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Group 14 Metallafluorenes for Lipid Structure Detection and Cellular Imaging †

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Abstract: Fluorescent compounds have been shown to be useful in probing lipid dynamics, and there is ongoing interest in nontoxic, photostable, and sensitive dyes. Recently, we evaluated a number of 2,7-disubstituted-alkynyl(aryl)-3,6-dimethoxy-9,9-diphenyl sila- and germafluorenes for their potential as cellular fluorescent probes. These compounds exhibit remarkable quantum yields in hydrophobic environments and dramatic increases in emission intensity in the presence of surfactants. Here, we show that they exhibit significant emission enhancements in the presence of small unilamellar vesicles and are nontoxic to *E. coli*, *S. aureus*, and *S. cerevisiae*. Furthermore, they luminesce in *S. cerevisiae* cells with strong photostability and colocalize with the lipid droplet stain Nile Red, demonstrating their promise as lipid probes.

Keywords: fluorescence; lipid; metallafluorene

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1. Introduction

The introductory understanding of the role of biological membranes is that they are barriers and are used to regulate transport and for energy processes. Only fairly recently has there been a developing understanding that membranes are dynamic in their lipid composition and properties, and that these local differences participate in cellular processes in a profound way and have been linked to disease states [1,2], including cellular stress [3].

Fluorescence spectroscopy is an accessible and powerful tool for the sensing of molecules and their behaviors, including binding interactions, conformational changes, and catalytic activities, in both *in vitro* and *in vivo* via cellular imaging [4]. Due to their ability to respond to changes in molecular environment, molecules that exhibit intramolecular charge transfer (ICT) or excited-state intramolecular proton transfer (ESIPT) are particularly attractive as probes of lipids, their interactions, and dynamics [5,6].

Probes commonly used for such purposes include Nile Red, dansyl, NBD [7], and F2N12S [6]. These vary with respect to relevant properties such as the excitation and extinction wavelength, extinction coefficient, working concentration (sensitivity), photostability, and quantum yield, all of which can impact their utility. When this is coupled with the rapidly expanding research area, it is no surprise that a call for more probes to meet expanding needs is prominently articulated [5].

Recently, we evaluated a small library of sila- and germafluorenes (metallafluorenes or MFs) containing alkynyl(aryl) substituents at the 2,7-position ([8,9]; Figure 1) for their potential as fluorescent probes of surfactants. These compounds are soluble and luminescent in aqueous solution and exhibit high quantum yields and dramatic emission enhancements in the presence of various surfactants (5–25-fold) [10]. These results suggest that MFs could have biological applications. Here, we examine the sensitivity, toxicity, and

photostability of MFs toward lipids both *in vitro* and *in vivo* and demonstrate the potential of these compounds as lipid probes. Indeed, they are sensitive to DOPC small unilamellar vesicles (SUVs) with significant fluorescence enhancements. These dyes show no toxicity to Gram-positive bacteria, Gram-negative bacteria, and yeast cells and demonstrate high photostability. When compared to the commercially available lipid droplet dye Nile Red, these MFs show strong colocalization with more punctate staining, demonstrating their potential as lipid probes.

