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LDRD Final Report on Imaging Self-Organization of Proteins in Membranes by Photocatalytic Nano-Tagging

John A. Shelnutt, Yujiang Song, Craig J. Medforth, Kevin R. Zavadil, and Darryl Y. Sasaki

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Abstract

We have developed a new nanotagging technology for detecting and imaging the self-organization of proteins and other components of membranes at nanometer resolution for the purpose of investigating cell signaling and other membrane-mediated biological processes. We used protein-, lipid-, or drug-bound porphyrin photocatalysts to grow *in-situ* nanometer-sized metal particles, which reveal the location of the porphyrin-labeled molecules by electron microscopy. We initially used photocatalytic nanotagging to image assembled multi-component proteins and to monitor the distribution of lipids and porphyrin labels in liposomes. For example, by exchanging the heme molecules in hemoproteins with a photocatalytic tin porphyrin, a nanoparticle was grown at each heme site of the protein. The result obtained from electron microscopy for a tagged multi-subunit protein such as hemoglobin is a symmetric constellation of a specific number of nanoparticle tags, four in the case of the hemoglobin tetramer. Methods for covalently linking photocatalytic porphyrin labels to lipids and proteins were also developed to detect and image the self-organization of lipids, protein-protein super-complexes, and membrane-protein complexes. Procedures for making photocatalytic porphyrin-drug, porphyrin-lipid, and porphyrin-protein hybrids for non-porphyrin-binding proteins and membrane components were pursued and the first porphyrin-labeled lipids was investigated in liposomal membrane models. Our photocatalytic nanotagging technique may ultimately allow membrane self-organization and cell signaling processes to be imaged in living cells. Fluorescence and plasmonic spectra of the tagged proteins might also provide additional information about protein association and membrane organization. In addition, a porphyrin-aspirin or other NSAID hybrid may be used to grow metal nanotags for the pharmacologically important COX enzymes in membranes so that the distribution of the protein can be imaged at the nanometer scale.

Introduction

Conventional techniques for producing nanoparticle tags (*e.g.*, laser deposition of GaAs at LLNL) for spatially locating proteins are not very compatible with common biochemical methods and *in situ* use. In particular, traditional quantum-dot conjugates (*e.g.*, with streptavidin[1]) are larger than most proteins and thus tagging may interfere with biochemical processes. These methods also require modification (*e.g.*, biotinylation) of the protein target (*e.g.*, an antibody to be bound to a cancer cell and then tagged).

We have developed a new, more direct photocatalytic[2] nanotagging technique for labeling components of biological systems for detection and imaging purposes. Photocatalytic nanotagging with metal or semiconductor nanoparticles grown *in vitro* and possibly in living cells is especially suited for imaging proteins and other components of membranes to determine their structural organization and interactions. Photocatalytic nanotagging also has great potential for providing new insights into *dynamic* biomolecular processes, especially membrane signaling and recognition processes. Among other advantages, the photocatalytic tagging method allows a small photocatalytic molecular tag (a tin porphyrin complex), which interferes less with membrane binding and other processes than a typical 10-25-nm quantum dot, to be carried by a protein during the biological process and, at a particular desired moment during the process, the metal nanotag is grown *in situ* by exposure to light. With this new nanotagging procedure, biological processes such as the self-organization of proteins and other membrane components (*e.g.*, lipid rafts), the transport of molecules through membranes, and the interactions of membrane surfaces with proteins and other species could be addressed by nanotag imaging and spectroscopy. Hybrids between a drug or pathogen molecules and the tin porphyrin can be made and bound to receptor sites, nanoparticles grown at these sites, and then imaged by fluorescence, electron microscopy (EM), or atomic force microscopy (AFM). In addition, the extra biochemical flexibility of our new nanotagging approach will allow molecular simulations to be used to guide the protein- or drug-modification steps that may sometimes be necessary to tag specific proteins. These computational and experimental studies can take advantage of genomic and proteomic information. For example, bioinformatics methods might be used to search for membrane-binding motifs and to guide the nanotagging of proteins of unknown structure and/or function. Molecular simulations can also help in exploring structure-function relationships for proteins and their cofactors and the structural effects of membrane interactions.

