Recent Advances from the National Cancer Institute Alliance for Nanotechnology in Cancer

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ABSTRACT **Nanotechnology will have great impact on how cancer is diagnosed and treated in the future. New technologies to detect and image cancerous changes and materials that enable new methods of cancer treatment will radically alter patient outcomes. The National Cancer Institute (NCI) Alliance for Nanotechnology in Cancer sponsors research in cancer prevention, diagnosis, and therapy and promotes translation of basic science discoveries into clinical practice. The Fourth Annual NCI Alliance Principal Investigator Meeting was held in Manhattan Beach, California October 20—22, 2009. Presented here are highlights from the research presentations at the meeting, in the areas of *in vitro* diagnostics, targeted delivery of anticancer and contrast enhancement agents, and nanotherapeutics and therapeutic monitoring.**

Nanotechnology has the potential to provide novel, paradigm-shifting solutions to medical problems.[1] In oncology, nanomaterials can enable targeted delivery of imaging agents and therapeutics to cancerous tissue; nanoscale devices enable multiplexed sensing for early disease detection and therapeutic monitoring. In recognition of this potential, the National Cancer Institute (NCI) launched its Alliance for Nanotechnology in Cancer in 2004 to fund and to coordinate research that seeks to apply advances in nanotechnology to the detection, diagnosis, and treatment of cancer. The program is highly translational and focuses on techniques capable of producing clinically useful procedures. The Alliance builds on the premise of multidisciplinary research, engaging technology developers: chemists, engineers, and physicists, as well as biologists and clinicians—the community capable of identifying the most pressing needs of clinical oncology that are not met with currently available approaches. Each year, the NCI sponsors a meeting of Alliance researchers to disseminate research results and to encourage collaborations. The Fourth Annual Principal Investigator Meeting was held in Manhattan Beach, California October 20—22, 2009 and hosted by the NanoSystems Biology Cancer Center at the California Institute of Technology, one of eight NCI-sponsored Centers for Cancer Nanotechnology Excellence.

Over the past 50 years, there have been great advances in understanding fundamental cancer biology. However, for the most part, these advances have not translated into greatly improved clinical outcomes. This is largely due to the highly toxic and nonspecific nature of most cancer therapeutics, which limits their use and effectiveness *in vivo*. Nanotherapeutics have the potential to actively target tumors, increasing treatment effectiveness while limiting side effects. This improved therapeutic index is one of the great promises of bionanotechnology.

The earliest nanovectors for drug delivery were liposomes, and the first cancer-specific nanotherapeutic was DOXIL, a liposomal formulation of doxorubicin approved by the FDA in the mid-1990s for treatment of Kaposi's sarcoma and now also indicated for the treatment of refractory breast and ovarian cancer. The use of a liposomal cage improved the pharmacokinetic profile of the hydrophobic doxorubicin, promoting accumulation at the tumor site and probably also triggering enhanced permeability and retention in the tumor's leaky vasculature. Abraxane, an albumen-bound formulation of paclitaxel for the treatment of metastatic breast cancer, was approved by the FDA in 2005. These formulations offer improvements in therapeutic index for existing drugs, but the next generation of nanotherapy for cancer is expected to use active targeting of tumor-specific cell markers to deliver entirely new modalities of cancer treatment, including triggered release

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of cytotoxic molecules, genetic material, heat, or cellular disruption.

Given that positive cancer outcomes are associated so closely with early detection, another important goal of cancer nanotechnology efforts is to improve diagnostic capabilities, through *in vivo* imaging contrast enhancement and *in vitro* device development. The use of superparamagnetic (SPM) nanoparticles as magnetic resonance imaging (MRI) contrast enhancement agents was first described almost 20 years ago, and researchers soon recognized that the differential uptake of these particles by cancerous and noncancerous cells could serve as a potential cancer diagnostic signal. Feridex, an injectable solution of SPM iron oxide nanoparticles, was introduced to evaluate liver lesions associated with alterations in the reticuloendothelial system (RES) in 1996. Initially, SPM contrast agents were passively targeted by interactions with the host RES and immune systems, but advanced materials now use active targeting of cells and tissues *via* antigen and cell receptor recognition. Nanoparticles with unique optical properties, such as fluorescent quantum dots and noble metal nanoparticles exhibiting surface plasmon resonance absorption, are also being investigated for targeted imaging of cancerous lesions. The emergence of advanced probes for molecular imaging technologies, such as positron emission tomography (PET), has been another important development in high-resolution imaging for cancer detection and diagnosis. Multifunctional nanomaterials that combine therapeutic and imaging capabilities, either through their innate properties (*e.g.*, magnetic nanoparticles for simultaneous MRI imaging and hyperthermia treatment) or by carrying both drug and contrast agent molecules, are currently widely sought.

