CHAPTER 7

APPLICATIONS: NANOBIOSYSTEMS, MEDICINE, AND HEALTH

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7.1 VISION FOR THE NEXT DECADE

Changes in the Vision over the Last Ten Years

Over the past decade, nanomedicine and nanobiology have undergone radical transformations from fantasy to real science. The days of discussing advances in this area in the context of "nanobots" are over, and systems and nanomaterials have emerged that provide major analytical or therapeutic advantages over conventional molecule-based structures and approaches. We have come to recognize that much of biology is executed at the nanoscale level, therefore providing a rational approach to using the structure and function of engineered nanomaterials at the nano-bio interface for interrogation of disease, diagnosis, treatment, and imaging at levels of sophistication not possible before (Nel et al. 2009). Fabrication of a host of nanostructures has been coupled with advanced chemical manipulation in order to impart biological recognition and interaction capabilities. Often, chemical manipulation results in nanomaterials that provide performance enhancement of therapeutics, imaging agents, diagnostics, and materials for tissue engineering and for basic science applications.

Thus, the foundation has been laid for creating systems that can revolutionize the fields of medicine and biology. Early work has provided significant evidence that the properties afforded by nanostructures offer not only different but also better ways of detecting, managing, treating, and in certain cases, preventing disease. Analytical tools have been invented that allow imaging and manipulation of biological structures in ways that would have been viewed as science fiction just a few years ago. These tools are dramatically accelerating the fundamental understanding of complex biological systems and providing a basis for understanding the rapid translational advances being made on the nanomedicine front. Through the application of nanomaterials in *in vitro* and *in vivo* biological systems, and with sophisticated tools to monitor such nano-bio interactions, it has become increasingly appreciated that such interactions are complex and warrant directed evaluation as we move forward in the next ten years. This chapter describes some representative examples of nanotechnology-based tools, materials, and systems that are having major impacts in both biology and medicine. The final several sections provide an international perspective on the impact of nanotechnology and nanobiosystems in health and medicine.

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Vision for the Next Ten Years

Bio-organic and synthetic chemistry are contributing to a new interfacial science that is of great importance to better understand biology at the nano-bio interface and to specifically design engineered nanomaterials or nanoscale systems that can fundamentally impact human health (Nel et al. 2009). (For a unique perspective on synthetic biology and nanotechnology see Section 7.8.2.) The nano-bio interface is defined as the dynamic physicochemical interactions between nanomaterial surfaces and the surfaces of biological components such as proteins, membranes, phospholipids, endocytotic vesicles, organelles, DNA, and biological fluids. The outcomes of these interactions determine nanoparticle uptake, bioavailability, and the possibility to carry out bio-physicochemical reactions that could be of therapeutic and diagnostic use. This includes the dynamic interactions that determine the formation of a protein corona that is specific to individual biological compartments, particle wrapping at the cell surface membrane, endocytotic uptake, and lysosomal as well as mitochondrial perturbation that can affect drug delivery and the safety of nanomaterials (Nel et al. 2009). Interactions between biological and nanoparticle components could, in reverse, lead to phase transformation, surface reconstruction, dissolution, and release of the surface energy from the nanoparticles. Probing these nano-bio interfaces at the biological level permits prediction of structure-activity outcomes based on nanomaterial properties such as size, shape, surface chemistry, surface charge, hydrophobicity, roughness, and surface coatings.

Through a more comprehensive understanding of the bio-nano interface, nanomedicine will mature into a higher-throughput and more predictable endeavor. This new branch of medicine will revolutionize the way medicine is practiced, create a new pipeline of diagnostic and therapeutic capabilities for the pharmaceutical industry, and catalyze extraordinary advances in molecular and cell biology. The cornerstones of nanomedicine are depicted in Figure 7.1, which delineates that substantially improved diagnosis, imaging, and treatment can be obtained through the use of nanomaterials and devices that are capable of performing analytical and imaging functions, targeted therapy and drug delivery, imaging plus delivery functions (theranostics), as well using nano-enabled material surfaces for tissue engineering.

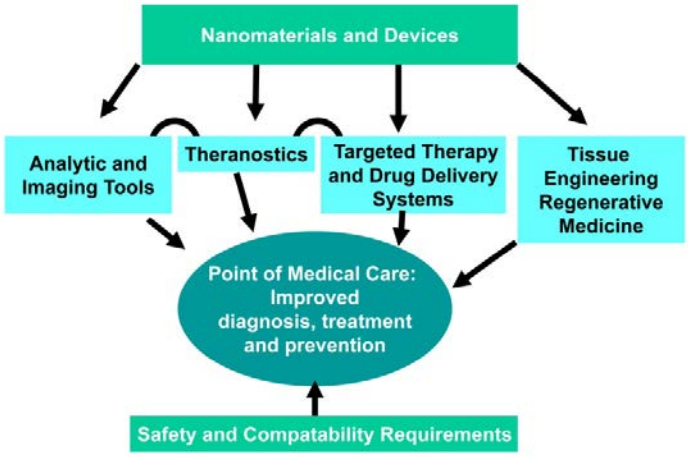

Figure 7.1. The cornerstones of nanomedicine (courtesy of A. Nel).

The most promising future nanoscience-based applications in medicine (Figure 7.2) are ultrasensitive and selective multiplexed diagnostics, drug delivery, targeted treatment of cancer and other diseases, body imaging, tissue/organ regeneration, and gene therapy. All of these applications combine engineering advances with improved strategies for manipulating biological systems. New approaches for drug delivery, imaging, and diagnostics will be refined and developed, and more sophisticated nano-therapeutics and diagnostics will supplement those already in clinical use or in clinical trials. To facilitate this development, it will be necessary to implement new manufacturing approaches. All new products must address stringent safety and compatibility standards that are being challenged by the novel properties of engineered nanomaterials and the potential that these may introduce new biohazards (Nel et al. 2006).

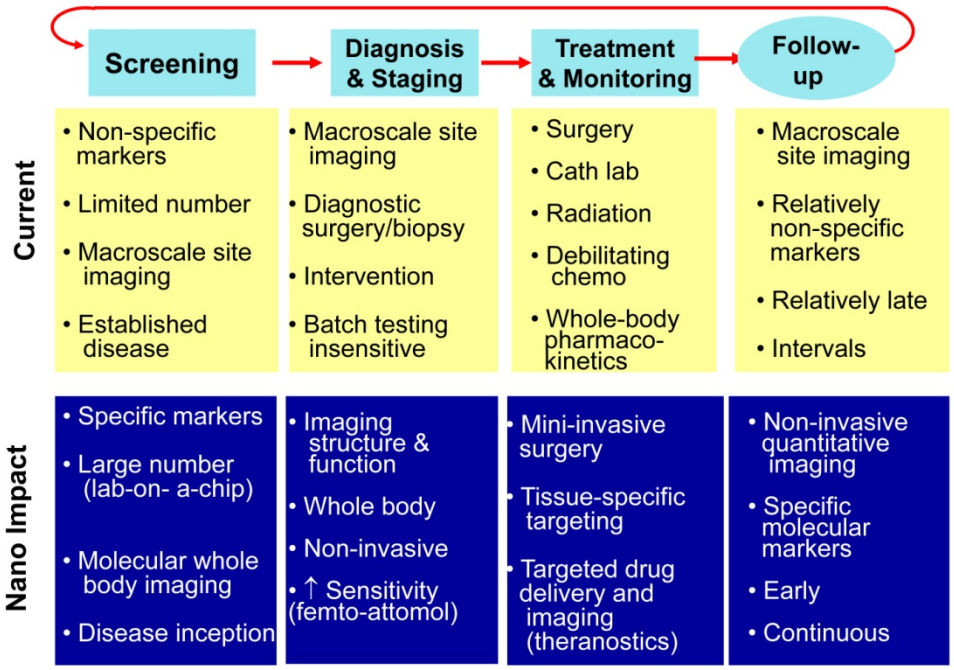

Figure 7.2. Proposed impact of nanotechnology on future clinical care. Modified from the European Science Foundation Forward Look report, *Nanomedicine* (European Science Foundation [ESF] 2005).

Nanotechnology is enabling the development of highly accurate *in vitro* and *in vivo* sensors, novel imaging contrast agents, and platforms for localized therapy (Grodzinski et al. 2006) (see also Section 7.8.3). Current sensors are largely *in vitro*; a reduction in sample size and multi-analyte detection capabilities continue to improve guidance for traditional therapies. In the future, diagnostics will provide earlier and more accurate bioanalyte detection capabilities, and localized nanotherapy will provide more effective treatments. Furthermore, advances in nanodiagnostic capabilities will allow for a more robust realization of personalized medicine, because disease phenotyping biomarkers will be used to direct appropriate patient- and disease-targeted therapies. Quantitative *in vivo* nanosensors will allow a leap in systemic diagnostics at multiple sites, and this capability together with localized and targeted nanotherapy will greatly increase treatment efficacy and serve to minimize side effects. Although *in vivo* diagnostics hold the greatest promise for the long run,

in vitro diagnostic approaches are more quickly translated to clinical applications, for several reasons: these include a less complex regulatory environment, greater familiarity and success with current sensor platforms, and more direct access to relevant clinical samples. The next step will be *in vivo* sensors that do not require systemic injection. Ultimately, as knowledge of specific disease biomarkers increases and therapeutics become more highly refined, the demand and necessity for systemic *in vivo* sensors will increase. Finally, continued advances in bioengineering and at the bio-nano interface will provide robust means to guide stem cell fate and to regenerate tissues.

