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Understanding the Design Principles of Living Systems at the Nanoscale

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Abstract: This is a summary of the Third Annual Cancer Nanobiology Think Tank hosted by the Nanobiology Program at the Center for Cancer Research at the National Cancer Institute-Frederick, National Institutes of Health. The Third Annual Nanobiology Think Tank was held in May of 2008 and was entitled "Understanding the Design Principles of Living Systems at the Nanoscale."

Key Words: Nanobiology, nanotechnology, cancer drug delivery, mechanical biology, single-molecule imaging, *in vivo* proteomics.

INTRODUCTION

The Third Annual Cancer Nanobiology Think Tank organized by Dr. Robert Blumenthal, Director of the Center for Cancer Research Nanobiology Program at the National Cancer Institute took place on May 13, 2008 in Frederick, Maryland. This year the think tank was devoted to the understanding of the design principles of living systems at the nanoscale. The invited speakers included Michael P. Sheetz, Professor of Cell Biology, Department of Biological Sciences, Columbia University who focused on cytoskeletal force and rigidity sensing mechanisms at a nanometer level and the integration of nanofabrication, bioinformatics, modeling, and cell biology. Jennifer Lippincott-Schwartz, Chief, Section on Organelle Biology, Cell Biology and Metabolism Program, National Institute of Child Health and Human Development, National Institutes of Health, described design principles of living systems at the nanoscale through her studies of the dynamics of membrane trafficking and the regeneration and functioning of intracellular organelles. Xiaowei Zhuang, Howard Hughes Medical Institute Investigator, Professor of Chemistry, Chemical Biology and Physics, Harvard University, discussed nanometer-resolution optical imaging approaches to real-time imaging of the interaction between nanoparticles (virions) and cellular structures. Finally, Jan E. Schnitzer, Scientific Director, Sidney Kimmel Cancer Center, presented elegant proteomic imaging of the endothelium and its caveolae *in vivo* with applications to targeted drug delivery and gene therapy.

The talks were complemented by discussion sessions and a series of poster presentations. The discussion sessions were focused on two general themes of how to advance the emerging field of nanobiology with particular interest in moving nanomedicine applications into practical use. The first discussion section led by Kuan Wang of the Laboratory of Muscle Biology, National Institutes of Arthritis and Musculoskeletal Skin Diseases, was a consideration of the involvement of the mechanical microenvironment in cellular behaviors such as cancer metastasis, stem cell differentiation and also in immune responses. The second discussion section led by Leonid Chernomordik, Section on Membrane biology, Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, centered on the important question of how to design delivery mechanisms to get therapeutic agents delivered to the appropriate targets in cells.

A. NCI Alliance and Nanotechnology

The Director of the NCI Alliance for Nanotechnology in Cancer, Piotr Grodzinski, started out the day by describing the National Nanotechnology Initiative (NNI) and how elements of the roadmap are helping to define the field by bringing together researchers from diverse backgrounds. The NCI Alliance for Nanotechnology in Cancer is a framework for multiple centers across the country as well as some complementary work that is done within the NIH intramural program. This includes eight Centers of Cancer Nanotechnology Excellence (CCNEs), twelve Cancer Nanotechnology Platform Partnerships (CNPPs), multiple training programs and the Nanotechnology Characterization Laboratory (NCL) at the NCI-Frederick which provides materials characterization and standardization to a wide array of researchers.

This is the third year of programs launched by the NCI Alliance for Nanotechnology in Cancer, and to date the work being done has focused on the development of improved methods for diagnosis, treatment, and monitoring of a wide variety of diseases. The success of this work is building the foundation for a new paradigm of therapy, where improved nanoscale approaches will be able to affect the development of disease in its very early stages and successfully reverse or significantly alter disease progression. The accomplishment of these goals will fundamentally change the current therapeutic paradigm for many diseases.

Two of the major goals of the NCI Alliance for Nanotechnology in Cancer are to bring technology into the realm of cancer diagnosis and treatment and to accelerate progress by bringing together physicists, engineers, biochemists and oncologists in large interdisclipinary centers. Snapshots from these centers include the work of Scott Manalis from MIT to develop an automated, multiplexed immunoassay with a high dynamic range for the clinical setting. Hongjie Dai of Stanford is developing single-walled carbon nanotubes that will monitor bio-events by producing electrical signals giving researchers very sensitive, specific and mutiplex detection systems for biological molecules.

