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Finding a single-molecule solution for membrane proteins

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Background

Another opportunity presents itself to honor the man, Irwin Clyde Gunsalus, who we all know as Gunny. As such, I felt it was appropriate to provide a bit of a background, which could easily be called "Lessons in Learning #2: How Science Works," to introduce Gunny's leadership and continual influence since my time in his laboratory. After this brief introduction we can do what Gunny really enjoys—talking about interesting new science!

It was a little over 30 years ago that I was introduced to Gunny and, shortly thereafter, found out who he was and, more importantly, is. An earlier tribute to Gunny's 70th birthday manifested itself as a collection of works titled *Experiences in Biochemical Perception*, edited by Nick Ornston and me and published by Academic Press in 1982 [1]. This volume turned out to be a wonderful documentation of the then recent discoveries by numerous scientists who had benefited from their association with Gunny. At the time this collection appeared, I was a lowly assistant professor at Yale, and really did not understand what the format for such a tributary volume should be, or even what type of material would be appropriate to include. Certainly I did not hope to put together a piece that would rival the quality of contributions that this issue of BBRC contains. I decided then to describe the process by which an Illinois physics graduate student, who came to the Midwest to continue spin resonance research under the tutelage of Charlie Schlicter (Haywood Blum, my undergraduate research advisor, was Charlie's graduate student and hence I had to continue the lineage) managed to even find the Department of Biochemistry. There was the detour through theoretical astrophysics to work on white dwarf stars with Fred Lamb, and the discovery of biological physics in the Illinois physics department through Peter Debrunner (my ultimate Ph.D. mentor), Hans Frauenfelder, and Eckard Münck. So in my contribution to the *Experiences in Biochemical Perception* volume I wrote a piece titled "From Stars to Electrons Through Cytochrome P450." In this I described the experience of finding the superhighway connecting the biochemists and physicists that existed at Illinois at the time. I thought it captured my novice excitement pretty well, something along the lines of C. P. Snow's "The Search." Well I can tell you that John Lipscomb, who contributes his recent pioneering post-Gunny work on non-heme oxygenases to this issue, was less than supportive of my attempt at writing a Gunny tribute. He accused me of an unbelievable level of pompousness to write such a personal account at such an early stage in my career, when I had just begun my own independent position and certainly had not earned the right to "tell stories." Well, I still haven't really earned the right to tell stories, and certainly not to rival the "Gunny stories" to which so many of the authors in this volume refer. My only comment regarding these "Gunny stories" is—they are all true! Many of these tales, however, become embellished with time, and in my case are folded into my own decaying memory. But this is how I recall the events. Note that a benefit of my earlier recounting of experiences in the Gunny laboratory is that I do not have to repeat the story of a physicist larva becoming the biochemist butterfly for this issue. Rather, we can just describe some of our recent interesting discoveries that build on decades of additional work on the reaction mechanism of Gunny's red enzyme—the cytochromes P450.

"Membranes! Theyre all in your head"

Bill Toscano (presenting his own beautiful recent work in this issue) was Gunny's graduate student when I

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went south of Green Street to learn biology and biochemistry. Bill was more the biologist and geneticist, and when he thought of branching out into cellular signaling for his postdoctoral work with Dan Storm, Gunny shouted: "Look, these enzymes [like adenylate kinase that Bill wanted to work with] are membrane-bound, and membranes are all in your head! You'll never really learn *how* these complicated systems *work*. You need a pure soluble system!"

This thought stuck with me for a long time. Gunny had given the world P450cam (or CYP101 as it is now called), a bacterial P450 system which was soluble, was produced to very high levels in *Pseudomonas putida*, and could be purified to absolute homogeneity with simple chromatographic steps. Although Jud Coon was the first to isolate and purify to homogeneity mammalian P450s, the thought in the Gunsalus lab was that the reconstituted systems from the endoplasmic reticulum were far from ideal since they could easily aggregate, and it was very hard to obtain them in the high yields necessary for structural and biophysical investigations by the out-of-control physics graduate students (such as Bob Austin, who writes in this issue). As Tom Poulos describes in this issue, the CYP101 protein provided the first precise three-dimensional structure of a P450 cytochrome. Indeed, many of the precise chemical and physical investigations of P450 mechanism continue to utilize this microbial P450 system. Certainly P450cam has paid my rent in the academic marketplace. Over the years, however, it became clear that this single P450 did not represent all of the important reactivity and component interactions that were present in the burgeoning number of eukaryotic P450s being isolated. I confess that some of Bill Toscano's ideas were finally beginning to creep into my head. Maybe reality set in when our American society of "biological chemists" became "biochemistry and molecular biology." Yes, perhaps there was some gold in these "membranes" about which Gunny cautioned. What about the membrane-bound P450s? What about all the other membrane proteins that are so critical to cellular function? What about the largest class of current drug targets—the G-protein-coupled receptors? What about the real machines of the living cell—the complex macromolecular assemblies that are involved in energy transduction and the processing of information and energy? Why couldn't we have a means for studying these membrane proteins with the same tools that are so powerfully applied to the soluble proteins? New methods were needed.