Advances in microfluidics combined with the development of DNA and protein microarrays greatly accelerated the genetic and proteomic analysis of cancer subtypes and the identification of markers of early or premalignant stage cancer and premetastatic disease. These technologies are being advanced to develop devices capable of rapid, multiplexed analysis of the molecular, genomic, and proteomic signatures of tissue or serum samples. The discovery and validation of markers of early stage cancers, premalignant growths, and metastasis would enable more rapid and effective treatment. Such diagnostic devices could also be used to monitor treatment efficacy and to rationalize therapeutic decision making to improve outcomes, to reduce side effects, and to circumvent treatment resistance.

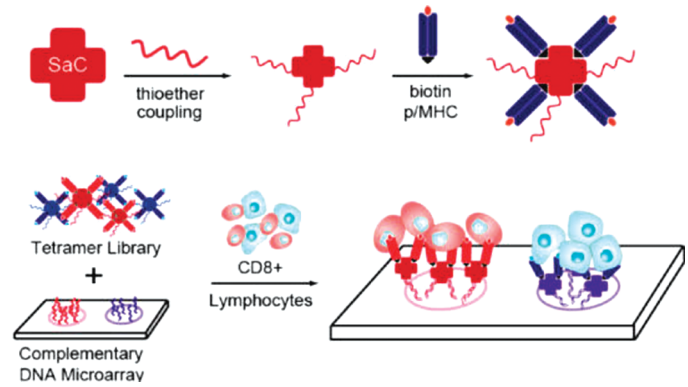


Figure 1. Self-assembled ssDNA-p/MHC tetramer arrays for multiplexed sorting of antigen-specific cells. The ssDNA-tagged p/MHC tetramers are produced by coupling ssDNA site-specifically to SAC prior to exposure to molar excess of biotinylated p/MHC monomers; p/MHC tetramer arrays are formed by pooling ssDNA-p/MHC tetramers of select specificity and hybridization to a complementary printed ssDNA microarray. T cells expressing the cognate TCR are detected by binding to the surface-confined tetramer. Reproduced with permission from ref 2. Copyright 2009 American Chemical Society.

Alliance Meeting Highlights. A presentation by Dr. James R. Heath of the California Institute of Technology titled "Analyzing Interactions between the Immune System and Cancer, with Clinical Applications to Glioblastoma Multiforme Diagnostics and Melanoma Immunotherapy" explored the intersection of *in vitro* diagnostics and therapeutics. Cancer immunotherapy boosts the immune system to suppress growth of the tumor without using toxic chemotherapeutics. One strategy is to collect nonspecific, cytotoxic T lymphocyte cells from a patient and to transfect the cells with a retrovirus carrying the gene for a tumor antigen-specific T cell receptor (TCR). The engineered cells are then grown in culture to $\sim 10^9$ cells and reinjected into the patient, where they target and kill tumor cells. The challenge the Heath group has undertaken is to develop a multiplexed platform to interrogate the antigens presented by the cancer cell peptide-major histocompatibility complex (p/MHC) and the corresponding antigen-specific T cells, enabling monitoring of adoptive cell transfer (ACT) immunotherapy.

