Engineering a Bio-orthogonal Compartment within a Living Cell

Background and Significance

Cellular viability demands the simultaneous execution of myriad functions that each requires a defined sequence of finely tuned enzymatic steps. In organic synthesis, it is exceedingly difficult to perform a defined sequence of reactions within the same vessel—yet it is possible within a cell. The difference is compartmentalization; cellular function can be spatially confined within separate organelles, enclosed by a lipid bilayer. A compartment that introduces a new function within a cell would be highly desirable.[1] To this end, we will design a new membrane-like structure using a bottom-up approach. This structure will be able to enclose the protein machinery required to impart this new function. This novel compartment must consist of biocompatible, bio-orthogonal (not interacting with the native cellular state) building blocks that spontaneously self-assemble after delivery across the cell membrane. To meet these requirements, we will exploit the 'fluorous effect': hydrocarbons, water, and perfluorocarbons are all mutually immiscible. The introduction of perfluorinated moieties into lipids renders them both lipophobic and hydrophobic, potentiating their segregation from the rest of the cell and self-assembly into a fluorous vesicle.[2] Additionally, the biocompatibility of fluorinated biomolecules has been demonstrated through the use of such molecules as reporter agents in bioimaging[3] and drug delivery,[4] and they have been extensively researched as a potential artificial blood substitute.[5] Thus, they are ideal building blocks for this project.

Innovation

Up to date, no research team has been able to engineer a compartment with a newly associated function within a living cell. In this manner, this proposal has three aspects which are original and innovative. First, bio-orthogonality in three dimensions is not as well established as it is in two dimensions. There are numerous examples of two-dimensional, bio-orthogonal organic reactions such as the Staudinger ligation, or various types of click reactions. Bio-orthogonality in three dimensions implies the self-assembly of building blocks without the cross-reactivity with other biomolecules within a cell. This type of self-assembly and segregation is likely a non-covalent interaction. Only few three-dimensional bio-orthogonal building blocks are known.[6] Most examples are protein-based architectures designed to contain and transport a specific type of molecule, none of which, however, possess an enzymatic function. Second, the co-localization of different types of fluorous biomolecules within a living cell has never been shown before. The fluorous effect is used for purification and separation purposes in organic synthesis, but the effect has not been harnessed to segregate fluorous from non-fluorous biomolecules in live cells. Third, the general concept of introducing a new function into a live cell by creating a new compartment which functions independently from the rest of the cell has not been realized so far.

Research Plan

Specific Aim 1. Synthesis of Building Blocks

1.1. Flourous Lipid (F-lipid): We have synthesized a previously reported **F-lipid**, and, using confocal microscopy, we have demonstrated its increased cell permeability in HeLa cells as compared to a non-fluorinated analog (Figure 1).[2] We will optimize the properties of the **F-lipid** by varying the structure in three ways: First, we will vary chain length and fluorous content of the **F-lipid** tail, and second, we will investigate head groups with greater similarity to naturally occurring lipids. Third, we will change the integrated structure of the fluorophore (shown in blue), to enhance photostability for super-resolution microscopy. We are currently synthesizing **F-lipids** conjugated to a SiR dye. This dye is known to be compatible with super-resolution techniques, especially stimulated emission depletion (STED) microscopy.[7] The fluorophore can be attached *in situ* in live cells utilizing bio-orthogonal transcyclooctene-tetrazine click chemistry.[8] This type of click reaction is extremely fast and thus minimizes background noise and reagent consumption.

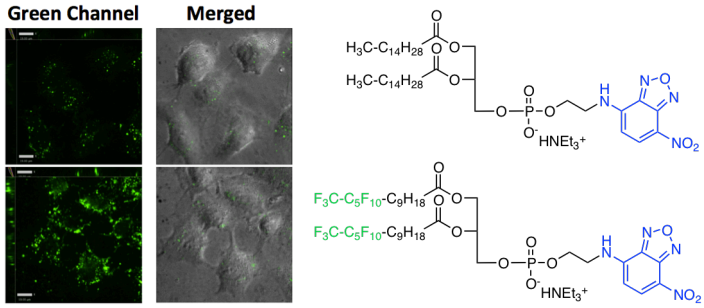

Figure 1: Fluorescence microscopy images of HeLa cells incubated at 10 uM with lipids shown. Microscopy images, left row: green channel, right row: merged with bright field. Carbon chains represented in *green* are perfluorinated, the fluorophore is shown in *blue*.