Diagnostic imaging is benefiting from advances in nanotechnology. Significant development by Xiaohu Gao and Shuming Nie [1] and also by Xavier Michalet and Shimon Weiss [2] is being accomplished in the use of quantum dots for *in vivo* diagnostic imaging of tumor tissues. Ralph Weissleder of Massachusetts General Hospital and Harvard Medical School and Jean de la Rosette of the University of Amsterdam Medical Centre, Netherlands, are developing nanoparticle-based detection of lymph node metastasis using dextran-coated iron oxide crystals. Developments are also being made in MRI detection using novel mechanisms such as proteolytic actuation of nanoparticle assembly by Harris, Ruoslahti, and Bhatia [3]. Otto Zhou from the Carolina CCNE is taking the old technol-

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gy of x-ray tubes to a new frontier in developing novel field emission x-ray sources based on the carbon nanotube field emission technology for *in vivo* cancer detection and treatment.

One important feature that nanoparticles promise to allow is multi-functionality [4]. Because nanoparticles are large enough to incorporate multiple functional groups available for derivatization, a single nanoparticle can combine targeting, delivery, and reporting into a single unit. By optimizing these functionalities in concert, it is possible to increase efficacy and decrease toxicity, while at the same time improving ongoing assessments of therapeutic success.

There are numerous examples coming out of the NCI Alliance for Nanotechnology in Cancer of new ground being broken in targeting, therapy, and imaging. Dendrimers have been used by James R. Baker, Jr., Director of the Michigan Nanotechnology Institute at the University of Michigan, to target delivery of methotrexate to tumor cells markedly decreasing toxicity and increasing antitumor activity and allowing for multi-functionality including therapy, targeting and imaging. Naomi Halas and Jennifer West of Rice University are developing a new class of nanoparticles that are based upon metal nanoshells but have highly tunable optical properties. These have potential in a variety of applications including drug delivery, imaging, immunoassays, and photothermal therapy.

Docetaxel-encapsulated nanoparticle-aptamer conjugates have successfully targeted prostate specific membrane antigen in the hands of Omid Farokhzad of Harvard and his colleagues. Researchers are also developing particles that can release drugs with a trigger that is remote. These particles according to Sangeeta Bhatia and Erkki Ruoslahti could not only detect tumors but then deliver drugs to the tumors in a controlled and even sequenced manner. A team at Northwestern, Milan Mrksich and Bartosz Grzybowski and a team at UNC, Rudolph Juliano and Muhammad Yousaf, are working to use non-traditional parameters such as changes in cell motility and cytoskeletal organization to understand and eventually target cancer metastasis.

There are also many examples of intramural NCI research and collaborations with the intramural program and extramural researchers including the development of drug delivery methods using phospholipids by Alok Singh (NRL) and Robert Blumenthal (CCRNP), structural protein modeling by Ruth Nussinov (CCRNP) and Carlos Aleman of Universitat Politècnica de Catalunya, Barcelona, Spain, carbon nanotube sensor development by Javed Khan (Pediatric Oncology Branch, NCI) and Romel Gomez (University of Maryland) and the monitoring of nanoparticle quality control by Sriram Subramaniam (Laboratory of Cell Biology, NCI), who has developed collaborative partnerships with industry to advance new technologies for 3D imaging on the nanoscale. Another potentially useful drug delivery method is nanoantibodies which are being developed by Dimiter Dimitrov and his group (CCRNP, NCI). Several companies have already expressed interest and a research proposal has been developed for a Cooperative Research and Development Agreement (CRADA) with one of them.

Clinical translation in nanotechnology is also escalating. Two important examples were given of nanoparticles that have made the transition to FDA approval successfully. The first was Abraxane from Abraxis Bioscience, paclitaxel bound to albumin. The second example of successful clinical translation presented was Doxil which is a liposome encapsulated doxorubicin. A common theme emerges when studying the pathways that these two drugs have taken and that is to adapt existing drugs and *in vitro* diagnostic tools or those that have successfully progressed to a relatively late stage in development before encountering problems and then using the tools available from nanotechnology to improve or resurrect the development of the drug. There are important advances on the horizon for *in vitro* diagnostic tools to evaluate clinical samples and several companies are preparing to file investigational new drug applications within the next year.