Nanotechnology is also enabling novel, specialized treatments for cancer. This will be a high-impact area of nanomedicine in the next ten years (see Section 7.8.1). In spite of historic advances in cancer biology and the development of ever more sophisticated chemotherapeutics, the outlook for patients with advanced cancers is still grim; for some cancers (e.g., brain, ovarian, lung, and pancreatic cancers), there have been no appreciable changes in life expectancy for the past 50 years. Several nanomedicine pathways can improve this outlook through: (1) fuller realization of the opportunities in personalized medicine for the identification and targeted treatment of sub-sets of patients with given cancer types, and (2) development of targeted therapeutics with higher efficacy and reduced side-effect profiles. Accompanying the increasing capabilities for nanoscale therapeutics to target treatments and limit adverse effects for cancer patients over the next 10 years should be regulatory changes that make the most promising candidates available to patients more rapidly. In fact, opportunities for nanotechnology in cancer treatment are many fold and are expected to have a significant impact. Over the next 10 years it should be possible to:

- Develop point-of-care nanodevices for early diagnosis and therapeutic response monitoring capable of using unprocessed bodily fluids with multiplexing and rapid analysis capabilities
- Develop diagnostic and post-therapy monitoring nanodevices for the detection and interrogation of circulating tumor cells and circulating tumor initiating cells
- Conduct successful clinical trials for nanoparticle delivery of siRNA molecules and other nucleic acid therapeutics
- Demonstrate novel nanoparticle-based drug formulations with significant improvement in targeting therapeutic windows as compared to free drug delivery
- Design particles to enable penetration of the blood-brain-barrier and enable more effective treatment of brain tumors
- Leverage nanotechnology-based studies of cell migration and cell motility for the development of anti-metastatic drugs
- Leverage nanotechnology tools to enable patient stratification for more personalized medicine
- Develop nanoparticle-based techniques to overcome the multidrug-resistance (MDR) mechanism
- Develop nanoparticle constructs capable of probing tumor microenvironments for tumor recognition and/or triggered drug release
- Implement nanotechnology-based techniques for intraoperative monitoring during surgery
- Develop theranostic multifunctional nanoscale platforms capable of interrogating the tumor microenvironment, subsequently administering therapy, and providing a readout of therapeutic efficacy

The impact of nanotechnology on cancer and other diseases depends on the design and construction of devices to diagnose, treat, and monitor disease at all stages. In addition, new tools and devices are needed to understand the processes behind the development and spread of a disease and to reverse or alter the progress of the disease.

In summary, the overall impact of nanotechnology on future point-of-clinical-care delivery will be multifaceted, with significant advances in patient screening, diagnosis, staging, treatment, and monitoring.

7.2 ADVANCES IN THE LAST TEN YEARS AND CURRENT STATUS

Nanobiosystems and nanomedicine are the most exciting and fastest-growing areas in modern nanotechnology research. Indeed, nanostructures are now being used in the development of powerful tools for manipulating and studying cellular systems, in ultrasensitive *in vitro* diagnostics that can track indications of disease at very early stages, in *in vivo* imaging agents that provide better contrast and more effective targeting compared to molecular systems, and in novel therapeutics for debilitating diseases such as cancer, heart disease, and regenerative medicine. Over the past decade, research and advances in these areas have occurred at an extraordinary pace, and looking to the future, these subfields of nanotechnology will experience a revolution in new capabilities. Major milestones over the past decade have included:

- FDA-approvals for the first nanotherapeutics (Alexis et al. 2010)
- FDA-clearances for the first nano-enabled *in vitro* diagnostic tools (Tang et al. 2007)
- The first siRNA human trials involving nanomaterial delivery systems (Davis et al. 2010)
- Development of intracellular probes for measuring the genetic content of a cell as well as its metabolic activity (Prigodich et al. 2009; Seferos, Giljohann, Hill et al. 2007; Zheng et al. 2009)
- Imaging of the sites of therapeutic targeting (theranostics) (De la Zerda et al. 2008; Kreen et al. 2008; Zavaleta et al. 2008; Major and Meade 2009; Song et al. 2009)
- Development of mechanized nanoparticles that are capable of stimulus-responsive release of guest molecules (Lu, Liong, Sherman, et al. 2007; Liong et al. 2008; Xia et al. 2009; George et al. 2010; Meng et al. 2010; Lu, Liong, Li, et al.; Meng et al 2010)

Although there are many nanotechnologies that have had significant impact in biomedicine, a few important examples are included below for illustrative purposes.

In vitro Diagnostics

The Bio-Barcode Assay

The medical field is increasingly anxious to adopt novel technologies that enable the accurate and early detection of disease-specific biomarkers so that timely and personalized treatments can be delivered. High priorities, moving in parallel with the development of new technologies to detect existing biomarkers, are the identification and validation of "low-abundance" biomarkers. Detection of disease- or patient-specific biomarkers at an early stage requires technology capable of measuring markers when in low abundance. The bio-barcode assay, pioneered by the Mirkin Group at Northwestern University, is a detection technology that provides significant sensitivity advantages over conventional methodologies such as the enzyme-linked immunosorbent assay (ELISA), without requiring costly, time-consuming, and technically demanding enzymatic amplification steps (e.g., those required by polymerase chain reaction, PCR, techniques) (Nam et al. 2003; Mirkin et al. 2004; Georganopoulou et al.

2004; Thaxton et al. 2005; Stoeva et al. 2006; Nam et al. 2004). The bio-barcode assay and variants of it are being developed for a number of disease processes where early detection is of paramount importance; it is suitable for subsequent adaptation to any number of others.

The bio-barcode assay is a sandwich assay involving two probe species that collectively function to detect and isolate a targeted biomarker and then amplify its presence at the time of assay readout without using enzymatic signal amplification (Scheme 1, Figure 7.3; Nam et al. 2003).

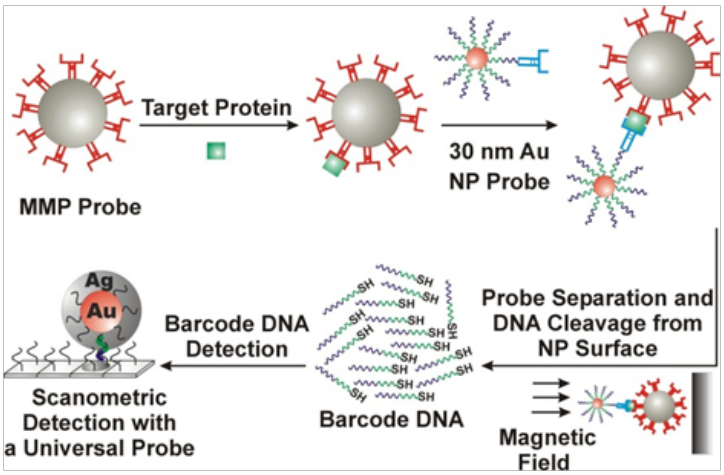

Figure 7.3. The bio-barcode assay for protein detection (Nam et al. 2003).

Illustrated in Figure 7.3 is the concept of protein detection (Nam et al. 2003); however, the bio-barcode assay can be adapted to detect any molecular target, for example nucleic acids (Nam et al. 2004), amenable to "sandwich" capture between the two assay probes. The first particle element is a micron-sized magnetic particle (MMP) surface decorated with target-specific recognition elements. Monoclonal antibodies and short complementary oligonucleotides are used for protein and nucleic acid biomarker targets, respectively. Mixing the MMPs with a solution (e.g., serum) containing the biomarker of interest results in specific target binding to the surface of the MMP. Magnetic separation isolates the bound target. Next, a solution containing gold nanoparticles (AuNP) is added to the MMP-target hybrids.

Importantly, the AuNP probes have two important functional elements. First, a recognition element is bound to the AuNP probe, which acts to sandwich the targeted biomolecule. Antibodies are used for proteins and oligonucleotide probes for targeted nucleic acids. Second, the AuNP has surface-immobilized bio-barcode sequences that are arbitrarily chosen as surrogate markers for the presence of the targeted biomolecule. Typically, the bio-barcode is 20, or so, nucleotides in length, providing approximately 4^{20} unique barcode identities. The barcode DNA does not participate in target recognition, but it is ultimately responsible for target amplification, identification, and quantification.

Following AuNP probe addition, the hybrid structures sandwiching the targeted biomolecule are separated using a magnet, the solution is washed free of unbound AuNP probes, and then the bio-barcodes are chemically released from the surface of the AuNPs. Released bio-barcode DNA is then added to a DNA microarray, where the barcodes can be sorted to specific array addresses and then detected using AuNPs surface-functionalized with oligonucleotides complementary to a universal bio-barcode tag. In a final step known as the Scanometric assay, the optical signature provided by AuNPs bound to bio-barcode DNA is greatly amplified through the electroless reduction of silver and/or gold catalyzed by the surface-bound AuNP

probes (Taton et al. 2000; Kim et al. 2009). Ultimately, the biomarker presence and quantity in the original solution is reflected by the presence and intensity of staining at the corresponding bio-barcode array address.

Initially demonstrated for the prostate cancer biomarker, prostate specific antigen (PSA)[30], the bio-barcode assay can be up to 10^6 times more sensitive than conventional ELISA assays for the same target (Nam et al. 2003). The bio-barcode assay sensitivity is mainly derived from the following:

- Target capture is highly efficient due to the homogeneous nature of the assay and because a large concentration of target-binding elements are present in the assay
- In addition to the recognition elements, AuNP probes carry with them hundreds of bio-barcode DNA strands, which lead to direct amplification of signal
- The scanometric assay, the first FDA-cleared nanoassay and the final step of the bio-barcode assay (Taton et al. 2000), has high sensitivity based upon the catalytic amplification of the optical signature of individual bio-barcode-bound AuNPs

A significant amount of work has been done to move from the initial proof-of-concept PSA nanoassay to clinical applications. As a marker for the early detection of prostate cancer, PSA suffers from a lack of specificity for prostate cancer and is controversial in this clinical setting (Andriole et al. 2009; Schroder et al. 2009). However, following surgical removal of the prostate gland (i.e., prostatectomy) for cancer, the presumed sole source of serum PSA has been removed. In the post-prostatectomy setting, assuming organ-confined disease, PSA serum levels fall to values that are undetectable using commercial ELISA-based technologies. Unfortunately, up to 40% of patients who undergo radical prostatectomy have prostate cancer recurrence, often first detected by a PSA that increases from undetectable to detectable and rising (Pound et al. 1999; Catalona and Smith 1994; Jang et al. 2006; Trapasso et al. 1994). Here, clinical data demonstrates that earlier salvage radiation delivered at the lowest possible serum PSAs leads to improved patient survival (Trock et al. 2008).

Accordingly, and building upon previous work demonstrating that more sensitive testing for PSA post-prostatectomy could provide substantial lead times in the diagnosis of prostate cancer recurrence (Yu et al. 1995), the Mirkin group utilized the bio-barcode assay to assess whether increased sensitivity would provide clinically useful information in the setting of prostate cancer recurrence (Thaxton, Elghanian, Thomas et al. 2009). In a pilot study, serial serum samples were collected prospectively from patients following prostatectomy. The serum samples had been tested using conventional assays prior to testing with the bio-barcode assay. The study included a mix of patients with and without prostate cancer recurrence, first demonstrated by a detectable and then rising PSA. Serum samples for interrogation with the bio-barcode assay had undetectable (<0.1 ng/mL PSA) PSA values as measured with conventional tests. However, the bio-barcode assay is over 300 times more sensitive than conventional ELISA assays, with a lower limit of detection of 0.3 pg/mL. Using the bio-barcode assay, PSA was detected in the serum of all patients, whereupon they could be stratified into three groups:

1. Low and non-rising bio-barcode PSA values (Figure 7.4A)
2. Initially low bio-barcode PSA values, but then rising values (Figure 7.4B), in some cases, that became detectable with conventional PSA assays (Figure 7.4C)

[30] PSA is a serum biomarker used to screen individuals for prostate cancer and also as a marker for prostate cancer recurrence following primary and secondary prostate cancer intervention (Pound et al. 1999).