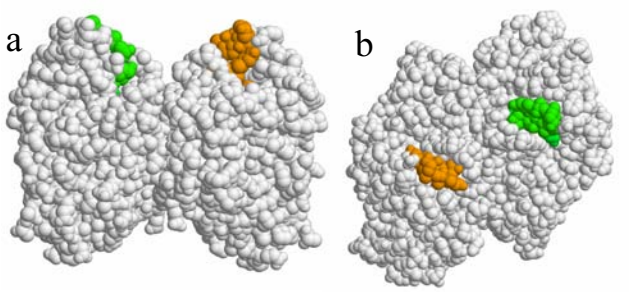

Figure 1. *Two views of human ferrochelatase, which is one of the systems targeted using nanotagging process. Green and orange structures are 3 cholate molecules bound to the two heme binding sites of the homodimer. The membrane would be at the top in (a) and horizontally oriented.*

Developing procedures for studying membrane self-organization and its role in recognition and signaling are the primary focus of our nanotagging studies. Self-organization in our definition includes protein association with intact lipid membranes, organization of proteins within and on membranes, and formation of microdomains such as rafts and caveolae. The latter are more rigid

and stable microdomains of the plasma membrane known to be rich in sphingolipids, cholesterol, and certain proteins. The relevant biological issues associated with membrane self-organization relate to the relative locations of various proteins and their association into large membrane-bound supercomplexes, the role of lipid rafts in this process, and the changes that occur in response to recognition and signaling events. In particular, for rafts, the determination of their size, number, and lipid/protein composition is required. Because the rafts are generally small (~25 nm in diameter), photocatalytic nanotagging of lipids or raft proteins (such as caveolins-1, -2, -3, and glycosyl-phosphatidylinositol(GPI)-anchored proteins) offers a new technique to help to answer these questions. Caveolins, which play an anchoring role in membrane rafts, are a prime target for photocatalytic nanotagging. Besides studying the structural role of raft proteins and lipids, photocatalytic nanotagging of raft proteins and lipids offers the possibility of investigating signaling functions, *e.g.*, pheromone signaling in yeast, in which the response is to form rafts that project an elongated tip that is enriched in Fus1p cell-fusion protein.

We first developed the nanotagging and imaging techniques that we plan to use in our investigations of protein self-organization in membranes by investigating proteins that do not need to be chemically modified in any way because they naturally bind the photocatalytic porphyrin molecules that will be used to grow the nanoparticle tags (e. g. ferrocheletase, Figure 1). Next, we proceeded to preliminary studies of protein- and drug-labeling methods applicable to all types of proteins and to the imaging of membrane model systems such as liposomes. In any of these cases, this is a photocatalytic molecule attached that is capable of growing a metal or semiconductor nanoparticle tag at the site where the catalyst molecule is bound. After the nanoparticles or quantum dots are grown *in situ*, the nanoparticles were imaged by transmission EM and optical techniques. We also used a series of liposomes made of functionalized lipid molecules as model systems to selectively detect divalent and trivalent metal ions.[3] This work has been published; herein we will not further discuss the details.

The nanoparticle tag is grown in the following way. First, the photocatalytic porphyrin is bound to the protein or membrane, most simply at a site where a natural porphyrin normally binds. Next, a metal salt is added to the solution along with a weak electron-donor molecule such as ascorbic acid, EDTA, nitrite, or a tertiary amine. When exposed to visible light, the photoexcited porphyrin is reduced by the electron donor, producing a porphyrin radical that is capable of reducing metal ions from solution. We have used this method in the photocatalytic reduction of gold, silver, platinum, lead, and other metal and semiconductor ions. Since the photoreaction is cyclic, each photocatalyst molecule can reduce thousands of metal ions from its surroundings as additional photons are absorbed. The zero-valent metals generated are then deposited onto the protein or membrane model near the porphyrin molecule in the form of a metal nanoparticle. These nanoparticles label the site of the photocatalyst because they are easy to image in a transmission electron microscope (TEM) due to the high electron density of the heavy metal nanoparticle. We also be able to make cadmium selenide quantum dots as tags using a similar procedure, thus permitting optical imaging of the system. Thus, these metal and semiconductor nanoparticles can serve variously as fluorescent labels and EM and Raman probes.

Accomplishments

A primary goal of our research is developing and demonstrating the feasibility and effectiveness of our photocatalytic nanotagging technique, and in particular the development of a nanotagging method that produces useful nanotags under solution conditions suitable for biological samples. We have now successfully demonstrated the utility of the nanotagging method by growing gold nanotags on tin-porphyrin-reconstituted hemoglobin (Fig. 2) and characterizing the tagged protein using transmission electron microscopy (TEM). In addition, we have incorporated commercially available photocatalysts into model membrane systems and labeled the membrane. Finally, we have developed 'second generation' photocatalysts that have structures optimized for covalent attachment to proteins and for specific localization in the model membrane system (i. e., porphyrins with lipid tails and ether head groups). Because proteins different than those originally proposed were used in accomplishing these first year tasks, we have generalized the original task statements in the following sections.