B. The Case for Mechanics in Cancer: Oncogenes are Critical for Mechanosensing and Determining Tissue Shape

Michael Sheetz, Professor of Cell Biology from the Department of Biological Sciences at Columbia University gave an intriguing introduction to a critical dimension in our understanding of the behavior of cells: mechanical biology. By including the phenomena that are important in the mechanical microenvironment of the cell, he sets the stage for improving our understanding of how cells respond to stimuli *in vivo*.

An under-utilized perspective in our approach to nanoparticle development is to consider the stretching of molecules as events on the nanoscale and to posit that these events in live cells are determined by the entirety of the dynamic microenvironment. An underlying question is what causes a cell to have a mechano-response to its environment that results in transformation into a cancer cell? Dr. Sheetz presented evidence that physical signals such as the shape of collagen fibers, the spacing of fibronectin, and protein unfolding lead to activation and events that are sensed by cancer cells as the initial transformation signal in a rapid period of time, i.e., seconds to minutes, whereas we are usually observing these phenomena on a time scale of hours. This suggests that we should question and understand force and rigidity in terms of cellular biology and that force should be considered as a cofactor in phenomena such as motor protein velocity, force sensors in ion channels, catch bonds in bacteria and cytoskeletal rearrangements.

Questioning how cells sense displacement and force opens up a unique frontier on an intermediary scale termed the mesoscale. Dr. Sheetz used a nice analogy comparing three divisions of scale to a car with the mesoscale being analogous to the engine at 20-1000 nm. This is the scale of actin motility. The smaller scale is the molecular scale of 0.1 – 20 nm, or the parts of the engine, for example filament assembly. The larger end of the scale is the car or the cellular scale at >1000 nm. A key advantage to focusing due attention onto the mesoscale is that tools developed on this level will be likely to function in different environments.

A very intriguing example was presented of a cell pulling in a collagen fiber in a hand-over-hand motion using a cyclic process until the fibril was compressed over the cell. The example suggested an engineering approach to consider processes such as this retraction as steps in a cycle of extension, guidance, attachment, contraction, release. This example underscored the necessity that the collagen be studied in a three-dimensional manner as opposed to simply in two-dimensions.

The work presented by Dr. Sheetz reveals a new frontier in understanding how cells react to stimuli, specifically mechanical stimuli occurring at the nano-scale. This reveals many questions including how does a change in mechanical force translate into a signaling stimulus? Sheetz and colleagues devised an elegant way to investigate this by mechanically extending a phosphorylation domain *in vitro*. Upon stretching, the team found a remarkable enhancement of phosphorylation with no apparent change in kinase activity. Next an antibody was raised to the stretched version of this tyrosine phosphorylation substrate and the location of the stretched version of the molecule within areas of the cell under higher traction forces was correlated with increased levels of phosphorylation suggesting that *in vitro* stretching results are applicable to the cell. This led to the proposal that molecules like tyrosine phosphorylation substrates can act as force sensors and can transduce mechanical signals into chemical signals such as phosphorylation leading to activation of signaling pathways [5]. Sheetz and colleagues took this one step further by stripping away the membrane and soluble proteins and then showing that stretching could still activate downstream signaling through the remaining complexes [6]. What is needed for a mechnical sensor to function? The putative list includes two points of attachment, a central phosphorylation domain, locally active kinase and a transduction mechanism.

The work of Dr. Sheetz and his colleagues shows the dramatic involvement that mechanical events play in the transformation of normal cells into cancer cells. It will take a full understanding of these events not only to be able to stop the cancer transformation but also to be able to build small machines that function with the level of robustness with which these processes operate. If we are able to reach this level of understanding, there are many important things that can be learned and utilized by future engineers including the creation of functional models, how to compartmentalize reactions, how to design kinetic limits on reactions, and details about linking different functions in time and space.