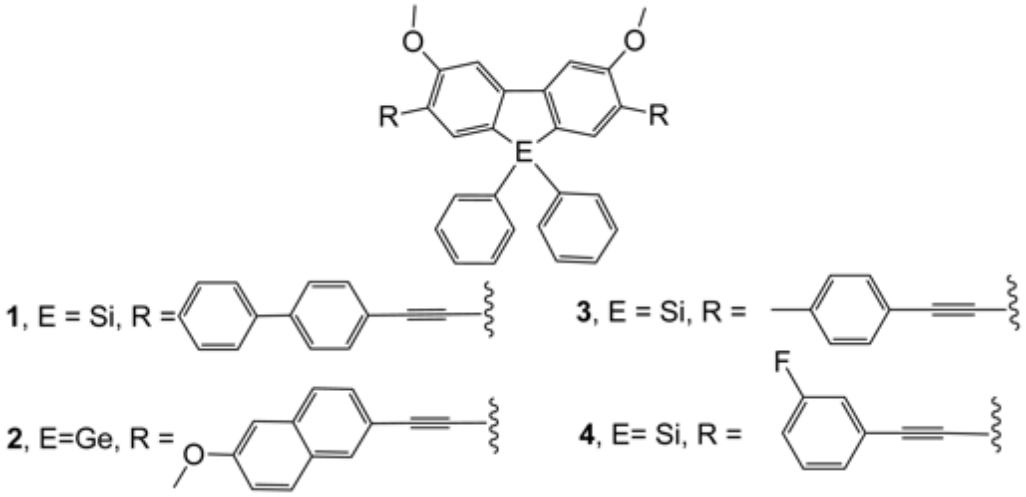

Figure 1. Structures of 2,7-disubstituted sila- and germafluorenes used in this study. **1** silicon based 4-ethynyl-1,1′-biphenyl substitutent; **2** germanium based 2-ethynyl-6-methoxynaphthalene substitutent; **3** silicon based 4-ethynyltoluene substitutent; **4** silicon based 1-ethynyl-3-fluorobenzene substitutent.

2. Materials and Methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Two-hundred-proof ethanol was purchased from Decon Labs (King of Prussia, PA, USA). DMSO and Nile Red were obtained from Millipore Sigma (Milkwaukee, WI, USA). p-Xylene was obtained from ThermoFisher (Waltham, MA, USA). All chemicals were of reagent grade and were used as received without further purification. Compounds **1**–**4** were synthesized as previously described using an appropriate alkynyl(aryl) precursor in a palladium-catalyzed Sonagashira cross-coupling reaction [8,9] and dispensed from stocks in DMSO as previously described [10].

2.2. Preparation of Small Unilamellar Vesicles (SUVs)

At 25 °C, a stock concentration of 4.2 mM DOPC was prepared by drying under inert gas and then resuspended in 10 mM Tris buffer. After 30 min, DOPC was sonicated for 27 min at 25 °C until cloudy. The DOPC-SUV solution was then passed through an Avanti Mini Extruder eleven times to make uniformly sized 0.1 μm DOPC-SUVs at 25 °C. DOPC-SUVs were then diluted to 0.1 mM in a quartz cuvette for fluorescence measurements [11,12].

2.3. Spectroscopy

Absorbance spectra were recorded on a Shimadzu 1800 (Kyoto, Japan) with slits (band-pass) set to 1 nm. Emission spectra were collected in an acid-washed quartz cuvette on a Fluorolog-3 (SPEX) spectrofluorimeter (Horiba Scientific, Piscataway, NJ, USA). The temperature was maintained at 25 °C with a thermostatted cell holder equipped with a magnetic stirrer. Emission spectra were collected with the indicated excitation wavelength and slits (bandpass). MF photostability in xylene was observed at the indicated emission maximum.

2.4. Microbial Toxicity

Culture tubes containing LB media or YPD media were inoculated with *Escherichia coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive), or *Saccharomyces cerevisiae*, respectively. Compounds **1–4** were added such that the final DMSO concentration was 2–10% and the MF at its solubility limit in the media. The tubes were incubated at either 37 °C (bacteria) or 30 °C (yeast) overnight and visually inspected for growth.

2.5. Confocal Laser Scanning Microscopy

Samples were prepared by smearing a small amount of cells onto a glass microscope slide and heat-fixed by passing the slide through a flame no more than 5 times. Then, **1–4** or NR was applied to heat-fixed cells at 15 μM and incubated at room temperature for 15 min for MFs and 10 min for Nile Red. Slides were then rinsed with 2–3 mL of deionized water, topped with coverslips, and sealed with clear nail polish. Cells were imaged with a Zeiss LSM 900 (Zeiss, Oberkochen, Germany) confocal microscope with an excitation wavelength of 405 nm. For photostability, the sample was illuminated with 1% laser power and images collected periodically.