Develop the photocatalytic methods needed for controlled growth of metal nanotags on proteins. This crucial task—the development of the nanotagging technique—has been completed. After extensive screening studies, we arrived at the nanotagging procedure described briefly here. The metal nanoparticle tags are grown in the following way. First, a metal salt (gold(I) thiourea complex) is added to buffered solution containing a weak electron-donor molecule (ascorbic acid). When exposed to visible light, the photoexcited porphyrin is reduced by the electron donor, producing a porphyrin radical that is capable of reducing gold(I) ions from solution. We have used a similar method to photocatalytically reduce silver, platinum, lead, and other metals and semiconductors. Since the photoreaction is cyclic, each photocatalyst molecule can reduce thousands of metal ions from its surroundings as additional photons are absorbed.

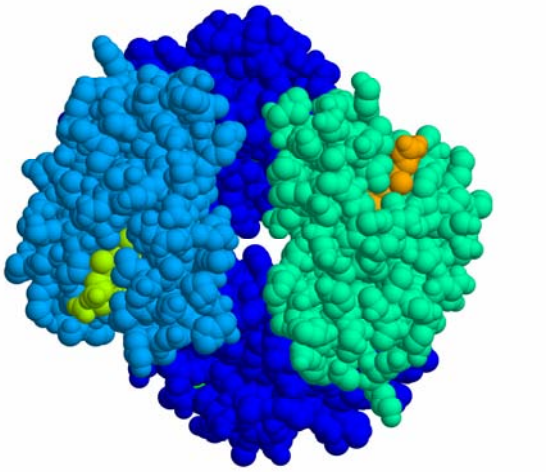

Fig. 2. *Model of tin-reconstituted Human Hemoglobin (α subunits in dark blue; β chains in light blue and green). Porphyrin molecules bound to the β chains of the tetramer are shown as blue and orange. We have made Sn-reconstituted hemoglobin, in which SnProtoP replaces the natural heme cofactors. Nanotags grow near the Sn-porphyrin sites, giving quadruples of Pt particles in TEM images. Distance across the tetramer is about 6 nm.*

The gold(I) thiourea complex, arrived at by an exhaustive search for suitable complexes, has provided us with the desired purely photocatalytic system for nanotagging. An important goal is to ensure the metal is deposited onto the protein surface near the porphyrin molecule in the form of a metal nanoparticle. We have previously used such an approach to deposit platinum nanostructures on surfactant assemblies, although not by a purely photocatalytic reaction. TEM images of the gold nanoparticles (2-7 nm) produced by this reaction are shown in Fig. 3a. The choice of the gold(I) oxidation state and thiourea as the complexing agent gives a solution that

does not absorb visible light and hence prevents photochemical reduction from competing with photocatalytic reduction of gold. Gold is also good choice for the nanotag because of its inert nature, deposition characteristics, its electron-microscopy and optical detection properties (gold nanoparticles are easy to image by TEM and also optically label the protein). Developments of this technique can be done by varying the solution conditions and porphyrin structure to grow smaller nanotags (perhaps <2 nm) and extending the range of applicability. We might also develop a procedure for photocatalytically growing cadmium selenide quantum dots and other tags using similar procedures, thus permitting simultaneous low-resolution optical and Raman imaging as well as high-resolution EM imaging.

Use photocatalytic nanotagging to label protein subunits and verify the precision and reliability of imaging using TEM. This task is complete. We have used the photocatalytic nanotagging technique described above to investigate protein subunits assembled into a multi-subunit protein complex in hemoglobin (Fig. 2). This is an essential step toward our ultimate goal of imaging the self-organization of membrane-bound and membrane-associated protein hierarchical superstructures. We elected to start with proteins that did not need to be chemically modified in any way because they naturally bind the photocatalytic porphyrin label that is used to grow the nanoparticle tags. Hemoglobin was used because we successfully substituted the photocatalytically inactive Fe(II)-protoporphyrin (heme) cofactor with the active analog Sn(IV) protoporphyrin (SnProtoP), which remains strongly bound to the Sn-porphyrin label. This leaves essentially no unbound Sn porphyrin in solution that might produce unwanted tags. The nanotagged self-assembled hemoglobin tetramer (Fig. 2) has four subunits labeled with SnProtoP, so we would expect to see four gold nanoparticles in the TEM images.