C. Mechanical Biology: Cancer Metastasis, Stem Cell Differentiation and Immune Responses

A brainstorming session was presented by Dr. Kuan Wang of the Laboratory of Muscle Biology from the National Institutes of Arthritis and Musculoskeletal and Skin Diseases. He commented that an important challenge of nanobiology research lies in the interdisciplinary languages and cultures that must be mastered across a broad spectrum of scientific disciplines and that the development of a research theme in nanobiology can and should be biology and disease-driven in order to develop the necessary tools, rather than just a search for applications of existing technology and engineering tools. Speaking about mechanical biology, Dr. Wang pointed out that the conceptual and technical challenges exist because biological tissues are soft composite materials that behave in a manner that is non-linear, non-equilibrium, non-isotropic and time-dependent. Successful approaches must then be multidisciplinary and technology and engineering-driven. For biologists interested in mechanical biology and mechanical medicine, a major obstacle currently is the lack of commercial high through-put, real-time, non-invasive and user-friendly techniques and probes. To achieve these goals, funding agencies must first be convinced of the high level of importance to the scientific community.

Dr. Wang discussed the history of skeletal muscle research and how the studies of muscle cells and giant elastic muscle proteins contributed to the emerging field of mechanical biology and single molecule nanomechanics. For instance titin, the giant muscle protein, has revealed details about extensibility and passive elasticity. The intrinsically disordered region of titin, PEVK, is an elastic force sensor and signal transducer that binds signaling proteins in a stretch dependent manner. The protein nebulin has produced a fascinating debate on whether it is acting as a “stretchable” molecular ruler or not. This debate has led to the development of sophisticated protein engineering techniques to tag proteins specifically designed for atomic force microscopy. One other technique discussed as needing further development is how to study protein-protein interactions under stress in a high-throughput format.

A discussion of current experimental methodology segued into two presentations of *in silico* techniques and how they are helping to fill in the gaps in our understanding. Jeff Forbes, a Staff Scientist at the National Institute of Arthritis and Musculoskeletal and Skin Diseases, presented the advantages that can be gained by using steered molecular dynamics and coarse grain simulations to understand how proteins respond to stretch at the atomic level. Such simulations produced surprisingly similar force profiles that are experimentally measured by atomic force microscopy. Two examples were given of systems that have been amenable to combinations of these types of studies: titin and fibrinogen. An interesting project was described in which a method called Go model is being used to systemically unfold all protein structures within the RCSB Protein Data Bank.

Buyong Ma, a Senior Computational Scientist from Ruth Nussinov’s laboratory in the Center for Cancer Research Nanobiology Program at the NCI-Frederick, discussed inroads that are being made into the creation of a virtual cell at the nanoscale. Examples of work in this direction include the E-Cell system created by the Mesoscopic Biology Project, Institute for Advanced Biosciences, Keio University. The developers describe this project as a software platform to model, simulate and analyze complex heterogeneous and multi-scale biochemical reactions systems like the cell. Another example is the Virtual Cell by the National Resource for Cell Analysis and Modeling at the University of Connecticut, which purports to allow experimentalists to create models, define cellular geometry, specify simulations and analyze the simulation results. A third example is MCell from the Salk Institute which is a Monte Carlo Simulator of Cellular Microphysiology. Indeed, the virtual human cell nucleus was presented at the Sixth Foresight Conference on Molecular Nanotechnology by Tobias A. Knoch, Christian Münkel and Jörg Langowski back in 1998.

An elegant example of the progress in simulations is the production of the complete structure of a satellite tobacco mosaic virus particle that was produced by Peter Freddolino, Anton Arkhipov, Steven Larson, Alexander McPherson, and Klaus Schulten at the Center for Biophysics and Computational Biology at the University of Illinois. This was an all-atom molecular dynamics simulation of a complete virus in a drop of salt water with the entire system simulating the dynamics of more than a million atoms. It is predicted that as we continue to see increases in computer power, we will be able to achieve nanoscale simulations of the cell. This can also be facilitated by partitioning cell components, parameterizing nanoparticles, and coupling systems elucidated by systems biology.

D. Single-Molecule and Super-Resolution Imaging of Bio-Molecules and Cells

Increasing developments in light microscopy are very important to advancing the understanding of the design principals of living systems at the nanoscale. Dr. Xiaowei Zhuang, Howard Hughes Medical Institute Investigator and Professor of Chemistry and Chemical Biology and of Physics at Harvard University presented a recently developed form of high resolution light microscopy called stochastical optical reconstruction microscopy (STORM). This is an important technique development, one of only a few, that is successfully beginning to fill a void between optical live cell microscopy at a diffraction-limited resolution of approximately 300 nm and the angstrom level studies at 0.1 – 1 nm resolution of X-ray crystallography, nuclear magnetic resonance, and electron microscopy.