3. Results and Discussion

3.1. Spectroscopic Studies

To assess their sensitivity to a biologically relevant membrane, the emission spectra of **1–4** were compared in the absence and presence of DOPC-SUVs. As shown in Figure 2, fold-enhancements range from two- to sevenfold, with **1** and **2** showing the most dramatic changes.

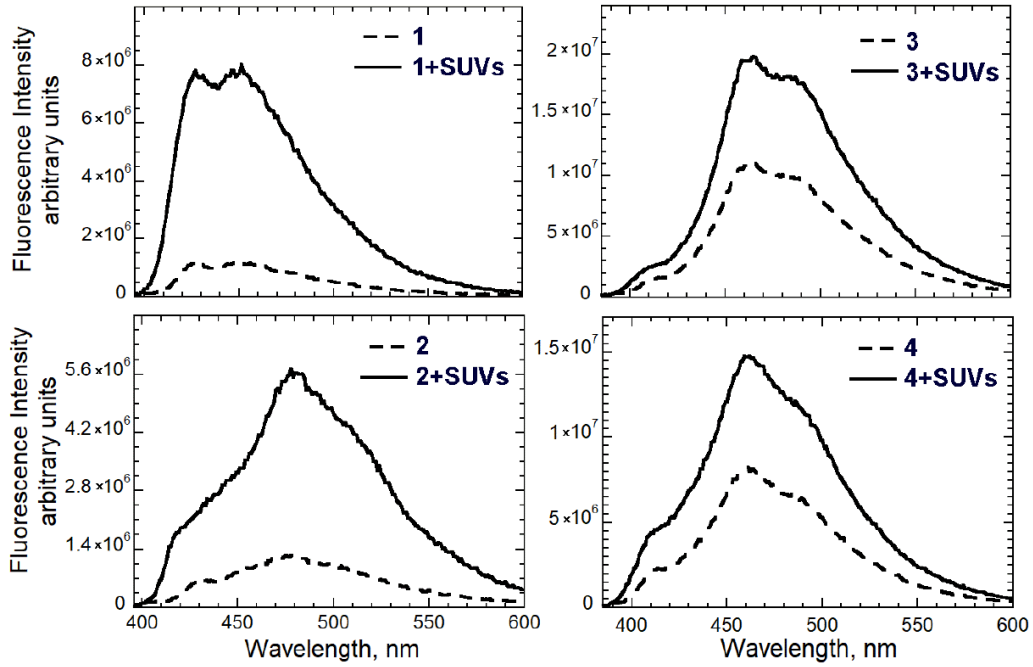

Figure 2. Emission spectra of MFs **1–4** (as numbered in Figure 1) in the absence (dashed) and presence (solid) of 0.1 mM DOPC-SUVs. Conditions: 1 μM MF, 0.1 mM DOPC, 10 mM Tris pH 8, 25 °C. The excitation wavelength was 387 nm and the slits (bandpass) set to 1.0 nm. Three minute incubation.

The photostability of these MFs was initially probed by observing the emission signal as a function of time in xylene, which is used to mimic the interior of membranes [13]. As summarized in Figure 3, these signals are remarkably stable over two hours of continuous excitation. Together, the responsiveness to SUVs and photostability in xylene indicate promise for MFs as probes of lipids *in vivo*.