After several attempts, we were successful in developing a procedure for extracting the four heme groups from hemoglobin and replacing them with SnProtoP. Briefly, the synthesis procedure for Sn hemoglobin was as follows. A human hemoglobin A solution was acidified with a solution of acetone and HCl, stirred to remove the heme groups, and then centrifuged to recover the apoprotein. The apoprotein was then re-suspended in acidified acetone and centrifuged again, with the pink supernatant being discarded. This process was repeated several times to remove remaining heme. The pellet of apoprotein was then dissolved in cold water and dialysed against phosphate buffer at pH 6.8 and then against Tris HCl buffer. The apoprotein was then centrifuged and filtered to remove precipitated protein. Finally, SnProtoP was added dropwise to a cooled and gently stirred solution of the apoprotein until the SnProtoP concentration was of 8 times the protein concentration. This mixture was incubated in the dark. The Sn hemoglobin was then chromatographed on a gel filtration column to remove excess SnProtoP. The Sn-porphyrin-reconstituted hemoglobin showed a somewhat red-shifted UV-visible absorption spectrum compared to the product-free SnProtoP in the same buffer solution, confirming binding of SnProtoP to the protein. The Sn porphyrins contained in the Sn-reconstituted hemoglobin were shown to be photocatalytically active in reducing methylviologen, and thus retained the ability to grow a metal or semiconductor nanoparticle tag near the active sites of the protein.

The growth of gold nanotags on Sn hemoglobin was accomplished using the procedure described above. After gold nanoparticles were grown *in situ* on Sn hemoglobin, the product was imaged by TEM. Fig. 3 shows an image of the gold nanoparticles grown using a Sn porphyrin (SnTPyP) in a SDS micellar solution (Fig. 3a) and with Sn-reconstituted hemoglobin (Fig. 3b). For the Sn porphyrin in micelles, we get only isolated gold particles. In contrast, for Sn-reconstituted hemoglobin, the nanoparticle tags are found in particle clusters consistent with the particles residing on the tetramers surface close to the bound porphyrin photocatalyst molecules in positions determined by the organization of the protein subunits (Fig. 3b). This study thus addresses the first level of protein self-organization information that is provided by nanotagging proteins, *i.e.*, the location and orientation of the nanotags indicate the corresponding arrangement of the protein subunits in the intact protein complex. The Sn hemoglobin results therefore validate our photocatalytic nanotagging approach. Reducing the size of the gold nanoparticles might provide more specific structural information and is a goal worthy of future study.

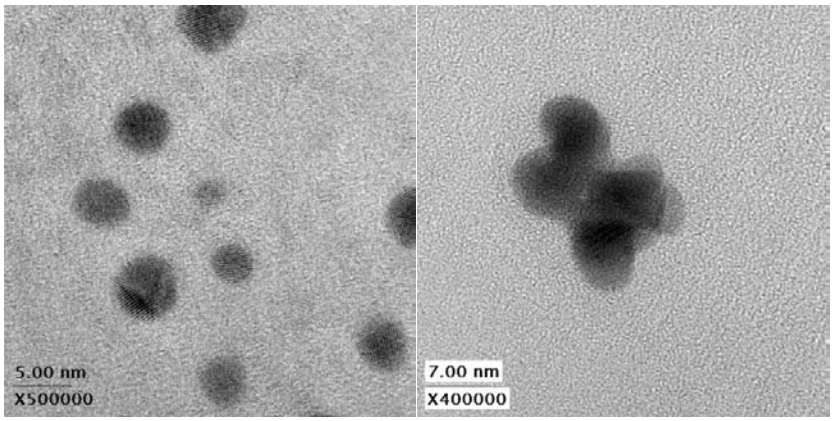

Fig. 3. *(a) TEM image of the isolated gold nanoparticles produced purely photocatalytically using the gold(I) thiourea complex and ascorbic acid as reductant in the presence of SDS. (b) A cluster of gold nanoparticles produced in a similar manner in the presence of Sn-reconstituted hemoglobin.*

We also had investigated ferritin and ferrochelatase using the nanotagging procedure. The ferrochelatase example demonstrates how nanotagging works to provide an understanding of protein self-organization in membranes. The biological function of ferrochelatase is to insert Fe^{2+} into protoporphyrin IX to make ferroprotoheme (heme) in the final step in heme biosynthesis. In mammals, this enzyme is associated with the periplasmic side of the mitochondrial membrane. Mutation of ferrochelatase or a breakdown in gene regulation leads to the genetic disease erythropoietic protoporphyria, which is the origin of the vampire myth. Ferrochelatase has a natural capacity for binding a metal-free protoporphyrin molecule and also binds metal protoporphyrin derivatives. Instead of the natural substrate, we bound a photocatalytic metal-protoporphyrin derivative at the active site of ferrochelatase subunits and used its photocatalytic ability to grow a tagging nanoparticle. Structural information was then be obtained by EM imaging of the nanoparticles, immediately providing information on the relative location of the two active sites on the protein homodimer complex (Figure 1). Of more interest from the standpoint of membrane self-organization, the nanotagging technique can provide structural information about the position of ferrochelatase relative to the membrane surface and relative to other proteins involved in heme biosynthesis. In particular, a putative transmembrane complex of ferrochelatase with protoporphyrinogen oxidase (PPO) is thought to exist. PPO precedes ferrochelatase in the heme biosynthetic pathway, and performs the oxidation of protoporphyrinogen to protoporphyrin. PPO is thought to be located across the membrane from ferrochelatase facilitating the exchange of the poorly water-soluble protoporphyrin between the