Dr. Heath's group has developed a p/MHC microarray, analogous to a DNA or protein microarray. Cysteine-engineered streptavidin (SAC) tetramers are prepared and labeled with single-stranded DNA (ssDNA) and then reacted with biotinylated p/MHC, forming high-affinity probes for antigen-specific T cells, as shown in Figure 1. Each SAC-p/MHC probe has a unique ssDNA label, and a microarray of complementary DNA (cDNA) strands is spotted onto a substrate. The spatial separation of the cDNA enables robust multiplexed separation of distinct T cell populations or nucleic acid cell sorting (NACS), as ssDNA-SAC-p/MHC conjugates assemble onto the preformed DNA microarray through interaction of ssDNA labels with corresponding cDNA, also shown in Figure 1. Cleavage of the DNA tethers by appropriate endonucleases enables selective release of captured T cells.[2] Having established that the NACS arrays outperform conventional spotting strategies in homogeneity, reproducibility, and sensitivity of T cell detection for human Jurkat cells, the group is now preparing a library of tetramers to poll diverse T cell populations.

Although NACS is a promising technology for T cell recognition, additional detection technologies are necessary to determine T cell activation status. To investigate this, the group developed a

single-cell barcode chip (SCBC) for multiplexed detection of cytokines secreted by T cells. The SCBC is based on DEAL (DNA-encoded antibody library) panels previously developed in Dr. Heath's lab,[3] in which capture antibodies are tagged with ssDNA and immobilized on cDNA microarrays, enabling spatial encoding of captured proteins. DEAL technology also forms the basis for the integrated blood barcode chip,[4] which uses on-chip hydrodynamic separation of plasma to enable multiplexed detection of proteins from whole blood, with a sensitivity comparable to enzyme-linked immunosorbent assay (ELISA).

Technologies developed in Dr. Heath's lab are currently being investigated in a clinical trial with melanoma patients, outlined by Dr. Caius Radu of the University of California, Los Angeles in his talk, "Novel Technologies for Advanced *In Vitro* and *In Vivo* Monitoring of Adoptive Cell Immunotherapies in Cancer". Single-parameter NACS is being used to detect transplanted transgenic T cells in patients, and multiplexed NACS is being used to track T cell response to multiple cancer antigens and look for evidence of immune recruitment by the transplanted T cells against the cancer. SCBC and integrated blood barcode chip (IBBC) are being used to probe the numbers and activity of the transplanted T cells and secreted cytokines. These *in vitro* measurements are being combined and compared with the results of *in vivo* PET images of immune activation to track therapeutic efficacy.

DEAL technology is also under clinical investigation for molecular and functional analysis of glioblastoma multiforme tumors. This work was presented by Dr. Paul Mischel of the University of California, Los Angeles in his talk, "Design Considerations for the Translation of *In Vitro* Diagnostic Technologies into the Clinic". Cells were first digested with collegenase, and the released proteins were analyzed by DEAL to investigate tumor heterogeneity in the glioblastoma system. The IBBC[4] is being used to test glioblastoma patients against a 36-protein panel, to distinguish responders from nonresponders to Avastin therapy, an $80,000 treatment with severe side effects that is effective in only a subset of patients.

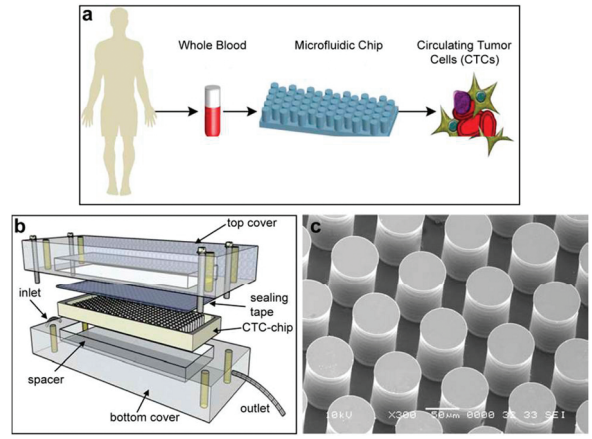


Figure 2. Microfluidic approach to isolate circulating tumor cells. (a) One-step process for point-of-care isolation of CTCs from peripheral blood. (b) Schematic of the manifold assembly. The microfluidic chip is sealed from above with a biological grade adhesive tape and placed in the manifold. (c) Scanning electron micrograph (SEM) of the micropost array. Reprinted from ref 5 with permission from Macmillan Publishers Ltd. Copyright 2007 Nature.