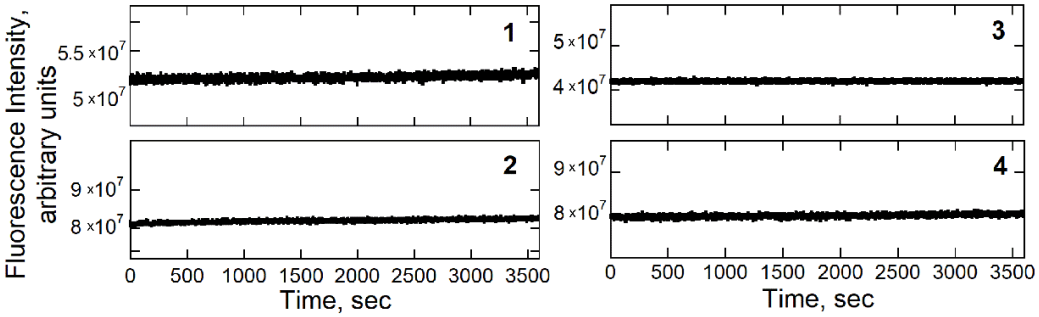

Figure 3. Photostability *in vitro*. Compounds **1**–**4** (as defined in Figure 1) were diluted into p-xylene and excited continuously. Conditions: 1 μM MF, 0.1–0.4% DMSO. **1**: excitation at 387 nm, slits 1 nm; **2**: excitation at 390 nm, slits 0.8 nm; **3**: excitation at 376 nm, slits 0.9 nm; **4**: excitation at 376 nm, slits 0.9 nm.

3.2. Microbial Toxicity Studies

To assess their potential for use in cellular imaging, MFs were screened for toxicity against microorganisms. For yeast, *E. coli* and *S. aureus*, no inhibition of growth was observed at the MF solubility limit in media (at least 50 μM).

3.3. Imaging of S. cerevisiae with Metallafluorenes

To determine if these MFs can be used to stain cells, **1**–**4** were introduced to yeast cells and subsequently imaged using confocal microscopy. Figure 4 illustrates that in all cases, the MF emission intensity is visible inside fixed yeast cells.

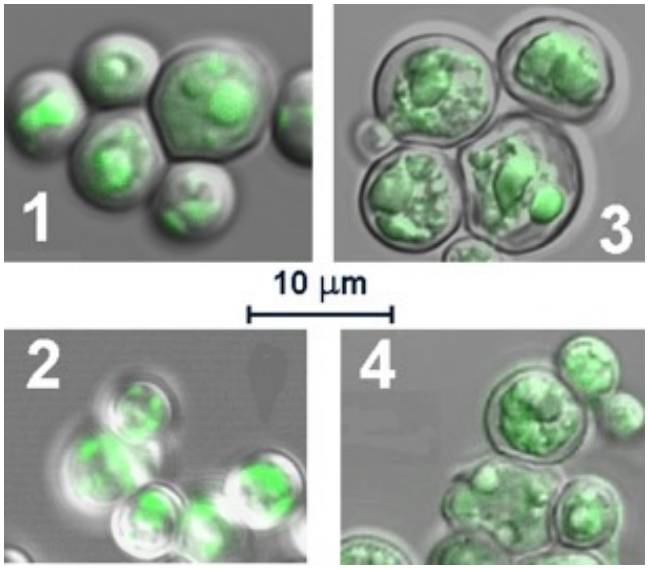

Figure 4. Confocal imaging of MFs **1**–**4** in yeast cells. Conditions: 15 μM MF as indicated, 63×. The excitation wavelength was 405 nm and scan range 400–600 nm. Numbers refer to MFs as defined in Figure 1.

To assess the MF photostability in yeast cells, excitation was applied and fluorescence was observed as a function of time. As summarized in Figure 5 for **1** and **2**, fluorescence persisted for over 2 min, with **2** showing greater photostability. See Supplemental Figure S1 for photostability studies of **3** and **4**.