3. Low bio-barcode values following secondary prostate cancer interventions that drove PSA values to undetectable levels as measured with conventional tests (not shown)

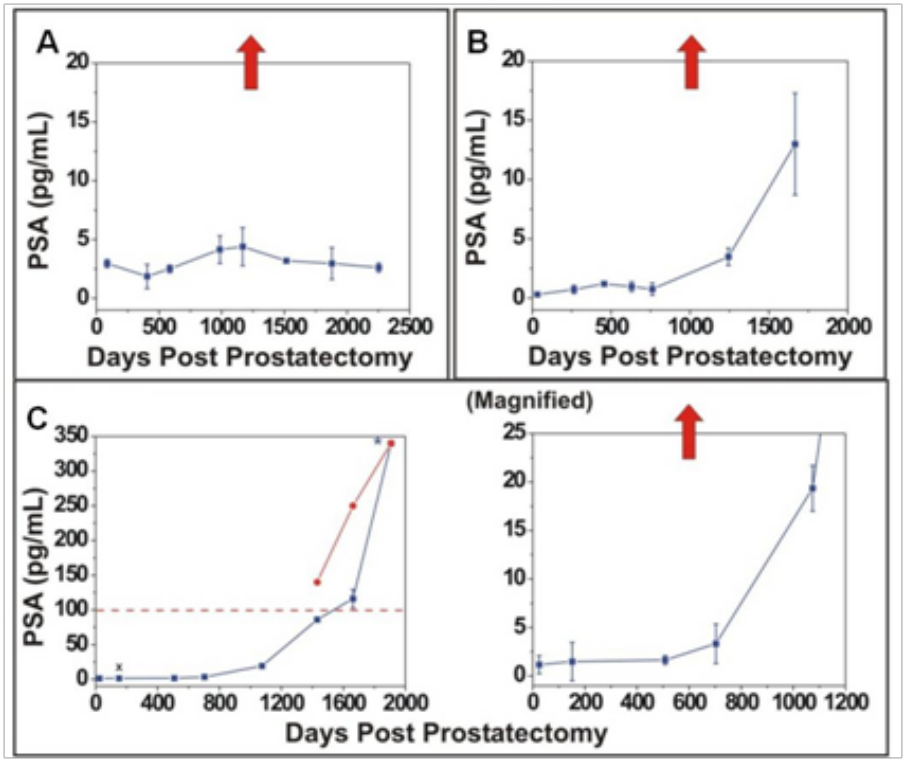

Figure 7.4. Bio-barcode PSA profiles. Following surgery (T = 0), the bio-barcode assay (■) clearly tracks PSA response to treatment at values well below the threshold of detection of current commercial PSA immunoassays (100 pg/mL, ●). Arrows (magnified plot) or dotted line indicate that values shown are below the limit of clinical detection of 1 ng/mL (from Thaxton, Elghanian, Thomas et al. 2009).

Overall, this pilot study concluded that by using the more sensitive nanotechnology-enabled analytical method, prostate cancer patients could be more accurately and more quickly risk-stratified following prostatectomy and closely monitored after undergoing secondary treatment.

Most recently, a commercial-research-use-only nanoparticle assay for PSA (Verisense PSA, Nanopshere, Inc., Northbrook, IL) with a comparable sensitivity to the bio-barcode assay was used in a similar analysis. That study was designed to validate the results of the pilot study and was conducted retrospectively using samples from over 400 patients who had undergone prostatectomy for clinically localized prostate cancer. Although these data are not yet published, the results of this much larger retrospective study corroborate those of the pilot study, and a significant case is being made for the initiation of a large, multi-institution prospective clinical trial to validate the technology and to assess its clinical value in the setting of prostate cancer recurrence.

Gold Nanoparticle-Based Nano-flares: Intracellular Probes

Discovery of the polymerase chain reaction (PCR) revolutionized nearly all aspects of basic science research and has profoundly impacted clinical medicine. Similarly, more contemporary quantitative PCR (qPCR) techniques allow for the detection and quantification of mRNA targets of interest such that relative changes in mRNA transcript levels can be

assessed under myriad conditions and from a wide variety of different cells and tissues. Due to the sensitivity and dynamic range of the technique, quantitative analysis can be performed on either single cells or large numbers of cells. However, one requirement for mRNA analysis using PCR or qPCR approaches is that the cells or tissue under study must be destroyed. That is, PCR requires cell lysis for solubilizing intracellular mRNA species. This severely and fundamentally limits qPCR as follows:

1. Removing mRNA targets from the friendly confines of the cell lipid bilayer membrane makes them susceptible to degradation by abundant and highly effective nucleases (e.g., RNase). Meticulous care is required for experimental success.
2. Cell lysis precludes the simultaneous assessment of protein and/or other small molecule changes in the context of ongoing changes at the mRNA level in real time and in live cells.
3. Experimental cells are no longer available for downstream applications.

A new class of intracellular diagnostic materials has been developed that addresses the above limitations (Prigodich et al. 2009; Seferos, Giljohann, Hill et al. 2007; Zheng et al. 2009). The synthesis of gold nanoparticles surface-functionalized with DNA oligonucleotides is shown in Figure 7.5. DNA AuNPs have unique properties that make them superior probes for complementary oligonucleotides of interest. In an extracellular diagnostic context, they demonstrate increased binding constants for targeted oligonucleotides, i.e., increased *sensitivity* (Lytton-Jean and Mirkin 2005) and exquisitely sharp melting transitions between perfectly matched and mismatched targets, i.e., increased *selectivity* (Elghanian et al. 1997; Storhoff et al. 2004). These properties enable point-of-care diagnostics for both proteins and nucleic acids.

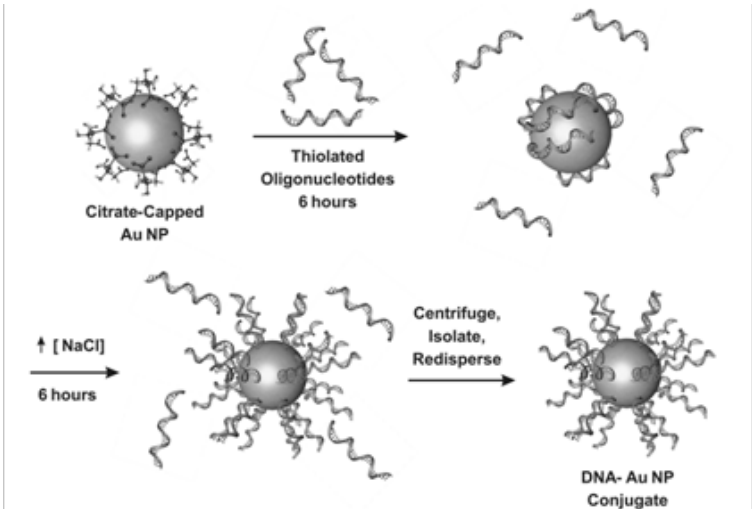

Figure 7.5. Synthesis of DNA AuNPs. Citrate-capped gold nanoparticles are mixed with alkyl-thiol modified oligonucleotides for 6 hours. The thiol adsorbs to the AuNP surface resulting in a loose association of DNA with the particle surface. Through a process of salt (NaCl) additions, the particles are 'aged' over 6 hours to achieve a dense loading of alkyl-thiol oligonucleotides to the surface of the AuNP. Centrifugation isolates the DNA AuNP conjugates from unreacted components (courtesy of Chad Mirkin).

Commercialized by Nanosphere, Inc., a number of FDA-cleared pharmacogenomic tests that detect single base changes in drug-metabolizing enzymes are providing real opportunities for personalized medicine at points of care. However, extracellular *in vitro* diagnostics are just a first glimpse at the impact that these unique probes will have in biomedicine. DNA AuNPs

have also shown extraordinary promise with regard to intracellular applications, including in the detection of target molecules and as a potentially new and potent class of therapeutics (Rosi et al. 2006; Giljohann et al. 2009). Here, DNA AuNPs have demonstrated:

- Universal and >99% uptake into cultured cells (over 40 cell lines and primary cells have been tested to date)
- Cellular uptake without the use of cytotoxic transfection agents
- DNA AuNP uptake is not inherently cytotoxic
- The unique properties of the particles stabilize surface-modified oligonucleotides against nuclease degradation (Seferos et al. 2009)
- Nanoparticle bound oligonucleotides can be used to both *detect* and *regulate* target genes at the mRNA level (Seferos, Giljohann, Hill et al. 2007, 2007b; Rosi et al. 2006)

Nano-flare technology (Scheme 3, Figure 7.6), pioneered by the Mirkin Group, is an elegant example of an intracellular application of these unique nanomaterials (Prigodich et al. 2009; Seferos, Giljohann, Hill et al. 2007). When bound to an AuNP, the fluorescent emission from a molecular fluorophore "flare" is effectively quenched by the close proximity to the AuNP (Nerambourg et al. 2008). Effective fluorescence quenching and the increased stability of the oligonucleotides on the surface of the DNA AuNPs (Seferos et al. 2009) leads to significantly lower background signal upon transfection (*vida infra*). Upon transfection into cells, the fluorophore-labeled oligonucleotide flares dissociate from AuNP surface-immobilized complements upon specific mRNA target binding, removing the quenching effects of the AuNP and resulting in a detectable increase in intracellular fluorescence. Because the increase in fluorescence correlates with the presence of specific mRNA targets (Figure 7.7), DNA AuNPs conjugated to fluorophore-labeled oligonucleotides act as intracellular target mRNA probes for assessing mRNA target changes which result from directed cellular perturbations (Seferos, Giljohann, Rosi et al. 2007).