In a plenary lecture titled "Molecular Analysis of Circulating Tumor Cells", Daniel Haber of Massachusetts General Hospital described a method to capture and to analyze circulating tumor cells (CTCs) from metastatic cancer patients. He hopes to use CTC analysis for early detection of cancer, noninvasive analysis of tumor genetics, and measurement of therapeutic response. CTCs are extremely rare, accounting for as few as 1 in 10^9 blood cells, but Dr. Haber, with collaborators Dr. Mehmet Toner and Dr. Sunitha Nagrath, has developed a microfluidic chip that can reliably collect CTCs from the peripheral blood of cancer patients. In the chip, CTCs undergo controlled laminar flow through an array of EpCAM functionalized microposts, shown in Figure 2c. Hydrodynamic calculations indicated that triangular array geometry would maximize CTC capture efficiency, and the optimum flow rate was determined using phosphate buffered solution spiked with lung cancer cells.[5] The optimized CTC chip was able to capture CTCs in the blood of patients with metastatic lung, breast, pancreatic, colon, and prostate cancer with purity from 5 to 50%. The group also performed molecular analysis of captured CTCs to search for known cancer markers. Serial measurements of CTC count during treatment for metastatic epithelial cancers revealed correlation between CTC quantity and clinical response,[5] suggesting potential clinical utility.

Having established CTC capture with their chip, the group investigated genetic analysis of tumors *via* captured CTCs. Some patients with non-small cell lung cancer have an epidermal growth factor receptor (EGFR) gene mutation associated with dramatic response to tyrosine kinase inhibitor therapy. However, drug resistance usually appears within 1−2 years of treatment, associated with a second EGFR mutation, T790M. Dr. Haber's group collected CTCs from 23 patients with EGFR mutant tumors before and during treatment with an anti-EGFR drug and was able to identify emergence of the drug-resistant T790 M mutant.[6] They were also able to correlate reduced progression-free survival with existence of T790 M mutants prior to initiation of therapy. These results indicate that CTC capture and analysis may be a powerful tool to monitor and to manage therapy and an integral part of personalized medicine for cancer patients.

In a presentation titled "Targeting of Contrast Agents and Therapeutics by Activatable Cell Penetrating Peptides", Dr. Roger Tsien of the University of California, San Diego presented his work using activatable cell-penetrating peptides (ACPPs) to localize imaging and therapeutic agents to cancerous tissue. It is well-known that polycation sequences on ligands can indiscriminately deliver cargo (*i.e.*, small molecules, pro-

teins, nanoparticles) to cells *via* the endosomal pathway. Dr. Tsien's group has shown this nonspecific cellular uptake can be blocked by the addition of cleavable polyanion sequences. Cleavage occurs at the target cell surface through interaction with surface enzymes, activating cellular uptake of the peptide and payload.[7] Peptides sensitive to matrix metalloprotease (MMP) overexpressed by cancerous cells had increased penetration into xenograft HT-1080 tumors and surgical samples of human squamous cell carcinoma.[7] The enzyme cleavage uptake pathway was confirmed by inhibition of ACPP uptake *via* MMP-2 and MMP-9 genetic knockouts and inhibitors.[8]

Imaging studies of xenograft tumors showed enhanced uptake and reduced background for ACPPs appended to labeled dendrimers (Gd for MRI, Cy5 for fluorescence), probably due to decreased renal filtration of the large dendrimer conjugates.[8] Pre- and post-operative MRI imaging coupled with intra-operative fluorescence imaging indicates that the ACPP-dendrimer-guided surgery left significantly fewer xenograft cancer cells than traditional surgery. Tumor-free survival following surgery was greater with molecular fluorescence image guidance, as well. Dr. Tsien also noted the possibility of counterlabeling nerve cells for surgery, decreasing the odds of adverse surgical effects due to inadvertent nerve damage. In addition, preliminary results show enhanced efficacy for ACPP formulations of DOXIL (liposomal doxorubicin).