Nanobiotechnology to the rescue!

It's hard to pick up a newspaper these days and not read something about the "nano" revolution and how it's going to cure all human ills. Of course, like most things, there tends to be grade inflation. All the people working on "micro"-scale things (a millionth of a meter versus the billionth of a meter the nano prefix implies) are suddenly looking a bit pale. This is why one often finds seminars with nano in the title and find the abscissa of graphs labeled "nano-something $\times 1000$." In our case, however, we discovered a process that operates at the true nanoscale and addresses the general problems of membrane proteins.

Through continuing efforts over the past few years, we have begun to answer the questions of membrane protein function by developing the means to generate soluble and monodisperse membrane proteins embedded in their natural phospholipid bilayer environment. All at the single molecule level. The secret is chemical self-assembly, the process that Nature uses all the time to generate the complicated molecular machines that provide the workhorses of cellular function. Our self-assembled nanometer-scale phospholipid bilayers, which we term Nanodiscs, have opened the door for a simple and reproducible way to understand how membrane proteins, as Gunny would say, *work*.

Self-assembly of phospholipids and membrane scaffold protein

While using the atomic force microscope (AFM) to image cellular macromolecular assemblies, I was looking for cellular systems on the size scale of 5–50 nm that carried out important biological functions and also had complex structures that could be potentially revealed by the AFM. After hearing a seminar by Ana Jonas in our department describing lipoproteins, we began a successful collaboration in the imaging and manipulation of the human high-density lipoproteins (HDL) [2]. Various HDL particles could be reconstituted in vitro by simply solubilizing phospholipids and the corresponding apolipoproteins with detergent and then removing the detergent. Various types of structures could be generated. It was known that the human HDL fraction goes through various transformations, from a lipid-poor aggregated state through a discoidal state to the more prevalent spherical form under the action of various plasma enzymes [3]. We generated many AFM pictures of the discoidal form of HDL and even used the AFM to pattern phospholipids on surfaces [2]. These discoidal nanoparticles were indeed a beautiful example of natural self-assembly. After the conclusion of our work with the human lipoproteins, it occurred to me that the same process might also self-assemble an integral membrane protein if it were included in the detergent-solubilized milieu, thereby generating a particle with the target incorporated into what is really a nanoscale supported lipid bilayer. In these discoidal structures, the amphiphilic helical nature of the apolipoprotein would render

the entire system soluble in aqueous solutions. But the naturally occurring lipoprotein comprising the majority of the HDL fraction, Apo-AI, was not designed by Nature for such a function, inasmuch as it contains amino acid sequences that form receptor binding domains as well as regions in the folded structure that are responsible for association with plasma enzymes such as lecithin-cholesterol acyltransferases. More problematic was Nature's incorporation into the Apo-AI coding sequence features that convey the plasticity found in the normal cellular formation of the prevalent stable spherical entities involved in the transport of cholesterol and cholesterol esters. If we wanted to generate an ideal "scaffold protein" to support these nanoscale supported lipid bilayers, we would need to do some drastic engineering of the human apolipoproteins. In addition, it was clear that we could not collect enough human plasma to make all the variants of Apo-AI that the exphysicist in me could imagine!