1.2. Fluorous Protein (F-protein): Three synthetic approaches will be pursued to generate **F-proteins**. The first involves a sortase-catalyzed transpeptidation,[9] the second is based on native chemical ligation,[10] and the third employs a transition-metal-catalyzed coupling strategy.[11] A variety of **F-proteins** with lipid tails of variable length and variable degree of fluorination will be produced. Particular attention will be paid to the production of fluorescent variants of **F-proteins**. As an alternative method for the generation of the **F-protein**, the incorporation of an unnatural amino acid (uAA) in genetically recoded *E.coli* will be pursued.[12] This uAA will either be fluorinated itself (i.e. trifluoroleucine) and a hydrophobic patch of the protein of interest will be substituted with the corresponding uAA, or the uAA will contain a bio-orthogonal handle and the fluorinated tail will be appended in a subsequent chemical ligation step.

Specific Aim 2. Orthogonality

In this part of the project, we will establish orthogonality between fluorinated and unfluorinated analogs. ***In vitro:*** In this phase of the project, we will employ atomic force microscopy (AFM) to visualize how fluorous content, chain length and head group variability will influence the morphology of lipid bilayers composed of mixtures of **F-lipids** and dipalmitoylphosphatidylcholine (**DPPC**), a naturally occurring (non-fluorinated) lipid. It has

been shown that **DPPC** and its fluorinated analog (**F-DPPC**) separate into two distinct phases.[13] Total internal reflection microscopy (TIRFM) will be used to explore temperature dependence of the phase separation between **F-DPPC** and a fluorescently labeled **DPPC** variant. Using this technique, **F-lipid** regions appear dark on a fluorescent background. We expect that **F-lipids** of a certain chain length will show optimal characteristics for cell permeability and phase segregation. The nature of the polar head group will likely have an even larger impact on the behavior of the lipid mixtures. Changing the head group to increase the resemblance to the natural phosphocholine will reduce structure-reactivity effects unrelated to the variable lipid tail under study.

In vivo: We will then explore the orthogonality of **F-lipids** in HeLa cells. Cells will be incubated with the dye-equipped **F-lipids** that showed promise for phase separation *in vitro.* Initial experiments will be performed with confocal microscopy; the most promising **F-lipids** will be analyzed with STED microscopy.

Specific Aim 3. Mechanism of Membrane Translocation

We will investigate if the **F-lipid** is transported across the cell-membrane via endocytosis and the observed **F-lipid** droplets are enclosed into vesicles, or if the **F-lipid** is transported via a different mechanism and the individual molecules self-assemble spontaneously after passage through the cell membrane. To distinguish between the two possibilities, we will study the temperature-dependence of **F-lipid** uptake using confocal microscopy, and we will use Dynasore, which is commercially available, to inhibit endocytosis.[14] The outcome of these experiments will instruct us about the nature of the observed **F-lipid** droplets and if they are topologically inside the cells or enclosed into lipid vesicles.

Specific Aim 4. Co-localization and Introduction of a New Function

In vitro: In this part of the project, co-localization of the optimized **F-lipid** and a variety of **F-proteins** will be the subject of study. AFM and TIRFM will be used to assess phase behavior of mixtures of **DPPC**, **F-DPPC** and a variety of **F-proteins** at a range of different temperatures.

In vivo: Visually distinct fluorescent variants of **F-proteins** will be delivered into HeLa cells containing orthogonal, fluorescent, **F-lipid** vesicles. If co-localization is observed using confocal and super-resolution microscopy, a selected fluorinated enzyme (**F-enzyme**) and an associated fluorogenic, fluorinated substrate (**F-substrate**) will be co-localized and **F-enzyme** activity will be monitored. The **F-substrate** will be designed such that reaction with the **F-enzyme** will convert it from a non-fluorescent initial state to a fluorescent product, which will allow reaction progress to be easily monitored by microscopy. At this point, we will have reached our goal of engineering a bio-orthogonal compartment with distinct activity. We will then start combining the functions of several **F-enzymes** with the aim of engineering an entire biosynthetic pathway.

This proposal was developed with the help of my advisor, Alanna Schepartz, who gave me the general instructions to develop a research plan to engineer a bio-orthogonal compartment within a living cell using fluorous biomolecules.

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