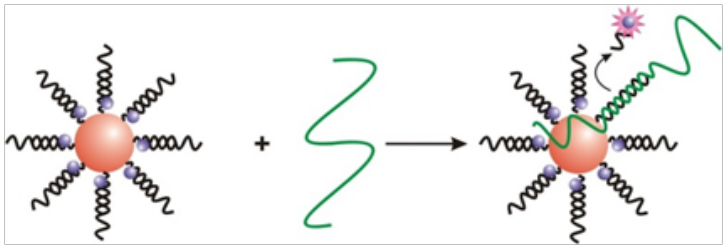

Figure 7.6. Nano-flares. AuNPs (large spheres) are densely surface-functionalized with thiolated oligos. Bound to the AuNP oligos are fluorophore (small sphere)-labeled short oligos (flare). Hybridization orients the molecular fluorophore sufficiently close to the AuNP such that effective quenching of the fluor is observed. Upon encountering an intracellular mRNA target (wavy line), the flare is displaced and the molecular fluorophore exhibits bright fluorescence (courtesy of Chad Mirkin).

Intracellular binding of target mRNA is further augmented by the unique properties of the DNA AuNPs (Elghanian et al. 1997; Storhoff, Marla, et al. 2004; Storhoff, Lucas, et al. 2004). Due to the ability for the nano-flare probes to readily enter all cell types tested to date, the Mirkin group proposes that the nano-flare agents can be used as a universal live cell target mRNA quantification platform with the potential for simultaneous protein or small molecule detection. Because cells are not destroyed, flow cytometric and light microscopy coupled with nano-flares may replace qPCR, while preserving cells for downstream applications.

Importantly, using either flow cytometry or confocal microscopy, there is no loss of sensitivity with reference to qPCR (i.e., single-cell analysis is possible with confocal microscopy), while also maintaining the analytical range to perform ensemble measurements averaged from the measurements of hundreds to thousands of measurements made on individual cells (flow cytometry).

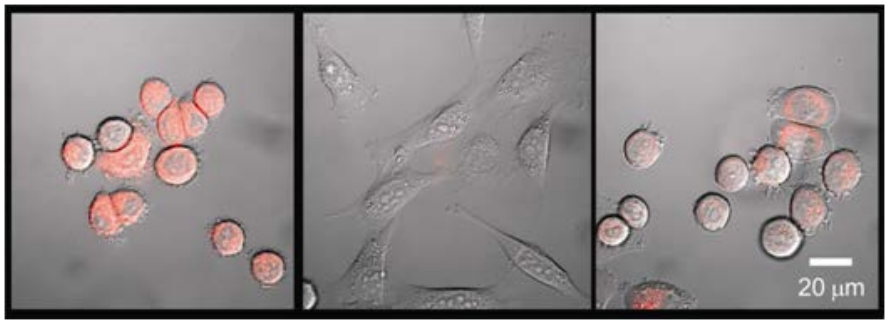

Figure 7.7. Intracellular nano-flares. A human breast cancer cell line (SKBR3, left and right panels) is shown after transfection with nano-flare particles specifically targeting the survivin mRNA sequence known to be expressed by SKBR3 cells. On the left, bright fluorescence is observed as the nano-flare sequence is complementary to the targeted survivin mRNA sequence. On the right, a control scrambled nano-flare sequence was added and minimal fluorescence is observed. The middle panel demonstrates the lack of survivin nano-flare fluorescence following transfection in a cell line that does not express survivin (mouse endothelial, C166) (courtesy of Chad Mirkin).

In vivo Imaging

Instrumentation: Carbon Nanotube-Based X-Ray Systems for Diagnostic Imaging and Radiotherapy

X-ray radiation is widely used today for *in vivo* cancer detection and for radiotherapy. For example, mammography is the most common modality for breast cancer screening, and over 50% of all cancer patients in the United States undergo radiation therapy. For x-ray based imaging and radiotherapy devices, there are constant demands to increase the resolution so that tumors can be detected at an earlier stage, to minimize the imaging dose, improve the accuracy of dose delivery, and minimize normal tissue damage during radiotherapy. The new carbon nanotube–based x-ray technology enables new imaging and radiotherapy devices with improved performance in these respects.

Utilizing the recent advances in nanomaterials, a new x-ray source technology has been developed (Figure 7.8). Carbon nanotubes (CNTs) instead of the conventional thermionic filaments are used as the "cold" electron sources for x-ray generation. The technology is capable of generating both temporally and spatially modulated x-ray radiation that can be readily gated and synchronized with physiological signals. The spatially distributed x-ray source array technology opens up new possibilities for designing *in vivo* imaging systems, especially tomography systems, with increased resolution and imaging speed, and expanded functionalities. By distributing the x-ray power over a large area, the technology can generate a significantly higher dose rate for certain radiotherapy applications. Since its invention, this nanotechnology-enabled x-ray source technology has moved from being a simple academic curiosity to commercial production. Its applications for cancer detection and treatment are being actively investigated in academic institutions and in industry.

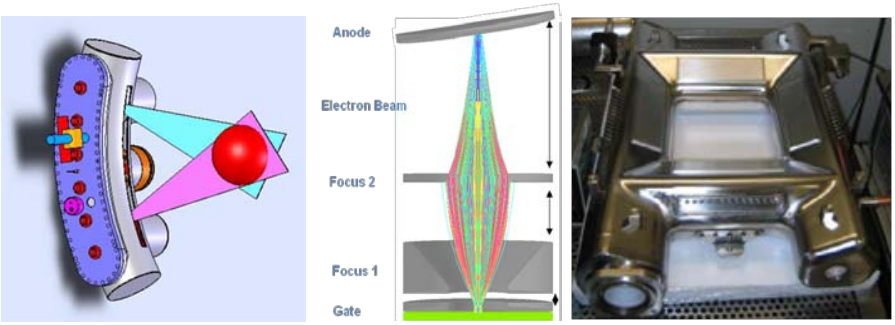

Figure 7.8. (*Left)* Schematics showing a CNT x-ray source array; *(right)* a square-geometry nanotube x-ray source array with 52 individually controllable x-ray beams, manufactured by XinRay Systems (courtesy of Otto Zhou, University of North Carolina).

Utilizing the electronic programmable capability of the CNT x-ray source, a physiologically gated, microcomputed tomography scanner has been developed. By synchronizing x-ray exposure and data collection with the non-periodic respiratory and cardiac motions, high-resolution CT (computed tomography) images with minimum motion blurs can be obtained from free-breathing mice. The scanner is now used routinely by a large number of cancer researchers at the University of North Carolina (UNC) (Figure 7.9).

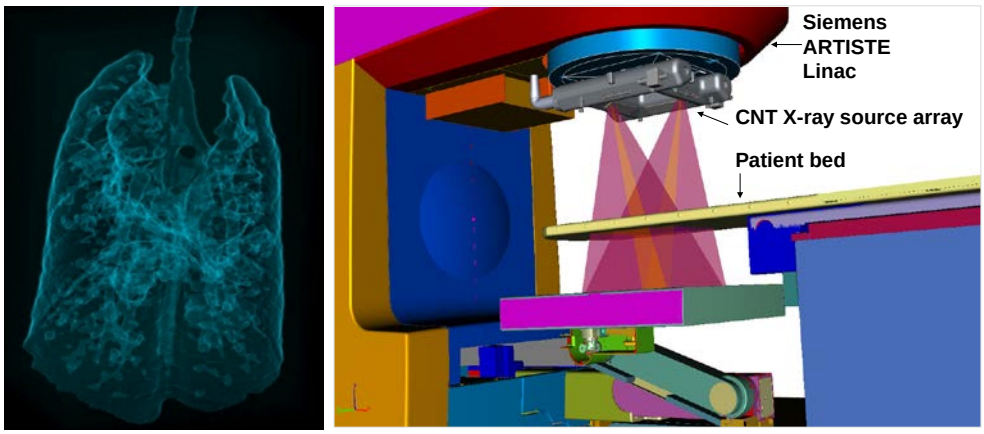

Figure 7.9. *Left*: Prospective-gated micro-CT image of a mouse lung tumor model (UNC data. Mouse model from Dr. B. Kim). *Right*: An illustration of a CNT x-ray source array mounted on a radiotherapy machine (image courtesy of J. Maltz of Siemens and P. Lagani of XinRay Systems).

Siemens and XinRay Systems developed a high-speed tomosynthesis scanner to provide real-time image guidance for radiation therapy using the distributed CNT x-ray source array technology. The device will enable oncologists to "see" tumors in real time during treatment and will allow more accurate radiation delivery. The scanner has been integrated with the Siemens Artiste treatment system. It is currently under testing at the UNC Cancer Hospital. Clinical trials are scheduled for 2010.

Molecular Imaging

Macroscale anatomical and physiological information acquired from diagnostic imaging techniques has successfully guided clinician prognosis. However, imaging at this scale overlooks the molecular processes that underlie most diseases. Molecular imaging has emerged in an attempt to exploit molecular markers for early diagnosis and tracking of

disease progression with the goal to significantly improve patient care. Molecular imaging can be defined as "the characterization and measurement of biologic processes in living animals, model systems, and humans at the cellular and molecular level by using remote imaging detectors" (Meade 2001).

Over the past decade versatile surface functionalization of nanomaterials has enabled the realization of additional functions, including the development of multimodal probes to utilize more than one imaging modality, and theranostic probes to achieve simultaneous drug delivery and diagnostic imaging. Some of the strategies in the design of new nanomaterials include development of synthetic procedures and surface functionalization with molecules of interest.

Controlling reaction conditions can generate nanomaterials of similar composition with distinct physical properties for a variety of applications. For example, a one-pot thermal decomposition reaction in the presence of polyethylene glycol and oleylamine yields water-soluble Fe_3O_4 magnetic "nanoflowers" of 39, 47, and 74 nm in size with high crystallinity (Hu et al. 2001). The nanoflower consists of a cluster of multiple Fe_3O_4 nanoparticles. The synergistic magnetism of the Fe_3O_4 nanoparticles increases the magnetic resonance image (MRI) contrast effect to be significantly higher than that of commercially available agents. Through modification of the reaction conditions in the nanoflower synthesis (e.g., stabilizing surfactants, reagent concentrations, reaction time, and temperature), ultrasmall Fe_3O_4 magnetic nanoparticles with tunable core size are produced (Hu et al. 2001). The synthesized 3, 4, and 5 nm ultrasmall nanoparticles have comparable MRI contrast effects to commercial agents. In contrast to commercial agents, the ultrasmall nanoparticles are expected to have the desirable pharmacokinetics for escaping the reticulo-endothelial system (RES) and increasing their circulation lifetime for molecular targeting (Neuberger and Wong 2005).