Work on cell-penetrating peptides was also presented by Dr. Erkki Ruoslahti of the Burnham Institute for Medical Research, University of California, Santa Barbara, in his talk, "Nanoparticle Routing *In Vivo*: Interactions with Cells and Tissues". Dr. Ruoslahti's group has developed a nanoparticle delivery system based on their research showing cell internalization and tissue penetration by peptides with a C-terminal arginine (or rarely, lysine) residue, that is, an R/KXXR/R motif.[9] Binding occurs only if the motif is located at the C-terminus of the peptide, which the group has termed the "CendR rule", but revealing internal CendR elements by proteolytic cleavage of end residues can activate non-CPPs into CPPs. Affinity chromatography studies showed that the CendR motif binds to the neuropilin-1 on target cells;[10] neuropilin-1 plays an important role in angiogenesis and regulating vascular permeability, so CendR peptides have enhanced tumor penetration capabilities.

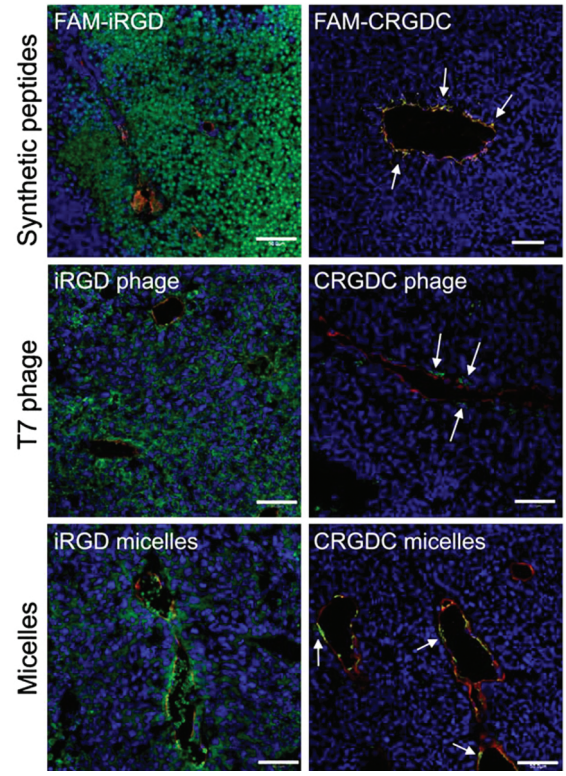


Figure 3. Confocal images of orthotopic 22Rv1 human prostate cancer xenografts from mice injected with the indicated peptides, phage, and micelles. iRGD was compared to a similar integrin-binding but nonpenetrating peptide, CRDGC. The circulation time was 2 h for the free peptides, 15 min for the phage, and 3 h for the micelles. Red, CD31; green, peptides, phage, or micelles; blue, nuclei. Arrows point to CRGDC compounds in or just outside the vessel walls, illustrating their targeting the tumor vasculature. Representative fields from multiple sections of five tumors are shown. Scale bars, 50 mm. Reproduced from ref 10 with permission from Elsevier. Copyright 2009 Elsevier B.V.

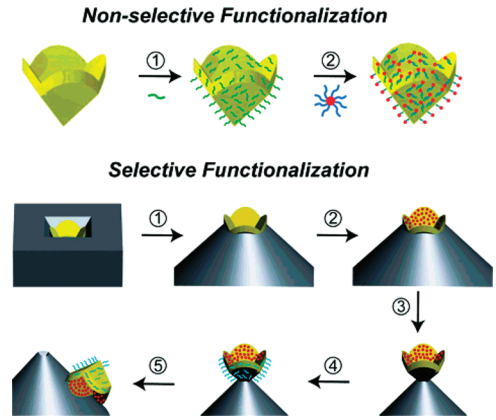
Dr. Ruoslahti's group has devised a tumor targeting strategy using a peptide, internalizing RGD or iRGD, with both CendR and RGD motifs. The RGD binds to αV integrins on endothelial tumor cells, followed by a proteolytic cleavage that exposes the CendR element for cellular uptake.[10] Studies of a fluorescein-labeled iRGD (FAM-iRGD) showed homing to and penetration of tumor tissue in xenograft models, even with micelle and phage cargoes, as shown in Figure 3.[10] Additional studies indicated increased delivery, penetration, and antitumor activity of iRGD-conjugated herceptin and DOXIL and increased efficacy of Abraxane without cardiotoxicity. Work with Dr. Michael Sailor at the University of California, San Diego has also shown that iRGD coupled to magnetic nanoworms (chains of magnetic nanoparticles ~80 nm long and 30 nm wide[11]) enhanced MRI image contrast.[10]