two enzymes. Determining the spatial relationship of these two proteins on the membrane is just one of the unanswered questions that the nanotagging technique should allow us to answer (see below). Other proteins in the heme biosynthetic pathway may also be part of this supercomplex, and their location on the membrane relative to ferrochelatase and PPO can be determined by photocatalytic nanotagging. Unfortunately, these studies were hampered by the weak binding of SnProtoP to these systems. For example, repeated attempts to bind SnProtoP to the active sites of the ferrochelatase dimer of both the V251L mutant and wild type enzymes resulted in inconclusive results as to whether the porphyrin was actually specifically bound to the protein. The porphyrin may have been bound to the protein, but the spectroscopic signature of binding was subtle enough to raise doubts. However this does not necessarily prevent us from using photocatalytic nanotagging to investigate this important system, as the use of photocatalysts covalently attached to the protein (see later) provides an attractive alternative method for nanotagging protein components.

Nanotagging a membrane model system. Since our ultimate goal is to study membrane self-organization and signaling processes, we investigated the nanotagging of unilamellar liposomes containing photocatalytic porphyrins within the lipid bilayer or anchored on the outer surface as model membrane systems. This is interesting from the standpoint of determining our ability to resolve nanotags on membranes in EM images. It also assesses the possibility of using liposomes as model membrane platforms for the planned membrane self-organization studies.

This task really has two parts—the first is the development of various methods to add porphyrin labels to lipid structures, including liposomes and natural membranes. We have taken two approaches to this subtask; the first approach is to investigate commercial available hydrophobic synthetic porphyrins that can be incorporated directly into the lipid bilayer of liposomes, and the second is to covalently attach porphyrins to lipid head groups to form novel porphyrin-lipid that can be incorporated into membrane. The second part of the task for each approach is to grow the metal nanotags and image these tags on the membrane using EM, which helps us understand the distribution of the porphyrins or porphyrin-lipid hybrids in the membrane. We will first describe results of the direct porphyrin incorporation into the bilayers of unilamellar liposomes, growth of platinum nanotags, and the imaging of the resulting nanotags by EM. Then, we will describe the synthesis of lipid-porphyrin hybrids and the imaging of the platinum nanotags produced by these photocatalysts.

Direct incorporation of porphyrin labels into lipid bilayers and growth of nanotags. Briefly, the metal nanoparticle tags are grown in the following way. First, a metal salt is added to buffered solution containing a weak electron-donor molecule (ascorbic acid). When exposed to visible light, the photoexcited porphyrin is reduced by the electron donor, producing a porphyrin radical that is capable of reducing metal ions from solution. We have used this photocatalytic method to reduce gold, silver, palladium, platinum, lead, and other metals and semiconductors. Since the photoreaction is cyclic, each photocatalyst molecule can reduce thousands of metal ions from its surroundings as additional photons are absorbed. The desired result is a metal nanoparticle formed at the site of the porphyrin molecule; the metal particle can be as small as 1-2 nm, the typical size of a small protein molecule, making it a useful EM tag that reveals the location of the porphyrin molecule. The goal is then is to label biological molecules of interest with photocatalytic porphyrin molecules and at some point, say after binding a porphyrin-labeled

enzyme to a membrane, use photochemical reduction to grow the metal nanotag whose location can be revealed by electron microscopy.

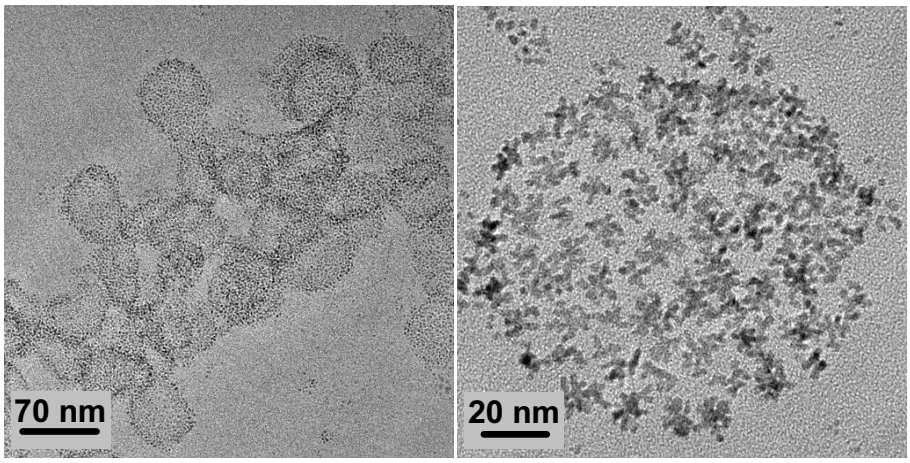

Fig. 4. *Nanotagged liposomes. (a) Pt nanoparticles grown on ~120-nm liposomes containing a high loading of SnOEP when exposed to intense white light. (b) Nanodendrite "flowers" tagging a ~140-nm liposome containing a high loading of SnOEP and Pt when exposed to intense white light for 30 minutes*