The properties of nanomaterials can be manipulated through surface conjugation to molecules with desired functions. Frequently, the cooperative effects between the nanomaterials and the attached molecules enhance the nanomaterial performance, such as in the conjugation of molecular gadolinium(III) complexes to nanodiamonds (Manus et al. 2010). The magnetic gadolinium(III) complex and non-magnetic nanodiamond are conjugated to yield a highly sensitive MRI contrast agent [Gd(III)-ND]. The immobilized Gd(III) complexes on the nanodiamond surface exhibit a 10-fold increase in MR signal enhancement over the free molecular Gd(III) complex, representing the second most potent Gd(III) contrast agent reported to date (58.8 $mM^{-1}s^{-1}$). The exceptional enhancement of the MRI contrast is likely attributed to the slower tumbling rate of the conjugated Gd(III) complexes, clustering of Gd(III)-ND in solution, and the unique hydration environment around the nanodiamond surface.

Utilizing the extensive surface modification potential of nanomaterials, complex systems with multimodal imaging capabilities have been achieved. The reported Cy3DNA–GdIII@AuNP multimodal probe consists of a gold nanoparticle with immobilized DNA on the surface decorated with Cy3 fluorescent dyes and Gd(III) complexes (Figure 7.10). The co-existence of magnetic Gd(III) complexes and Cy3 fluorescent dye enables the detection of Cy3DNA–GdIII@AuNP conjugates by MRI and confocal microscopy (Figure 7.11). The high sub-cellular resolution of confocal microscopy verifies the intracellular delivery of Cy3DNA–GdIII@AuNP also visualized by MRI. Since AuNPs serve as CT contrast agents, these nanoconjugates are promising multimodal probes for MR, fluorescence, and CT. This system is one of the latest examples of nanomaterials that possess multiple characteristics for molecular imaging, including increased MR contrast sensitivity, intracellular delivery, and multimodal capability.

The nanomaterial field has made great strides in the development of MR contrast agents for molecular imaging. Advances in synthetic procedures and complex surface modifications are among the many strategies to generate nanomaterials with desired properties. Simultaneous achievement of high sensitivity probes with optimal pharmacokinetics, intracellular delivery, multimodality, and theranostic capabilities is becoming a reality.

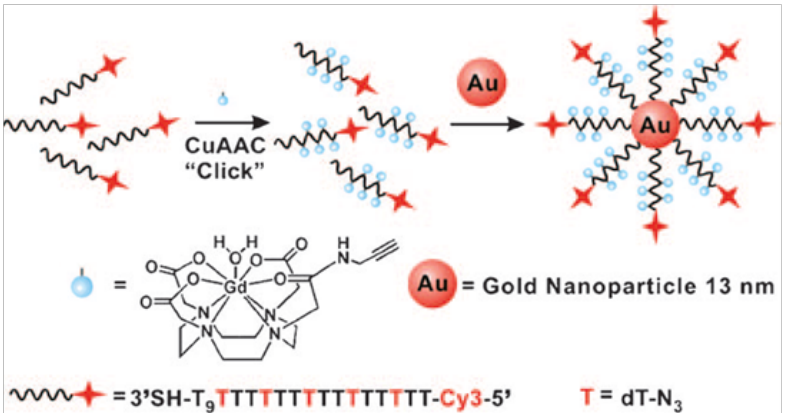

Figure 7.10. Schematic of Cy3-DNA–GdIII@AuNP conjugates. Gd(III) complexes are covalently attached to fluorescent DNA using click chemistry and conjugated to Au nanoparticles. The resulting nanoconjugate is stable, multimodal (MR, fluorescence, and CT), and cell permeable (Song et al. 2009).

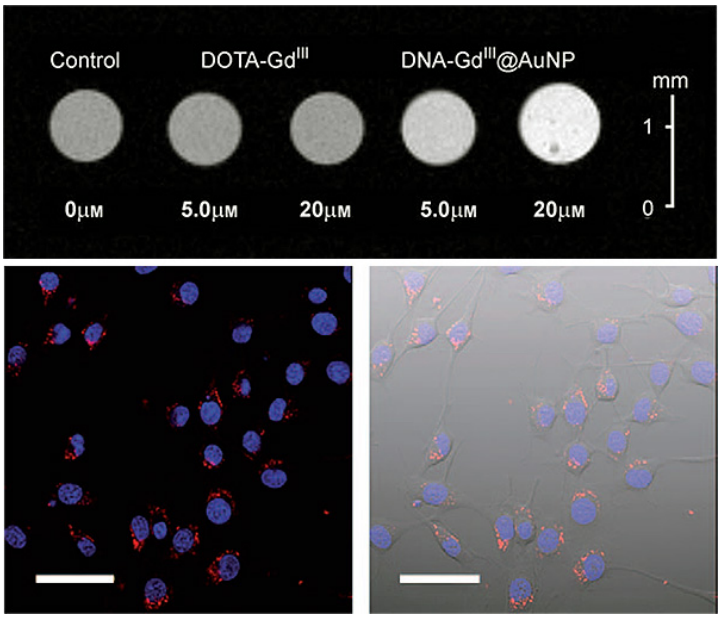

Figure 7.11. *In vitro* MR and confocal fluorescence micrographs of NIH/3T3 cells incubated with Cy3-DNA-Gd(III)@AuNP conjugates. MR images (*top*) were acquired at 14.1 T and 25°C on cells incubated with 5 μM and 20 μM nanoconjugate with comparison to a molecular control agent, Gd(III)-DOTA. For confocal imaging (*bottom*), the cells were incubated with 0.2 nM nanoconjugate. (*Bottom left*) merged DAPI and Cy3-DNA-Gd(III)@AuNP channels. (*Bottom right*) Overlay with the transmitted light image. Scale bar is 50 μm (from Song et al. 2009).

Nanoimaging for Cancer

Nanoscience applied to cancer research is critical to the future of eliminating cancer and is making a significant impact on cancer diagnosis and management in revolutionary ways already. With its capacity to provide enormous sensitivity, throughput, and flexibility, nanotechnology has the potential to profoundly impact cancer patient management. *Ex vivo* diagnostics used in conjunction with *in vivo* diagnostics can markedly impact future cancer patient management by providing a synergy that neither strategy alone can offer. Nanotechnology can significantly advance both *ex vivo* diagnostics through genomic/proteomic nanosensors and *in vivo* diagnostics through nanoparticles for molecular imaging. A technology that is amenable to detection of small tumors is based on the Raman Effect, inelastic scattering of light by molecules. The Raman Effect is very weak, producing low signals. However, the signal to noise ratio is typically very high due to the lack of autofluorescence. This relatively inexpensive and easy to use imaging technology can achieve better depth and spatial information than existing diagnostic imaging methods. To improve the utility of this technology further, gold nanoparticles coated with small molecules that enhance the Raman effect by increasing inelastic scattering of light are being developed (surface-enhanced Raman scattering, SERS). These same nanoparticles are also functionalized with molecules that allow them to home in on the tumor (Keren et al. 2008; Zavaleta et al. 2008; Zavaleta et al. 2009). Small animal imaging instruments have already been developed to detect these nanoparticles in living subjects, and this technology currently is going through FDA evaluation for its use as an endoscopic Raman imaging and screening tool for human colorectal cancer.

The sensitivity of the technique is a hundred times higher than that of fluorescence using quantum dots. Depending on the molecule on the surface of a gold nanoparticle, each nanoparticle produces a unique Raman signal, allowing for simultaneous detection of 10–40 different signals from a living subject; hence, the Raman technique's pM sensitivity and multiplexing capabilities are unsurpassed. However, its limited depth of light penetration hinders direct clinical translation. Therefore, a more suitable way to harness its attributes in a clinical setting would be to couple Raman spectroscopy with endoscopy. It was recently reported that flat lesions in the colon were five times more likely to contain cancerous tissue than polyps detected by conventional colonoscopy. The use of an accessory Raman endoscope in conjunction with locally administered (e.g., via spraying), tumor-targeting Raman nanoparticles during a routine colonoscopy could offer a new way to sensitively detect dysplastic flat lesions and very small tumors that otherwise would be missed. Topical application of SERS nanoparticles in the colon appears to minimize their systemic distribution, thus avoiding potential toxicity and supporting the clinical translation of Raman spectroscopy as an endoscopic imaging tool.

It is not currently expected that targeting tumors with nanoparticles will entirely replace current procedures used to detect cancer, but the hope is that the combination of blood biomarkers will indicate the presence of early-stage disease, and an imaging study will follow in the hope that it will lead to detection of relevant disease. Towards this goal, researchers at the Stanford University's Center for Cancer Nanotechnology Excellence Focused on Therapy Response (CCNE-TR) have worked together for five years to examine panels of biomarkers that are relevant and predictive of disease in ovarian, prostate, lung, and pancreatic cancer and now functionalizing their nanoparticle-based imaging agents to detect these biomarkers. While all of these technologies are still in their infancy, the next generation of instruments—for example, those that would replace mammograms using photoacoustic imaging (van de Ven et al. 2010)—will be seen imminently.

In vivo molecular imaging has a great potential to impact cancer by detecting disease in early stages, identifying extent of disease, personalizing treatments by theranostics or targeted therapy, and measuring molecular effects of treatment.

Therapeutics

Targeted Nanoparticle siRNA Delivery in Humans

Work leading to the first targeted nanoparticle delivery of siRNA in humans, the drug CALAA-01, was begun by the Davis Group at the California Institute of Technology in 1996 (Davis 2009). Their goal was to develop a multifunctional targeted cancer therapeutic that would enable the systemic administration of nucleic acids. Their self-stated design methodology was a systems approach to a multifunctional colloidal particle—a rationally derived nanoparticle therapeutic employed before the term "nanoparticle" was widely used. The initial drug schematic (Scheme 4, Figure 7.12) presented the desired components of an envisioned therapeutic: (1) a cyclodextrin-containing polymer (CDP) core that spontaneously self-assembles with nucleic acids yielding small colloidal particles less than 100 nm in diameter, (2) a targeting ligand providing for tumor cell specificity and uptake, and (3), an appreciation of endosomal acidification as a mechanism for particle disassembly and endosomal escape of the therapeutic nucleic acid (Davis 2009). This CDP system was initially envisioned for use with plasmid DNA; however, because the association of the nucleic acid with CDP is based upon electrostatic interactions, it was understood that the approach could be a general one for candidate nucleic acid therapeutics. In addition to platform generality, the platform components were also chosen due to their amenability to scale-up and manufacture.