In a session devoted to clinical translation of nanotechnology, Dr. Mark E. Davis of the California Institute of Technology discussed his experience bringing a nanoparticle formulation of siRNA to clinical trials in his presentation, "In the Clinic with Nanoparticles Containing Nucleic Acids". Generally, siRNA-based therapies use localized delivery, due to rapid clearance of siRNA from the system. Dr. Davis and Dr. Antoni Ribas of the University of California, Los Angeles, performed the first targeted siRNA therapy in patients, as part of a phase 1 trial that began in May 2008. Their construct is a cyclodextrin nanoparticle

(CALAA-01) with a human transferrin (hTf) ligand for delivery to solid tumors. The siRNA targets the ribonuceotide reductase subunit 2 (R2) gene. The drug formulation is a cyclodextrin-containing polycation CDP/AD-PEG/AD-PEG-Tf in one vial, to be combined with siRNA in another vial in the pharmacy.[12] The final construct is ~70 nm in diameter, with a molecular weight of ~10^8 g/mol, and contains ~10 000 CDP molecules, 2000 siRNA, 4000 AD-PEG, and 100 AD-PEG-Tf.

Prior to the start of the phase 1 trials, Dr. Davis was concerned about complement activation, due to the charged particle surface and previous evidence of hypersensitivity reactions to PEG. In addition, nucleic acids can cause cytokine storms. Nonhuman primate studies indicated that 3 and 9 mg siRNA/kg doses were acceptable, but that 27 mg/kg caused renal impairment. Antibodies were raised at low titer to the Tf targeting ligand, but these did not affect pharmacokinetics. Extensive preclinical studies were conducted in primates, rats, dogs, and mice, in single dose and multidose, multicycle regimes, due to the novelty of the therapeutic. Measured outcomes included serum chemistry, hematology, blood pressure and respiration, organ weight, blood pharmacokinetics, cytokines, histopathology, complement, and histamine. The next steps are phase II and phase III trials of therapeutic efficacy. It is not clear when these trials will be held; Dr. Davis is charting an entirely new path here since the FDA has not approved a nucleic acid formulation to date.

Dr. Tayyaba Hasan of Harvard Medical School and Massachusetts General Hospital gave a presentation titled "Optically Activated Nanoconstructs: Therapeutics and Diagnostics" about her group's work with nanoparticle agents for photodynamic therapy (PDT). In PDT, light interacts with a photosensitizer, triggering release of heat or reactive oxygen species from the photosensitizer and killing nearby cancer cells. The photosensitizer may also fluoresce, enabling imaging for diagnosis, guided biopsy resection, or therapeutic monitoring. However, following PDT and many other cancer therapies, vascular endothelial growth factor (VEGF), a major regulator of angiogenesis important to cancer progression and metastasis, is known to be upregulated. The increasing VEGF level can promote metastasis. Avastin, a humanized, monoclonal antibody, recognizes and blocks VEGF, but VEGF release by cancer cells during PDT is distributed too widely in the extracellular matrix to be cleared by Avastin. Avastin itself is cell-impermeable and therefore requires a vehicle for intracellular delivery to target and to block VEGF prior to release.