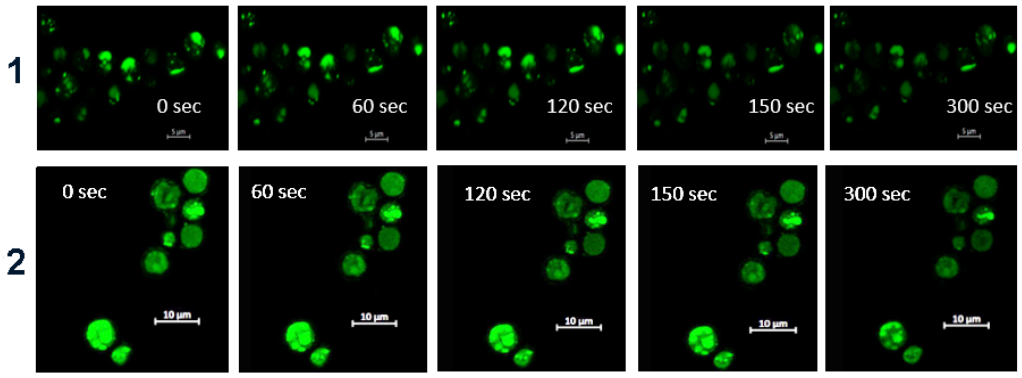

Figure 5. Photostability of **1** and **2** in yeast cells. See Methods for details. *S. cerevisiae* were stained for 15 min with 15 μM **1** or **2** and then imaged periodically during continuous excitation. Magnification is 63×. Numbers at left refer to compounds as defined in Figure 1.

Finally, to determine where these MF localize in yeast, we costained with Nile Red, a well-known lipid droplet stain [14]. As shown in Figure 6, **1** yields more punctate images and colocalizes with this probe, demonstrating clear specificity for *S. cereviseiae* organelles, including the vacuole and possibly lipid granules. See Supplemental Figure S2 for a colocalization study of **2** and **4**.

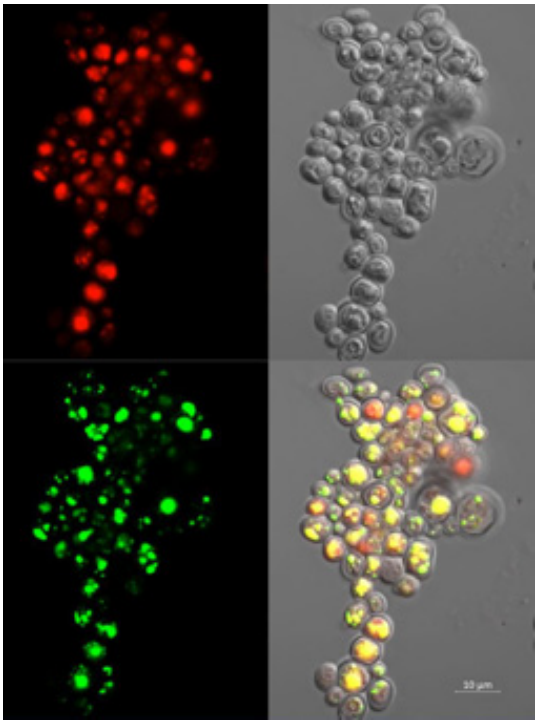

Figure 6. MF **1** colocalizes in yeast with Nile Red. Red, Nile Red; green, **1**; top right, transmitted light; bottom right, yellow indicates colocalization. 15 μM probe at 63× magnification.

4. Conclusions

We show here that these metallafluorenes have good photostability and are sensitive to lipid structures *in vitro*, demonstrating impressive fold enhancements in the presence of SUVs. Furthermore, they are non-toxic to cells and can enter cells and colocalize with Nile Red, a lipid probe. In addition, the higher extinction coefficients of MFs and competitive quantum yields [10] make them more sensitive. All of these observations bode well for the application of MFs as lipid probes both *in vitro* and *in vivo*. The synthetic scaffolding of these MFs provides convenient tuning of desired properties by changing the 2,7 substituent.

This feature facilitates designs that incorporate optimal solubility, emission spectra, dipole moment, and solvatochromism for specific applications.

5. Patents

WO/2020/210416; PCT International Patent Application No.: PCT/US2020/027355.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/CSAC2021-10455/s1, Figure S1: Photostability of 3 and 4 in Yeast Cells, Figure S2: 2 and 4 Colocalize with Nile Red in Yeast.

Author Contributions: Conceptualization, C.M.D., H.J.S. and S.J.J.-N.; investigation, H.J.S. and S.J.J.-N.; resources, C.M.D., J.B.-W., S.M.G. and W.O.; data curation, S.J.J.-N.; writing—original draft preparation, C.M.D. and H.J.S.; writing—review and editing, C.M.D. and S.J.J.-N.; supervision, C.M.D.; project administration, C.M.D.; funding acquisition, J.B.-W. and C.M.D.; H.J.S. and S.J.J.-N. contributed equally to the preparation of this manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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