Since our ultimate goal is to study membrane self-organization and signaling processes, a necessary goal of our work is to ensure the metal is deposited onto the protein or lipid surface near the porphyrin-label molecule in the form of a metal nanoparticle. We have investigated the nanotagging of unilamellar liposomes containing photocatalytic porphyrins within the lipid bilayer as a model membrane system, with the goal of resolving nanotags on membranes by EM and discovering the distribution of porphyrin labels within the bilayer. Fig. 4 shows a pair of TEM images of liposomes that have been labeled with tin(IV) octaethylporphyrin (SnOEP). Because of its hydrophobicity, SnOEP necessarily resides within the lipid bilayer of the liposomes. Although the surfactant itself cannot be seen in these images, the circular patterns of platinum metal nanoparticles (Figs. 4a) and nano-flowers (Fig. 4b) clearly delineate the liposomal structures and show the effectiveness of this nanotagging technique.

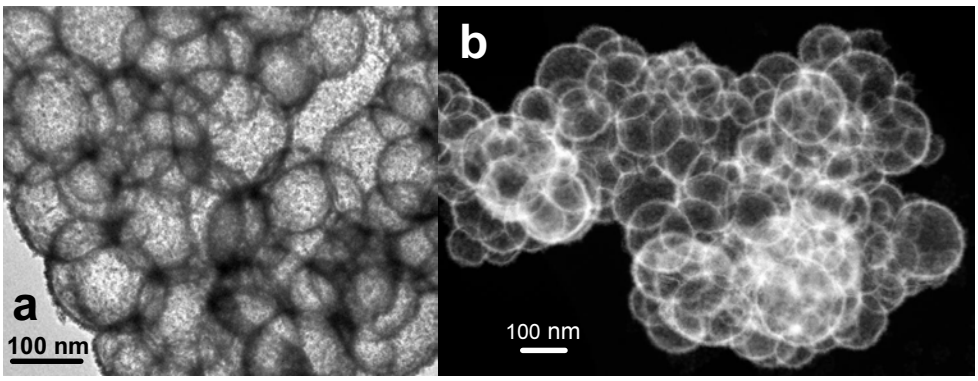

Fig. 5. *(a) High resolution TEM image of platinized liposomes in which individual nano-flowers are evident as networks supporting the spherical structure of the liposomes. (b) HAADF scanning-TEM image of dendritic platinum nanotag networks in platinized liposomes.*

The autocatalytic growth that occurs on the photocatalytically initiated Pt seed particles was originally viewed as a drawback to the use of platinum for nanotagging purposes. However, we have recently found some advantages to using Pt, as we can grow small 2-dimensional dendritic "flowers" as nanotags to any desired diameter by controlling the amount of platinum complex available for growth of the

photocatalytically generated seed particles. These flower nanotags provide new insights into the distribution of porphyrins across the surface of the SnOEP-labeled liposomes. If the SnOEP growth centers were aggregated or phase separated within the membrane such that the porphyrins were not randomly distributed, we would expect patches of platinum rather than evenly distributed nano-flowers. This is clearly not the case. Indeed, if the Pt flowers are grown to a size where they just touch and grow together with neighboring nano-flower tags, then a rigid network of the Pt nano-flowers is formed and this network preserves the spherical structure of the liposome even upon drying on a TEM grid (Fig. 5). This result suggests that the individual porphyrins are scattered over the entire surface, boding well for our ability to nanotag porphyrin-labeled lipids and proteins in membranes and then resolve the protein structures at the nanoscale using these liposomal models. The results also suggest that labeling lipids with photocatalytic porphyrins will allow raft formation to be imaged at the nanoscale by EM.