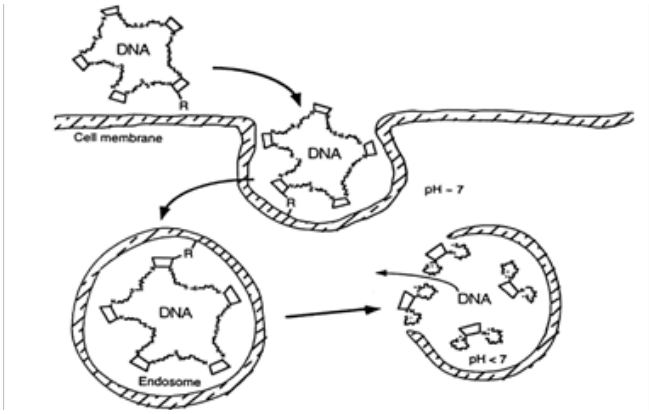

Figure 7.12. Scheme 4: Envisioned nanoparticle delivery strategy that eventually became CALAA-01 (Davis 2009).

CALAA-01 is an embodiment of this initial vision. CALAA-01 ultimately evolved to include a number of key components that spontaneously assemble into therapeutic nanoparticles ~70 nm in diameter (Davis 2009). In addition to the CDP particle core and the siRNA payload, the surface of the formed nanoparticles is decorated with (1) adamantane-polyethylene glycol (AD-PEG) for drug stabilization in biological matrices, (2) adamantane-PEG-transferrin (AD-PEG-Tf) for tumor-specific targeting and cellular uptake, and (3) imidazole residues to titrate the decrease in endosomal pH upon cellular uptake and promote the endosomal escape of the otherwise sequestered nucleic acid drug. Adamantane is a small molecule that binds tightly and forms an inclusion complex with cyclodextrin on the surface of the formed nanoparticles, thus, displaying PEG and Tf.

Shortly after the conjugate therapeutic was developed, investigators found that the nanoparticles could be formed by self-assembly through simultaneous component reconstitution and mixing (Pun et al. 2004; Bartlett and Davis 2007). This finding gave rise to a unique two-vial formulation strategy (Scheme 5, Figure 7.13) which allows for the rapid and straight forward self-assembly of the nanoparticle delivery system components (CDP, AD-PEG, AD-PEG-Tf) with siRNA, at the point of care (Bartlett and Davis 2007).

This formulation provides for siRNA solvation immediately prior to reconstitution with the nanoparticle delivery system components. This is an advantage because siRNA is highly unstable when solvated, but, following the self-assembly process, is protected from nuclease degradation. In addition, this two-vial formulation allows for the molecular delivery of system components of CALAA-01 to be separated from siRNA and tested separately for safety in animal models prior to introduction in humans (Heidel et al. 2007). The composition of the formed nanoparticle therapeutic following mixing of the separated components has been well-characterized from the standpoint of size and molecular composition (Bartlett and Davis 2007).

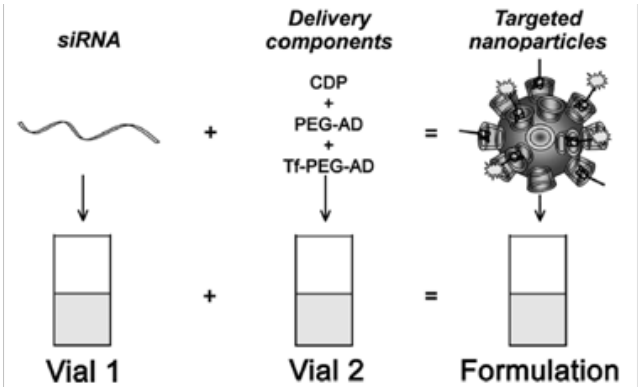

Figure 7.13. Formulation strategy for CALAA-01 (Davis 2009).

The initial *in vitro* and *in vivo* demonstration of the CDP-based siRNA delivery system was published in 2005 using a disseminated murine model of Ewing's sarcoma (Hu-Lieskovan et al. 2005). In these studies, the siRNA targeted the breakpoint of the EWS-FLI1 fusion gene, which is an oncogenic transcriptional activator in TC71 cells positive for EWS-FLI1 and for the transferrin receptor (Hu-Lieskovan et al. 2005). In addition to *in vitro* inhibition of their targeted gene product, TC71 cells transfected with firefly luciferase and injected into NOD/SCID mice served as a model system of metastatic Ewing's sarcoma where tumor dissemination and treatment efficacy could be assessed using bioluminescent imaging. In this murine model, investigators administered their targeted, CDP-based siRNA delivery particle and demonstrated anti-tumor effects and target-specific mRNA down-regulation (Hu-Lieskovan et al. 2005). Further studies provided evidence that the CDP-based delivery system does not illicit an innate immune response (Hu-Lieskovan et al. 2005), and that active targeting to the transferrin receptor enhances tumor cell uptake (Baratalett et al. 2007). With these promising animal results, a solid foundation was provided for moving the CDP-based siRNA therapeutic platform to the clinic.

In the case of CALAA-01, siRNA-targeting ribonucleotide reductase subunit 2 (RRM2) was identified (Heidel et al. 2007). In addition to potency, siRNA-targeting RRM2 demonstrated complete sequence homology in mouse, rat, monkey, and humans, which allowed for a single siRNA to be used for conducting preliminary studies in all animal models. Targeting RRM2,

Davis and colleagues confirmed effective protein knockdown with concomitant reduction in tumor cell growth potential in a subcutaneous mouse model of neuroblastoma (Bartlett and Davis 2008).

Building upon the above experiments and drawing closer to the initiation of clinical drug testing, Davis and colleagues performed the first study showing that multidosing of siRNA, in the context of the CDP-based nanoparticles, could be done safely in a nonhuman primate (Heidel et al. 2007). This study demonstrated that dosing parameters that were well tolerated were similar to those that had demonstrated anti-tumor efficacy in mouse models. Furthermore, reversible toxicity was observed in the form of mild renal impairment at high dose; however, extrapolations from mouse model efficacy studies suggested that the therapeutic dosing window would be large.

In May of 2008, CALAA-01 became the first targeted delivery of siRNA in humans (Davis 2009). Details of this study, and others focused on nanoparticle therapeutics, can be found at http://www.clinicaltrials.gov. In Phase I studies, patients were administered CALAA-01 to assess drug safety. Patients were administered CALAA-01 by way of intravenous infusion on days 1, 3, 8, and 10 of a 21-day cycle. Importantly, in a recent study by the Davis group (Davis et al. 2010), the authors demonstrate in tumor tissue taken from patients with melanoma after systemic administration of CALAA-01that CALAA-01 effectively targets RRM2 through an RNAi mechanism of action. Certainly, the updated results of clinical trials with CALAA-01 are eagerly anticipated.

Many more nanoparticle therapeutics are on the horizon and will follow CALAA-01 into the clinical setting. As a pioneering nanoparticle therapeutic, much can be learned from CALAA-01. For instance, scrupulous detail to the design, fabrication, characterization, and quality control of CALAA-01 provided a solid platform for moving the drug forward in preclinical and clinical trials. In addition, the choice of a species-generic but target-specific siRNA sequence targeting RRM2 provided direct access to multiple animal models, ultimately for translation to humans. Finally, testing in a wide variety of preclinical animal models provided the data necessary to anticipate safe dosing parameters in humans and dictated drug-specific safety monitoring protocols in humans. Building upon CALAA-01, it is anticipated that what is now an "infant" field will ultimately provide new therapeutics, based upon advances in nanotechnology that will provide for significant improvements in human health management.

Nucleic Acid Functionalized Gold Nanoparticles for Therapeutic Use

The DNA AuNP platform described above with regard to *in vitro* extra- and intra-cellular diagnostic applications is also being developed as the next generation of therapeutic nanoparticles for regulation of gene expression. As previously noted, the AuNP serves as a platform for oligonucleotide attachment (functionalization) and allows for a high density of oligonucleotides to be loaded onto the surface of each gold nanoparticle (approximately 80 strands on a 13 nm particle) (Giljohann et al. 2007; Demers et al. 2000) (see Figure 7.5, above). The conjugate can include a number of sequence modifiers, including releasable fluorophores, for easy detection, as was demonstrated in the nano-flare example. This high local density of DNA and the ability of the nanoparticles to engage in multivalent interactions are hypothesized to be the origin of a number of unique properties associated with this class of materials, including their target-binding properties and propensity for cellular transfection (Giljohann et al. 2009; Giljohann et al. 2007). These particles are readily internalized by cells (Giljohann et al. 2007), demonstrate enhanced binding to complementary targets (Giljohann et al. 2009), are stable to nuclease degradation (Rosi et al. 2006; Giljohann et al. 2009), and are not inherently cytotoxic. The conjugates remain intact and function to inhibit protein

expression for at least several days in an intracellular environment (Rosi et al. 2006; Giljohann et al. 2009). Accordingly, these conjugates act as *single-entity agents* capable of simultaneous transfection and gene regulation. Furthermore, DNA AuNPs bound to complementary targets exhibit unusually sharp melting transitions, in some cases over 1°C, when compared with unlabeled or fluorophore-labeled DNA probes (Elghanian et al. 1997; Storhoff et al. 2004; Mirkin et al. 1996). This greatly increases the selectivity and specificity for perfectly matched target sequences versus those with nucleotide mismatches.

Introduction of genetic material (e.g., DNA and RNA) into cells and tissues holds significant promise for therapeutic and diagnostic applications (Lebedeva and Stein 2001). However, development of nucleic acids into viable intracellular diagnostic or therapeutic agents has faced challenges with regard to (1) stable cellular transfection, (2) entry into diverse cell types, (3) toxicity, (4) agent stability, and (5) efficacy (Lebedeva and Stein 2001). Initial evaluation of DNA AuNPs as therapeutic agents was driven by the unique properties of the nanoconjugates realized in the context of extracellular diagnostic applications. For intracellular therapeutic use, DNA-AuNPs were discovered to be an innovative way to efficiently transfect antisense oligonucleotides for regulating gene expression; importantly, the particle itself acts as the antisense agent and is not just a carrier of nucleic acids (Rosi et al. 2006; Figure 7.14). Furthermore, AuNPs surface-functionalized with siRNA were also synthesized and demonstrated to regulate intracellular gene expression (Giljohann et al. 2009). Significantly, through the course of these experiments, the collective properties of the nucleic acid–functionalized AuNP was investigated in the context of addressing the problems associated with conventional gene regulation techniques. Ultimately, the dense loading of DNA on the surface of the AuNPs, so vital in the context of extracellular diagnostics, provides significant advantages of the DNA AuNP system over conventional intracellular gene regulation strategies.

