-1531198).