Dr. Zhuang and colleagues have engineered STORM to take advantage of the on-off switching that is made possible with photoactivatable fluorescence probes. There are three groups who simultaneously developed the use of photoactivatable probes to dramatically push forward resolution in light microscopy. Dr. Zhuang’s group, first reported STORM in Rust, *et al.*, Nature Methods, in 2006 [7]. Dr. Lippincott-Schwartz, in Betzig, *et al.*, reported the method photoactivated localization microscopy (PALM), in Science in 2006 and Hess, *et al*, reported, fluorescence activation localization microscopy (FPALM) in Biophysical Journal, also in 2006 [8, 9].

STORM is based upon imaging of photo-switchable fluorescent probes to temporally separate the otherwise spatially overlapping images of individual molecules, allowing the construction of high-resolution images. The imaging process includes multiple imaging cycles. In each cycle, only a sparse subset of the fluorophores is switched on, by weak activation light for example, such that each of the active fluorophores is optically resolvable from the rest. This allows the position of these fluorophores to be determined with nanometer accuracy. Over the course of many activation cycles, the positions of numerous fluorophores are determined and used to construct a super-resolution image. Initial studies were performed using a model system of probes placed at pre-determined distances on DNA molecules. The distances measured by STORM showed strong correlation with the probe distances which the researchers had designed and fabricated.

One of the next intriguing steps in the development of STORM was to add more colors. Dr. Zhuang's lab discovered a family of photoswitchable probes, each consisting of a photo-switchable cyanine dye reporter and an activator dye that facilitates the photoactivation of the reporter. Many different dyes will serve as reporters and activators so it was possible to use a combinatorial approach to build up a repertoire of color probes that could be successfully employed using a limited number of lasers. Cross-talk between the probes was found to be low usually at less than 10-20 %. Clusters can be resolved in this way at a few tens of nanometers. Dr. Zhuang showed comparisons between what is resolvable using widefield fluorescence microscopy and what is well-resolved by multi-color STORM including the striking resolution of clathrin-coated pits amongst microtubules, some images even revealing a center that appears hollow as is expected for the structure of the clathrin-coated pits [10].

The next progression of the STORM technique is to move into three dimensional space. This challenge is being addressed using the bias of a cylindrical lens. When a molecule's Z position is changed, there will be an elliptical difference in its image that will yield information in the Z axis without any need to scan. One striking example of the utilization of this principle was an image that was cut close to the membrane of a clathrin-coated pit. In this image, it is possible to resolve the spherical shell of the pit with a resolution of 21 nm in the X-axis, 22 nm in the Y-axis and 52 nm in the Z-axis. This is ten-fold better than can be accomplished using confocal scanning microscopy without the need to scan the sample [11].

Dr. Zhuang explained that barriers to future improvement of this method are all about the number of photons emitted by the probes, the label size and the labeling efficiency. The theoretical resolution limit that can be calculated is 2-3 nm using 6000 photons. Important things to consider for the further advancement of microscopic studies of living systems at the nanoscale are development of small-sized labels including genetically encoded tags and improvements to available cameras so that imaging capture speeds can be increased to keep up with imaging technology.

E. Mitochondria, Cell Cycle Control and Cancer

Jennifer Lippincott-Schwartz, Chief, Section on Organelle Biology, Cell Biology and Metabolism Program, National Institute of Child Health and Human Development, National Institutes of Health, presented elegant studies that utilize the sophisticated light microscopy techniques that her laboratory has developed to reveal the story of how cell cycle control is related to mitochondria by an important checkpoint at the G1-S boundary. This checkpoint is important for the propagation of mitochondrial information from the mother to the daughter cell. There are two extreme phenotypes in mitochondrial cell cycle morphology and these are the fission-dominant mitosis phenotype which shows distribution of divided mitochondria dispersed evenly around the dividing cell and the fusion-dominant G1-S stage in which mitochondria are fused together into a network that maximizes the mitochondrial volume. There are three characteristics that are keys to mitochondrial physiology: matrix and electrical continuity, membrane potential, and ATP generating capacity. It was possible using advanced light microscopy to determine the levels of all three of these key components.