Scheme 1. Non-selective and selective functionalization of gold pyramidal shells. Free-standing gold nanopyramids can be decorated uniformly by (1) assembling thiolated single-stranded DNA to their surfaces and (2) hybridizing to their complements attached to gold colloids. Template-bound pyramids can be differentially modified by (1) etching the silicon template, (2) functionalizing the inner surfaces with one molecule, (3) exposing the outer faces by etching the template, (4) functionalizing with a different molecule, and (5) releasing the pyramids. Reproduced with permission from ref 16. Copyright 2007 American Chemical Society.

Dr. Hasan's group used nanocells,[13] lipid bilayer nanoparticles containing the photosensitizer benzoporphyrin, to target tumors passively for PDT using the enhanced permeability and retention effect. The photosensitizer core was surrounded by an Avastin layer and poly(ethylene glycol) shell, for a total diameter of ~170 nm. Using Alexa-fluor-labeled Avastin, they were able to confirm intracellular delivery of the drug. Dr. Hasan's group previously developed a technique for *in vivo* imaging of secreted VEGF during PDT and Avastin therapy using hyperspectral imaging.[14] Studies of an orthotopic pancreatic cancer model showed enhanced delivery of Avastin to pancreatic tumors following tail vein injection with nanocells. Compared to conventional Avastin PDT therapy, there was also a significantly reduced local tumor burden and reductions in both the frequency and extent of distant metastases in the lungs, liver, and lymph nodes.

A novel biomarker detection technique was presented by Dr. Teri Odom of Northwestern University in a talk titled "Wide Field Imaging of Nanopyramid Probes To Detect Biomarkers". Since current immunohistology techniques are poorly quantified and typically only ~80% reliable, Dr. Odom has focused on noble metal nanopyramids developed in her lab as countable biomarker tags. The nanopyramids are fabricated in a process called PEEL (Scheme 1): **p**hase-shifting photolithography; **e**tch; **e**lectron-beam deposition; **l**ift-off,[15] with dimensions from 80 to 500 nm and tip sizes as small as 2 nm. The pyramid surfaces can be selectively functionalized, as shown in Scheme 1.[16] The pyramids were studied using dark-field microscopy and spectroscopy to correlate pyramid orientation and structure (size, shape) and scattering properties, which are complex and highly structure dependent. For pyramids larger than 250 nm, the scattering spectra and image intensity are orientation dependent (tip-up or tip-down).[16] Exploiting these properties, Dr. Odom's group has developed a wide-field imaging technique that enables rapid and reliable determination of nanopyramid location, distribution, and orientation on substrates and *in vitro*.

Dr. Odom's group is now investigating the use of antibody-labeled nanopyr-

Although numerous novel materials have been developed for *in vivo* use, more detailed understanding of the pharmacokinetic and toxicological properties of these materials needs to be developed.

amids to image cancer cells and studying the optimal pyramid size, shell thickness, and tip geometry (sharp or blunt) for heat generation for PDT.[17] The combined theranostic properties of the nanopyramids should enable quantification of the PDT process, including the number of pyramids per cell, their orientation, and associated therapeutic efficacy.

LOOKING FORWARD

The field of cancer nanotechnology has developed rapidly in recent years. Among Alliance researchers alone, there have been over 1000 papers published in peer-reviewed journals and more than 200 patents disclosed or filed since 2005, and there are currently eight clinical trials associated with Alliance-funded work. The challenge now is to continue successful translation of the technological achievements in the field into broad clinical practice. Although numerous novel materials have been developed for *in vivo* use, more detailed understanding of the pharmacokinetic and toxicological properties of these materials needs to be developed. This is perhaps the largest barrier to rapid translation of large numbers of nanotherapeutics to the clinic. New devices are expanding the panel of biomarkers and signatures at our disposal for cancer diagnosis and progression prediction and improving the limits of detection for known markers, but validation of new markers and measurement reproducibility across platforms are serious issues that have not been resolved.

The successes described here were accomplished through collaborative efforts between engineers and physical and life scientists, leading to the development of technologies and early clinical solutions that promise to improve cancer detection and treatment. Forging new partnerships with clinical researchers and practitioners is the next step necessary to fulfill the promise of cancer nanotechnology and to change radically the experience of having cancer in the 21st century.

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