The formation of the fully coated nanostructures which retain the spherical structure of the liposomes also provides a way of verifying the one to one correspondence between the embedded porphyrin labels and the derived nanotags in liposomes and other membrane model systems. If such a correspondence exists, we would expect that the number of Pt dendrites on a liposome would be equal or less than the number of porphyrin molecules in the liposome. The number of nanotags can be less than the number of porphyrins either because two porphyrins may be close enough together to generate a single seed molecule or because some nanotags do not connect to the others and may be lost from the Pt network that is observed on the TEM grid. Nevertheless, we would expect the number of nanotags and the number of porphyrins to be similar. We have used two methods to estimate the number of nanotags on a 120-nm average liposome, one based on the average size of the nano-flowers required to join to neighboring nanotags to form a network, and another based on counting nano-flowers within a known surface area. Both methods give estimates of the number of nanotags per 120-nm liposome of between 550 and 750. The number of porphyrin molecules in an average-sized (120-nm) liposome can also be estimated. Based on average cross-sectional areas of 0.37 nm^2 for cholesterol and 0.65 nm^2 for DSPC and the known lipid concentrations, we can obtain the concentration of liposomes. Then, using the known concentration of SnOEP we can estimate the number of porphyrins per liposome (approximately 2000). Thus, the number of nanotags is similar to, and smaller than the number of porphyrins in the liposomes, suggesting that there is approximately a one to one correspondence between the two. This result provides crucial support for the use of the photocatalytic technique for nanotagging applications in membrane and membrane model systems.

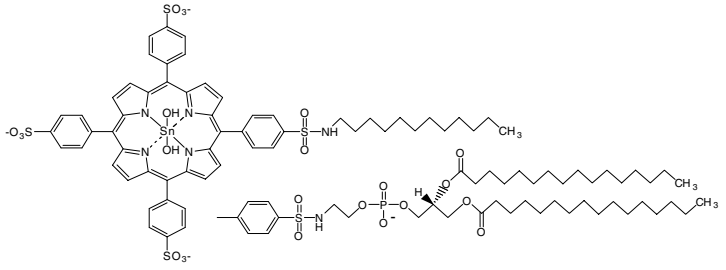

Fig. 6. *Photocatalytic porphyrin-lipid conjugates produced by coupling a porphyrin sulfonyl chloride with an aminolipid. The conjugates are abbreviated as SnTPPS-C12 and SnTPPS-DSPC. The dihydroxide complex is shown; water can be a ligand also at low pH.*

<u>Lipid-porphyrin hybrids for labeling and growth of nanotags.</u> Fig. 6 shows photocatalytic porphyrin-lipid hybrids that we have synthesized and incorporated into membranes for studying raft formation in liposomal model systems. To date, we have synthesized porphyrin hybrids using a simple synthetic amine lipid

(C12:0) and we are partway through the synthesis of biologically derived phosphoethanolamine (C16:0) lipid. Both lipids are attached to a polar porphyrin headgroup based on a commercially available sulfonic acid substituted porphyrin (SnTPPS). The attached photocatalytic porphyrin labels the location of the attached lipid in a membrane. Growth of platinum nanoparticles by the porphyrin reveals the location of the lipid molecule in the membrane. This study represents the first data for photocatalytic porphyrin labels that should reside exclusively on the outside surface of the membrane because of the charged polar substituents (SO_3) on the porphyrin ring.

Hydrophobic porphyrin labels such as SnOEP produce sheet-like Pt dendrites in liposomes, most likely because the SnOEPs reside in the bilayer and the metal grows between the lipid layers of the liposome. The structures shown in Figs. 4 and 5 are illustrative of such dendritic Pt sheets. In contrast, porphyrin photocatalyst anchored to the outer surface of the membrane by interactions with the bilayer are expected to produce globular Pt dendrites, much like those observed when SnOEP is incorporated into sodium dodecylsulfate (SDS) micelles. Fig. 7a shows the Pt globular dendrites are obtained by shining bright incandescent light on a solution of the dodecyl lipoporphyrin (SnTPPS-C12) shown in Fig. 6 even without liposomes. It appears that the lipoporphyrins self-aggregate to form small micelles, as indicated by the cloudiness of the lipoporphyrin solution, and this is consistent with the large size of the Pt dendrites observed when compared to a comparable concentration of SnOEP in SDS-micellar solution. Apparently, Sn lipoporphyrin itself forms small micelles that act together to grow fewer seed particles, resulting in larger globular dendrites than might otherwise be expected.