Matrix and electrical continuity were measurable by photobleaching experiments. If the mitochondrial membrane is bleached, one can determine by recovery whether there is exchange or not of the membrane components and exchange indicates continuity of the membrane. It was determined that the G1-S stage matrix is continuous at this stage in the cell cycle.

In order to study membrane potential, tetramethylrhodamine (TMRE) was utilized. TMRE is a cell permeable cationic dye which is accumulated into the mitochondrion in proportion to the membrane potential. Using a two photon laser it was possible to selectively ablate the fluorescence in a portion of the mitochondrial membrane and then to observe whether or not there was a propagated depolarization. This indicated electrical connectivity and was further evidence of the continuity of the matrix membrane.

ATP generating capacity was another important hallmark of mitochondrial physiology. ATP production is maximal at G1-S and mitochondria serve as a checkpoint at this stage in the cell cycle. Dr. Lippincott-Schwartz made a strong case that we should pay attention to the role of the mitochondrion in cell cycle regulation and cancer.

F. *In Vivo* Proteomic Imaging of Endothelial Caveolae: A Gateway for Nanoparticles into Tissues & Tumors

Jan Schnitzer, M.D., Scientific Director and Professor of Cellular and Molecular Biology at the Sidney Kimmel Cancer Center presented his extraordinary story of using proteomics to search for a gateway into tissues and solid tumors using endothelial caveolae. Important caveats in nanoparticle design were brought to light. One is that there has yet to be a strategy for targeted delivery that has transitioned successfully into the clinic. The reason for this is that most techniques rely upon passive delivery with ninety plus percent of particles accumulating rapidly in the liver and spleen. Although some tumors have a characteristic "leakiness" that could help to overcome delivery issues, this leakiness is not homogeneous.

There are three endothelial barriers that need to be considered in the design of nanoparticles. The first is termed continuous and is restrictive including the blood brain barrier. The second endothelial barrier is discontinuous and constitutes the liver, spleen and bone marrow. This barrier is permeable with large gaps and is very easy to target, however, it is exactly these characteristics that make these tissues the nemesis of delivery objectives to other areas. Finally the third type of barrier is the fenestrated barrier. This barrier resembles 70 nm circular windows and has very high water content. Tumors have all three of these types of endothelial barriers, however, they are quite heterogeneous.

It was noted that this issue is not only important in nanoparticle drug delivery but also in methods such as the discovery of tumor markers by *in vivo* screening with phage libraries [12]. The overwhelming accumulation in permeable endothelium is a very important consideration in phage screening *in vivo*. Within 60 s, >90 % of phage will accumulate in the liver effectively eliminating 90 % of the possible library generators.

With respect to drug delivery, the key obstacle is getting in to the site where the therapy is needed. To address this problem, Dr. Schnitzer used the approach of targeting the vascular endothelium using proteomic mapping. The surface of the vessels was "painted" and caveolae were clipped off and characterized analyzing the protein, creating a database called Avatar and then validating the protein components. The importance of validation was stressed as requiring a minimum of 20-fold enrichment and a comprehensiveness that includes multiple methods and techniques. Caveolae were studied in multiple organs and tissues. Of 8000 proteins in the rat proteome, 1800 non-redundant proteins were identified in the plasma membrane with 500 proteins in caveolae and 100-200 of these were tumor specific proteins.

One of the proteins identified by the proteomic analysis was used to generate an antibody, EC-APP, for the purposes of using targeted transcytosis as a tool for delivery. A huge amount was localized into the parenchyma of tissues by gold staining in a very short period of time (~10 min). Dynamic intravital microscopy showed live imaging of caveolae actively pumping antibody across the endothelium; a striking finding as this pump is working against a concentration gradient. There was no accumulation seen without targeting and without caveolae. Rapid active targeted transcytosis with penetration in lung tissue only was verified by SPECT CT and the pharmacokinetics showed many fold increase in accumulation [13].