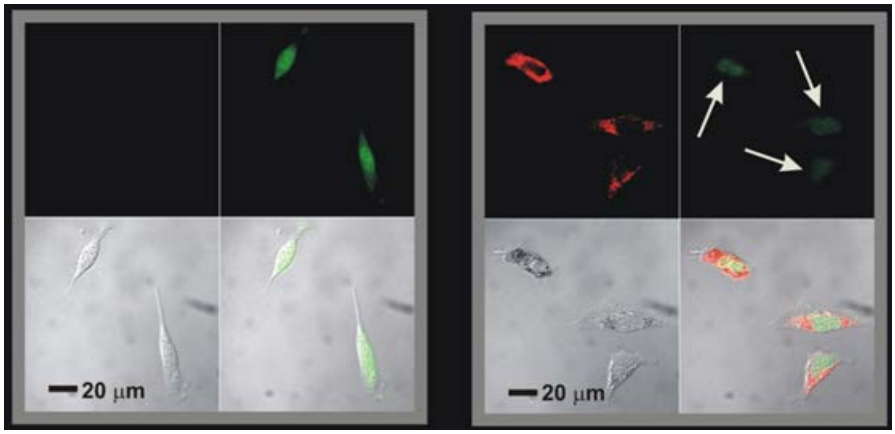

Figure 7.14. Antisense DNA AuNPs. (*Left-hand group of images*) Mouse endothelial C166 cells expressing green fluorescent protein (GFP; light areas, untreated). (*Right-hand group of images*) Cy5-labeled antisense GFP-AuNPs transfected into C166 cells downregulate the expression of GFP (Rosi et al. 2006).

Currently, the siRNA- or DNA-AuNP therapeutic platform is being applied to a wide variety of candidate diseases from brain cancer to heart disease, where the unique properties to the platform may overcome the significant hurdles standing in the way of successful disease treatment. In order to more rapidly commercialize this technology and drive it from the bench to the bedside, Professors Mirkin and Thaxton of Northwestern University co-founded AuraSense, LLC.

Biomimetic High-Density Lipoproteins

With consideration given to mortality and worldwide prevalence, the significance of atherosclerosis is profound (Getz and Reardon 2007; Josi et al. 2008). Atherosclerosis is a chronic infiltrative and inflammatory disease of the systemic arterial tree caused by excess circulating cholesterol (Lusis 2000; Spagnoli et al. 2007). Cholesterol is not soluble in the aqueous milieu of the human body, thus it travels by way of dynamic nanoparticle carriers known as lipoproteins (LPs) (Lusis 2000). The main LP carriers of cholesterol are low-density lipoprotein (LDL) and high-density lipoprotein (HDL). High LDL levels promote atherosclerosis and are associated with an increased risk of cardiovascular disease (Kannel and Wilson. 1995; Kannel et al. 1971). Therapeutic LDL lowering has been shown to reduce cardiovascular disease mortality (Zambon and Hokanson 1998; Ballantyne et al. 1997; Andrews et al. 1997). Conversely, HDL is well-known to promote reverse cholesterol transport (RCT) from sites of peripheral deposition (macrophage foam cells) to the liver for excretion (Joy and Hegele 2008; Brewer 2004; Degoma et al. 2008). Accordingly, high HDL levels inversely correlate with cardiovascular disease risk (Brewer 2004).

There is intense interest in therapeutic strategies to harness the beneficial effects of HDL (Joy and Hegele 2008; Conca and Franceschini 2008; Kontush and Chapman 2006; Singh et al. 2007; Watts et al. 2008) in order to address the substantial cardiovascular disease burden that exists despite LDL-lowering therapies. HDL is a dynamic serum nanostructure that matures from nascent form to a mature spherical form; in both forms, the surface components are (1) APOA1, (2) phospholipids, and (3) free cholesterol. In the maturation process from nascent to spherical forms of HDL, free cholesterol is esterified through the action of lecithin:cholesterol acyl transferase (LCAT) where the cholesteryl esters are added to the core of HDL due to increased hydrophobicity. This process increases the size of the particle, which then takes on a spherical shape. In order to overcome the biological steps required to fabricate a biomimetic HDL nanostructure from the bottom-up, biology needed to be replaced, to some extent, by nanotechnology. Specifically, by using a 5 nm diameter AuNP as the core of the biomimetic HDL, the biological steps required to fabricate biomimetic HDLs with the surface chemistry of natural mature spherical HDL could be bypassed (Thaxton, Daniel, Giljohann et al. 2009). As proof of concept toward the development of nanoparticles as novel tools and therapeutic agents for studying and treating atherosclerosis, a synthetic HDL biomimetic nanostructure (HDL AuNP) was fabricated collaboratively by the Thaxton and Mirkin research groups, and its cholesterol-binding properties were measured (Thaxton, Daniel, Giljohann et al. 2009; Scheme 6; Figure 7.15). The size, shape, and surface chemistry of the synthetic version closely mimicked that of natural mature spherical HDL (Table 7.1).

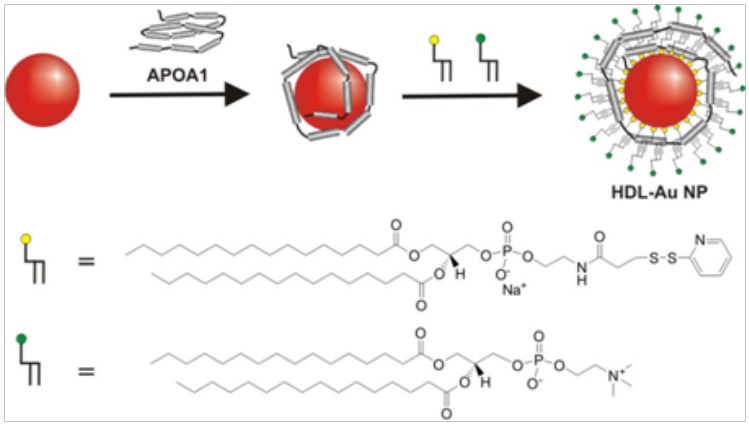

Figure 7.15. Synthesis for templated, spherical HDL AuNPs (Thaxton, Daniel, Giljohann et al. 2009).

Table 7.1. Hydrodynamic diameter of HDL conjugates

Particles	Hydrodynamic Diameter (nm)
Au NP (5 nm diameter)	9.2 ± 2.1
Au NP + APOA1	11.0 ± 2.5
Au NP + APOA1 + phospholipids	17.9 ± 3.1

As discussed, transport of cholesterol to the liver by HDL is a key mechanism by which HDL protects against the development of atherosclerosis (Joy and Hegele 2008). Thus, determining if HDL-AuNPs bind cholesterol is important for determining the potential of these structures as therapeutic agents. By using a cholesterol analog, the measured binding constant for cholesterol to HDL AuNPs was measured and found to be 3.8 nM (Figure 7.16). Interestingly, there is little information regarding the K_d (dissociation constant) of natural HDL for comparison purposes. Accordingly, HDL AuNPs are key biomimetic nanostructures that can be used as reference points for further comparison. Current research activity is centered around the biology of HDL AuNPs, both *in vitro* and *in vivo*.

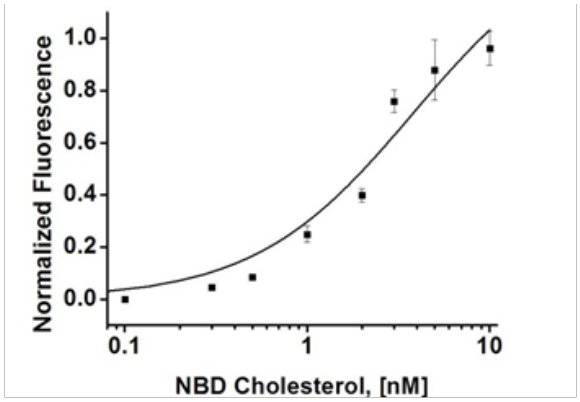

Figure 7.16. Binding isotherm of a fluorescent cholesterol analogue to HDL-AuNPs. NBD cholesterol was titrated into a 5 nM solution of HDL-AuNPs. The fluorescence vs. NBD-cholesterol concentration was used to calculate the K_d of HDL-AuNPs (courtesy of C.S. Thaxton).

Mechanized Nanoparticles for the Delivery of Therapeutic Agents

Arguably, chemical systems are at their best when they are robust and "smart." Imagine a device for the delivery of an anticancer drug specifically targeted to cancer cells that involves a rugged nanoscale container endowed with nanoscale antennae and associated machinery. One type of delivery system developed by the Stoddart Group at Northwestern University in collaboration with Professors Zink, Nel, and Tamanoi at the University of California Los Angeles (UCLA) consists of mesoporous silica nanoparticles 200–500 nm in diameter. Interspersed on the surface of the nanoparticles are different models of nanomachinery that can be actuated chemically (via pH change), biochemically (via enzyme action) or photophysically (via light) (Lu, Liong, Sherman, et al. 2007; Liong et al. 2008; Xia et al. 2009; George et al. 2010; Meng et al. 2010; Lu, Liong, Li, et al.; Meng et al 2010). Silica is an attractive material to incorporate into such devices on account of it being rigid, chemically inert, and optically transparent.

Historically, molecular machinery very often has been based on bistable rotaxanes (Saha et al. 2005; Klichko et al. 2009), which are mechanically interlocked molecules that consist of a ring component that can be induced to move along a stalk component attached to the outside of the nanoparticle at one end and terminated by a large stopper that prevents the ring from leaving the stalk at the other end. These stalks are bestowed with a recognition element that is close to the surface of the mesoporous nanoparticle. Thus, when a macrocycle such as cyclodextrin or cucurbit[n]uril is complexed to this recognition element near the surface, it acts as a gatekeeper and retains the cargo (i.e., a drug or imaging agent) in the mesoporous channels of the nanoparticle. When it is moved away from the surface, the gates are opened and the contents of the pores (cargo) can be released into the surrounding medium. The macrocycle gatekeeper can be moved from the surface of the nanoparticles by either of two basic mechanisms: (1) nanovalve systems that incorporate a second recognition element in the stalk of the rotaxane that allows the gatekeeper macrocycle to slide away from nanoparticle surface, allowing the cargo to escape from the pores, or (2) snap-top systems that incorporate a cleavage point in the stalk of the rotaxane such that the stopper can be removed in response to a specific stimulus, allowing the gatekeeper macrocycle to slip off the stalk entirely, thus freeing the cargo from the pores of the mesoporous silica nanoparticles (MSNP). Figure 7.17 shows the basic design of the nanovalve/snap-top mechanized nanoparticle system.