Gunny always taught not to let your ignorance keep you from doing something new. In the mid-1980s I wanted to use the new powerful tools of genetic engineering to modify mammalian proteins to reveal their mechanisms of action. But the laboratory of this physicist had no idea how to clone and express such proteins in *E. coli.* So I thought since we did not know how to do this thing called "cloning," why not just build the gene from scratch and incorporate all the signals that would make the bacteria produce the modified protein of interest? Gene synthesis techniques are now common, but more than one "real" molecular biologist who visited my laboratory in the 1980s said this approach would never work. Even though it was hard, time-consuming, and expensive, the gene synthesis approach did indeed "work" and we ended up being the first to express the HIV protease in *E. coli* and generate huge quantities of sperm whale myoglobin, rat liver cytochrome b_5, and human hemoglobin that provided the foundation for many biophysical investigations in many laboratories. Well, if this worked for these mammalian metalloproteins, why not use the same methods to make self-assembling "scaffold proteins" that would form our Nanodiscs? Again, this approach worked better than we had hoped.

Gene synthesis, expression, and biophysical characterization of nanodiscs

We reasoned that a supporting scaffold protein that would solublilize lipid bilayers into a minimum energy discoidal shape could be generated by alpha helix elements, constructed with polar residues on the outside, facing solvent, and non-polar residues on the inside, facing the lipid acyl chains. In addition, punctuation marks, consisting of glycine or proline residues, could be placed periodically to break the semi-rigid alpha helix construct, thereby introducing turns in the overall scaffold. Our first gene constructs were made by standard recombinant DNA techniques and turned out to express as much as a gram of these "membrane scaffold proteins" (MSP) per liter of *E. coli.* Nice to know the old gene synthesis strategy still worked!

The idea was then to place a mixture of phospholipids and MSP in a non-equilibrium solubilized state, for instance, using detergent or high hydrostatic pressure, and then move the system to equilibrium by gentle removal of the perturbant. This initiates a process of self-assembly, wherein the phospholipids and MSP find each other and generate a discoidal phospholipid bilayer encircled by the MSP. The resulting little membrane fragment is truly a nanoscale object—10 nm in diameter and 5.5 nm high—and is soluble by virtue of the amphiphilic nature of the MSP. This is schematically illustrated in Fig. 1 and is fully described in a recent publication from our laboratory [4].

What is amazing in this nanoscale self assembly process is the overall precision. Fig. 2 illustrates calibrated size exclusion chromatographic analysis of the assembly that we call, and have trademarked, Nanodiscs. Yelina Grinkova documented the precise nature of the assembly by radioactively labeling the phospholipids. A nominal 10-nm-diameter Nanodisc, assembled with dipalmitoylphosphotidyl choline, contains 160 phospholipids—plus or minus two.

In designing the scaffold proteins that would self assemble phospholipids into Nanodiscs, we knew from the beginning that we would want to be able to control the

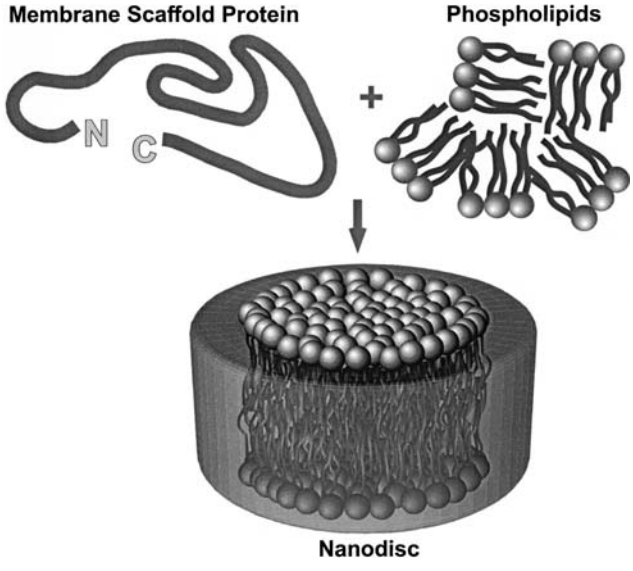

Fig. 1. Schematic showing the self-assembly of membrane scaffold proteins and phospholipids to form Nanodiscs. The resulting discoidal membrane structure is nominally 10 nm in diameter and 5.5 nm in height.