An interesting detail regarding this type of targeted transcytosis is the issue of size restrictions. Preliminary experiments suggest that there are significant limitations based upon size and so far a cutoff of about 100 nm seems likely. However, the elimination of liver scavenging may also help because it could be that larger particles simply have slower kinetics of entry and the liver pulls these larger particles out of circulation so quickly that the chance for uptake is effectively eliminated. This problem may be relevant to quantum dots which are fairly large. Dendrimers were mentioned as an intriguing alternative because one can use 5th generation dendrimers that are small, ~5.3 nm, yet contain ~128 functional sites.

Dr. Schnitzer is involved with one of the Cancer Nanotechnology Platform Partnerships (CNPPs) in the NCI Alliance for Nanotechnology in Cancer. He is the principal investigator on a large grant from the National Cancer Institute to map the vasculature utilizing newly developed techniques in genomics and proteomics. This work promises to produce a wealth of important targets that will allow for selective diagnostics and therapeutics to be developed using advances in nanotechnology.

G. How do We Get (Therapeutic) Stuff into Cells?

The day concluded with a productive brainstorming session led by Leonid Chernomordik, and entitled “How do we get (therapeutic) stuff into cells?” Joseph J. Barchi, Jr., Senior Scientist in the Laboratory of Medicinal Chemistry at the Center for Cancer Research, NCI-Frederick, brought up the importance of ligand density and binding. Multivalency is a component that many nanotechechnologies provide as a clear advantage to standard modes of therapy. However, Dr. Barchi warns that the potency and hence the efficacy need to be optimized for different targets. Some interesting examples were given one being selectins and leucocyte rolling: if the density is too high, the leucocyte will roll on by.

An important question emerged which is whether we will be able to achieve greater standardization in methods to measure binding data. Currently, researchers perform microarray analyses, however, these are based upon different chemistries and hence result in data that is difficult to compare productively. Surface plasmon resonance suffers from the same issue in that different chemistries are used and different data are produced and again it is difficult to do any cross-comparison. A very important parameter that needs to be defined for new nanoparticles is what is the optimal ligand number to achieve the greatest efficacy and the field will be propelled in answering this question by the development of standardization in binding techniques.

Kamran Melikov, Staff Scientist at the Laboratory of Cellular and Molecular Biophysics in the National Institute of Child Health and Human Development, NIH, also gave a short presentation highlighting not only the importance of considering different modes of entry into the cell but also how will the entry site be repaired after the drug or treatment modality has entered the cell. Different modes of entry are: 1) endocytosis, 2) membrane fusion, whether receptor-mediated or pH-induced, 3) destabilization by hydrophobic peptides and 4) endosomal swelling. One caveat for designing particles to enter via endocytosis is that the drug or therapeutic modality must be designed to escape the endosome if it is to reach targets other than endosomal targets. Membrane fusion is an interesting option but facilitation often requires large protein complexes. There is a great deal of information regarding membrane destabilizing peptides in the literature, however, it is still unclear whether these peptides are leading to entry directly via the plasma membrane or not and the efficacy of delivery is highly dependent on the size of the cargo.

The discussion then turned to shear force and the possibility of wounding a cell with ultrasound or by some other means to facilitate delivery. It was noted that if one uses ultrasound to wound a cell, the plasma membrane will be removed and also that if there is no gradient of calcium, there still will not be an influx even with the wound present. If it is possible to produce wounds in the cell, it must be addressed how these wounds will then be healed. Red blood cells have been found to reseal without machinery however, most cells will most likely require exocytosis, endocytosis or the membrane edges will have to reseal.

One of the major issues in nanobiology and in studying the design principles of living systems at the nanoscale, is imaging. The discussion centered on two important questions. One was whether we need the super-resolution of 20 nm for *in vivo* situations. It was noted that for many applications, pushing the limits down from 300 nm to 20 nm would result in too much data in a live imaging situation. It was also made clear that the bottlenecks to improvements in super-resolution imaging in cells are not currently the same as the bottlenecks that exist for development of *in vivo* imaging and that it will take development in both areas before we can say whether the two methodologies will coalesce. Another important question that arose is whether it is worth studying methods of delivery and therapy in cell culture. The need to study nanobiology within the complex microenvironment of the tissue in which cells reside was made evident during the think tank both in the case of targeting the vasculature and also in the important role that mechanosensing plays in the transformation to cancer. There was also a consensus among many who agreed that vital information can still be gained from experiments in cell culture especially in studying the details of biophysical mechanisms such as membrane fusion and the organization and function of intracellular organelles.

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