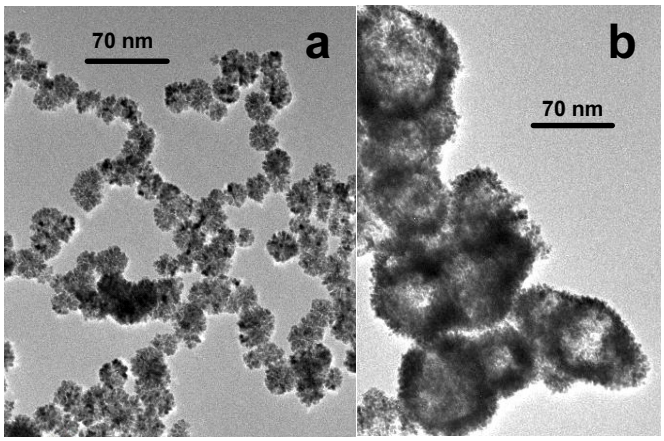

Fig. 7. *(a) Globular Pt dendrites produced by illumination of micelles formed by SnTPPS-C12 and (b) platinum dendritic structures coating DSPC/cholesterol liposomes containing SnTPPS-C12.*

Fig. 7b shows the resulting Pt growth after addition of unilamellar DSPC/cholesterol liposomes to the solution of the Sn lipoporphyrin. We expect the three negative charges of the porphyrin head group of the lipids in Fig. 6 to repel other similarly labeled lipid molecules, leading to even dispersal of the porphyrin labels over the liposomal surface. Since we now suspect that dendritic sheet growth depends on seeds initiating Pt growth between the bilayers, we might also expect the porphyrin head groups anchored to the surface to grow globular dendrites on the liposomal surface. The platinum 'fur' on the liposomes shown in Fig. 4b is entirely consistent with this model of the nanotag growth process.

Our next undertaking is to add a porphyrin such as tetra(N-methylpyridyl)porphyrin with four positively charged N-methyl groups in a spatially complimentary arrangement that can neutralize the negative charge of the SnTPPS headgroups, causing the headgroups to bind together and the attached lipids to form rafts. Rafts composed of porphyrin-labeled lipids would be expected to

photocatalytically grow distinctive extended islands of platinum globular dendrites rather than evenly coating the liposomes. The ability to grow these extended Pt nanostructures in addition to simple nanoparticle tags is a distinct advantage of our photocatalytic nanotagging technique, providing a novel method for obtaining relative locations of labeled molecules.

Investigate synthesis methods for covalently labeling proteins with porphyrins. We wanted to prepare proteins with photocatalytic porphyrins covalently linked to them; numerous ways to selectively label proteins with fluorescent markers are known. In analogy, we have investigated a general synthetic method for labeling the lysine sidechains of proteins with photocatalytic porphyrins as shown in Fig. 8. This method uses the same commercially available tin porphyrin (SnTPPS) shown in Fig. 6 that is used for the lipid labeling. In this case, the sulfonyl chloride is converted to an activated sulfonate ester that can react with free amine groups on the protein of interest. The synthesis of the pentafluorophenyl ester of SnTPPS is in progress.

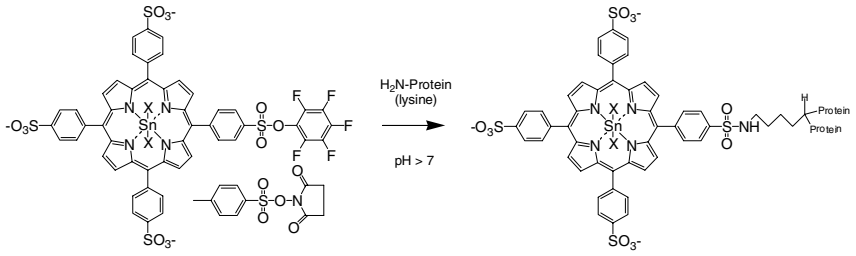

Fig. 8. *Synthesis of photocatalytic porphyrin-lysine hybrids of proteins by formation of sulfonamide linkages using activated sulfonylesters. X = OH, OH_2.*

The basic techniques and procedures for photocatalytic nanotagging are now at hand and ready to be applied to the assembly and organization of porphyrin-labeled proteins and lipids in membrane systems, and especially to the roles of recognition and signaling in membrane self-organization, should funding become available. Self-organization in our definition includes protein association with intact lipid membranes, organization of proteins within and on membranes, and formation of microdomains such as rafts and caveolae (more rigid and stable microdomains of the plasma membrane composed of sphingolipids, cholesterol, and proteins). Because the rafts are generally small (~25 nm in diameter), porphyrin-labeling of lipids or raft proteins (such as caveolins-1, -2, -3, and glycosyl-phosphatidylinositol (GPI)-anchored proteins) followed by photocatalytic growth of metal nanotags for EM imaging offers a novel technique for studying these systems. Caveolins, which play an anchoring role in membrane rafts, are thus a prime target for future photocatalytic nanotagging studies. Photocatalytic nanotagging of raft proteins and lipids might be used to investigate signaling functions, such as pheromone signaling in yeast, in which the response is to form rafts and project an elongated tip that is enriched in Fus1p cell-fusion protein. These biochemically complex studies are now feasible with the development of our reliable and general photocatalytic nanotagging procedures.

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