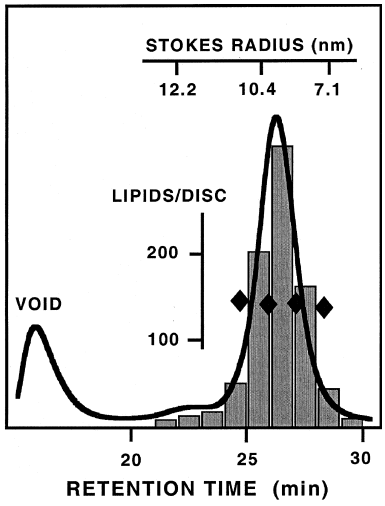

Fig. 2. Calibrated size exclusion chromatography on a Superdex 200 (Pharmacia) column illustrating the precision of the self-assembly reaction that forms Nanodiscs.

diameter of the resulting soluble membrane structure. An obvious solution was to make the belt longer—much as this author has had to increase his belt size since the grand association with Gunny. Fig. 3 illustrates a schematic of how this could be easily accommodated by simply increasing the number of "sausage links" in the encompassing belt. These various sized Nanodiscs are useful in controlling the self-assembly of receptors, which have varying oligomerization states, as well as the incorporation of larger membrane-protein assemblies into these soluble structures such as the photosynthetic reaction center (to make nano-light harvesting complexes) and chemotactic motors (to generate little gobots). Although we are developing Nanodiscs for use in the full atomic resolution structure of membrane proteins, it was in a Nanocolloquium (notice that even in the 1970s the Illinois group foresaw the "nano revolution") organized by Gunny and Hans Frauenfelder (contributing in this issue) where the following adage was introduced: "Use tools that give you only the resolution that you need to answer a given question." Solution X-ray scattering is such a tool. Through the work of Ilia Denisov, an independent research scientist in my laboratory, and Anne Lazaridies of Duke University, we were able to characterize completely the size and topology of Nanodiscs (Fig. 4).

The real beauty of the Nanodisc self-assembly process, however, is that one can simply and reproducibly incorporate membrane protein targets into these structures. The incorporated membrane protein then finds itself in a native-like environment with the stability and activities that are present in vivo. For example, in a very recent publication, Tim Bayburt has shown that the general class of 7-transmembrane helical proteins can be assembled into Nanodiscs [5]. Even more unanticipated, Natanya Civjan has shown that pre-purification of the membrane-protein target is not necessary! She was able to take various clones of plant and insect cytochrome P450s that Mary Schuler's laboratory at Illinois heterologously expressed and go directly from these expression systems into a soluble and active single nanoparticle system [6]. Gunny seemed particularly happy with when I told him of these results, not only because the Schuler-

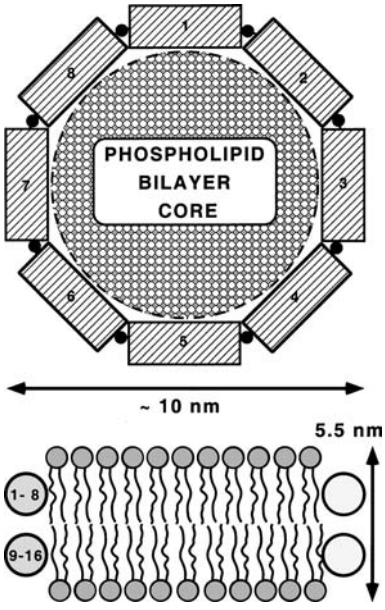

Fig. 3. Schematic diagram of engineered membrane scaffold proteins used to generate encircle and solubilize the supported phospholipid bilayer of Nanodiscs.

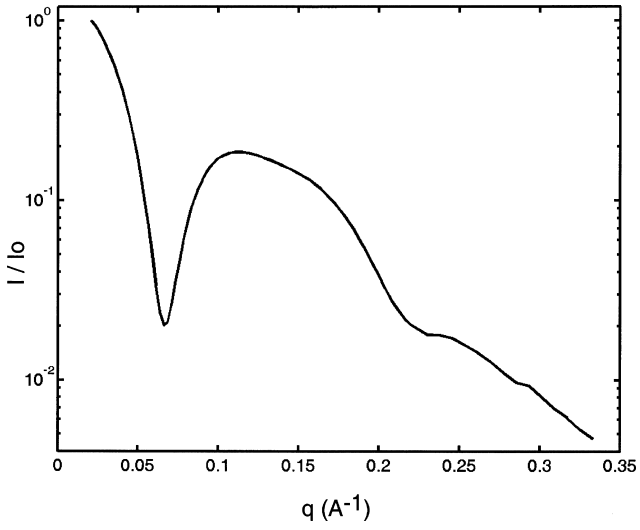

Fig. 4. Solution X-ray scattering of Nanodiscs. Nominal 10-nm-diameter discoidal bilayers generated from MPS1 and dipalmitoylphosphatidyl choline, as described in [4], are subjected to 15-keV X-rays from the Argonne Advanced Photon Source DND-CAT and the log of scattered X-ray intensity is plotted against the scattering vector $q = (4\pi/\lambda)\sin(\theta)$.

Sligar collaboration again yielded fruit but also that now the plethora of uncharacterized membrane-bound P450 systems were open for additional detailed physical and chemical characterization. After all, the lowly mustard plant has 270 P450s.

So where does all this lead? These little nanoscale membrane bilayer systems obviously have great potential for drug discovery and drug delivery. Gunny seemed mildly supportive when I started a company, Nanodisc, Inc., to begin commercialization of these discoveries. The learning curve for this endeavor has been pretty steep for a novice academic. More interesting, though, is the future basic scientific questions that Nanodisc technology can now address. For the Nanodiscs themselves, we have now in hand an excellent system to begin to understand the dynamics and pathways of complex biological self-assembly. The living cell certainly doesn't labor to generate the complex multi-component architectures that provide function. Rather, it relies on the natural selection of molecular recognition events to give rise to self-assembly. This is a buzz word of the "nano" phase that science is now immersed. In my laboratory, Tim Bayburt and Ilia Denisov are unraveling the physics of this process in Nanodiscs by equilibrium and time-resolved structural methods.

I mentioned at the beginning that I would not repeat my earlier comments written for Gunny's 70th birthday. However, in the closing paragraph of that volume [1], I indicated that my future scientific thrusts would include epithelial physiology and the study of membrane channels. Well, here we are 20 years later. Despite the beautiful and pioneering work that defined the three-dimensional structure of various membrane channels, I was frustrated by the difficulty in meeting Gunny's criteria: "pure protein, in quantity." With Nanodiscs, we have the tool to incorporate single copies of multi-component channels and transporters in a soluble entity. Through collaborations with Timp, Adesida, and Lyding at Illinois, and the Nanoscale Science and Engineering Center at Northwestern University headed by Chad Mirkin, we are beginning to put Nanodiscs on patterned surfaces, across precisely drilled nanometer diameter pores, and interfaced with semiconductor substrates. The era of the single molecule patch clamp is upon us.

It's appropriate for all of us to congratulate Gunny on his 90th birthday celebration. The many authors of this tribute all describe particular lessons and experiences that he bestowed upon them. My great thanks is simple. Gunny taught by his own actions that Science is fun. Follow your nose. Forget peer awards and society's definition of success. Do what you find interesting and enjoy daily the process of discovery. As I recall his saying: "Nature is subtle, but not malicious... She will reveal Her secrets with just a little inquisitiveness." We thank Gunny for showing us this road with the added benefit that, along the way, one meets like folks who share this vision and become lifelong friends.

Acknowledgments

I have endeavored in the text to acknowledge the individuals who contributed to the new science that was described. During the past 25–30 years since being in Gunny's laboratory, I have been fortunate to have many outstanding "partners-in-fun": graduate students, post-doctoral associates, research professors, technicians, and collaborators. In particular, my secretary for more than a decade, Aretta Weber, fields the calls, faxes, and e-mails from Gunny and is sometimes even able to translate them! I also thank John Lipscomb, who visited us in Seal Harbor, Maine, where I do most of my serious writing, and took time from our cycling, rock climbing, and sea kayaking to restrain my reminiscences so I don't make the mistake I did for Gunny's 70th tribute. In addition to the people, my laboratory has benefited from over 25 years of continuous funding from the National Institutes of Health. It began with Gunny dropping off an NIH grant application packet on John Lipscomb' s and my desks in the early 1970s together with some quip like: "Well if you are going to continue to spend money you had better get some—put together an NIH grant to pay yourselves." We did, and lo and behold it was funded. We thought that Gunny probably rewrote the entire thing, but a secretary told us later that he just signed the thing and sent it in. In addition to NIH funding, we have enjoyed recent NSF support, particularly through the Illinois Functional Genomics Center (Mary Schuler, PI), the Northwestern Nanoscale Science and Engineering Center (Chad Mikrin, PI), and through collaborations with Joe Lyding and Greg Timp through their "Protein Logic" initiative.

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