Recently, nanovalve systems have been developed that can release cargo by either raising the pH (Angelos et al. 2008) or lowering the pH (Kashab et al. 2009) of the system, which shows potential for use in a biological setting. Various snap-top systems have been developed also, including one that can release its cargo upon exposure to an esterase enzyme (Patel et al. 2008). Currently, the Stoddart group is working on developing additional snap-top systems that can release cargo in response to specific enzymes that are over-expressed by tumor tissue, which will potentially provide new diagnostic and treatment options for various cancer types.

Drs. Zink, Nel, and Tamanoi at the Nano Machine Center at the California NanoSystems Institute at UCLA have developed a pH-sensitive MSNP that is capable of delivering doxirubicin to an acidifying intracellular compartment in cancer cells from where drug release to the nucleus takes place when the pH drops below 6 (Meng et al. 2010). The drug is retained in the nanoparticle at physiological pH (7.4) of the blood, which theoretically means that drug release can be controlled to prevent systemic side effects while being used to target its delivery to cancer cells, e.g., by the attachment of surface ligands such as folic acid (Liong et al. 2008).

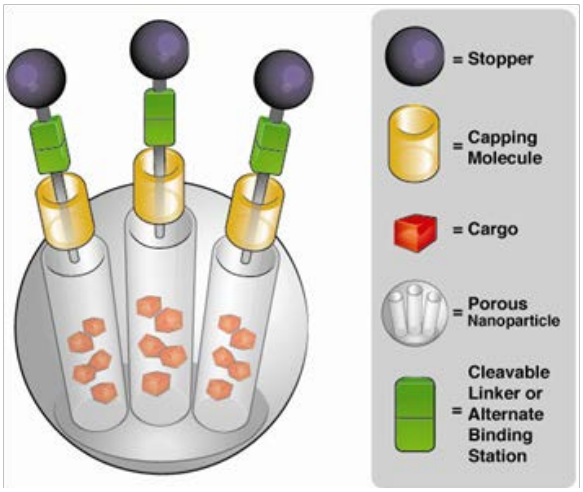

Figure 7.17. Graphical representation of a mesoporous silica nanoparticle (SiO_2, average diameter of 200 nm) adorned with a layer of [2] rotaxanes on the nanoparticle surface. Cargo can be released from the pores of the nanoparticles by either forcing the capping molecule to move to another binding site of the rotaxane stalk (nanovalves), or by cleaving the rotaxane stoppers off the stalks completely (snap-tops)(courtesy of F. Stoddart and A. Nel).

In addition to the mechanized features of the MSNP that allow on-demand drug delivery, this particle platform has been endowed with additional design features to emerge as a multifunctional therapeutics and imaging platform. First, attachment of ligands such as folic acid has allowed the particles to be targeted to breast or pancreatic cancer cells; second, super-paramagnetic iron oxide nanocrystals were encapsulated inside the MSNP, allowing magnetic resonance imaging, with the possibility of using the particles for theranostics (Liong et al. 2008). Third, the UCLA group has recently demonstrated that it is possible to deliver doxirubicin from the pores simultaneous with the release of a siRNA species that targets the Pgp (permeability glycoprotein) transporter that is involved in doxirubicin and other chemotherapeutic agent resistance (Meng et al. 2010). This was accomplished by attaching polyethylenimine to the negatively charged particle surfaces, allowing secondary but stable attachment of siRNA while still keeping the pores available for electrostatic attachment of doxirubicin. This has allowed this dual delivery system to be used for partial restoration of doxirubicin sensitivity in a squamous carcinoma cell line that has been selected for doxirubicin resistance due to overexpression of Pgp (Figure 7.18). Fourth, additional surface functionalization with polyethylene glycol (PEG) and other steric hindrants has allowed modification of MSNP biodistribution, with reduced reticuloendothelial system (RES) uptake but with enhanced electron paramagnetic resonance (EPR) spectra in subcutaneous xenografts in mice. All of the above features, combined with a demonstration of safety during intravenous injection, in addition to recovering most of the injected silica in the urine and feces within days, establishes this system as a versatile drug delivery platform (Lu et al. 2010).

Functionalized MSNPs show great promise for being the preferred method of administering therapeutics in the not-too-distant future. Perhaps the most attractive property of these delivery systems is their inherent modularity that allows easy interchange between the various components of the functionalized MSNP. This arises from the piece-wise synthesis that is employed to assemble the delivery systems, so that various epitopes can be attached, such as targeting moieties or fluorescent tags, to further tailor the properties of the nanoparticles. Furthermore, MSNPs are known (Lu et al. 2007) to be capable of being

endocytosed by live cells to deliver a cargo of imaging agents or therapeutics to the lysosomal compartment of the cell (Meng et al. 2010). The endocytotic uptake is dramatically enhanced by surface ligation or attachment of a cationic group (Liong et al. 2008; Xia et al. 2009). Moreover, long-aspect-ratio MSNPs show dramatically enhanced uptake by macropinocytosis through an apparent outside-in as well inside-out communication system that involves filipodia and the cellular cytoskeleton. *In vivo* studies have also shown that these delivery systems are nontoxic due to mostly unreactive components such as the silica nanoparticles and the cyclodextrin capping molecule; *in vitro* studies have also not shown any adverse effects upon exposure to these vectors (Xia et al. 2009; Meng et al. 2010; Lu et al. 2010). This evidence supports the notion that functionalized MSNPs can and will be used as weapons in the fight against deadly diseases.

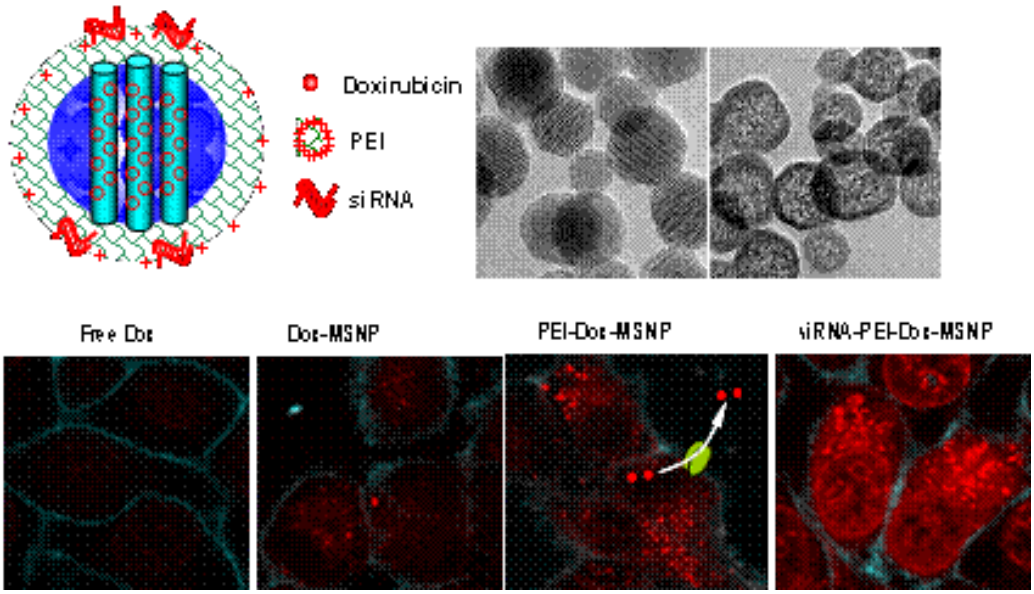

Figure 7.18. Contemporaneous drug-plus-siRNA delivery to a squamous carcinoma cell line (HELA), which exhibits doxirubicin resistance due to overexpression of the Pgp drug transporter, which also expels doxorubicin (Meng et al. 2010). By utilizing mesoporous silica nanoparticles (MSNP; TEM image in *top middle*) coated with a cationic polyethyleneimine (PEI) polymer (*top right*), it is possible to attach Pgp siRNA to the particle surface (*top left*). This arrangement leaves the MSNP pores open for electrostatic binding of doxirubicin. The HELA confocal series (*bottom images*) shows the fluorescent doxirubicin located in the particles (dots) after uptake into acidifying endosomes. This series further demonstrates that whereas doxirubicin trapped in the particles is taken up in larger amounts than the free drug, essentially all of the doxirubicin being released from within the endosome by protons (interfering with the drug's electrostatic attachment) is expelled by the Pgp transporter in the image of the PEI-Dot-MSNP superimposed on the confocal picture. If, however, the drug is delivered concurrent with the siRNA, which knocks out Pgp expression, the released drug is retained in sufficient quantities to enter the nucleus where it induces apoptosis as shown in the image labeled siRNA-PEI-Dot-MSNP. Thus, dual delivery can overcome cancer drug resistance (courtesy of A. Nel and F. Stoddart).

PRINT Nanoparticles as Therapeutics

The DeSimone Group at the Carolina Center of Cancer Nanotechnology Excellence at UNC invented PRINT (*Pattern Replication In Non-wetting Templates*) technology, which enables the design and manufacture of precisely engineered nanoparticles with respect to particle

size, shape, modulus, chemical composition, and surface functionality (Figure 7.19; Euluss et al. 2006; Gratton et al. 2008; Petros et al. 2008).

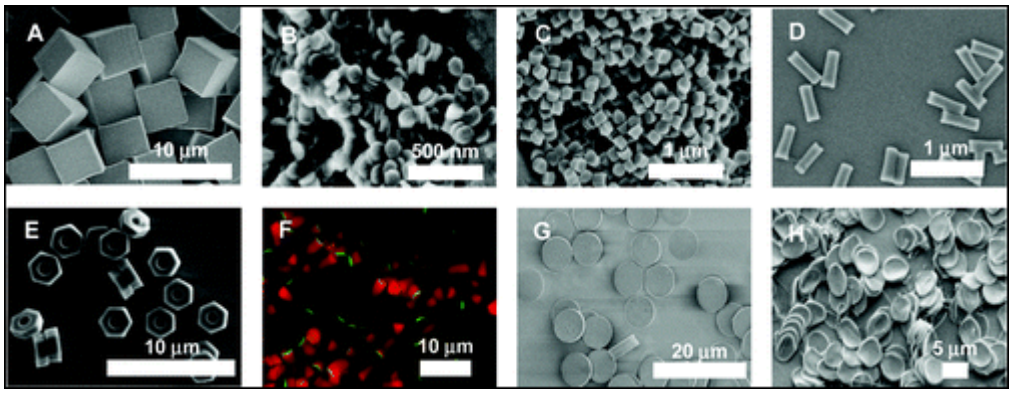

Figure 7.19. PRINT is an innovative approach to fabricate nanoparticles with precise control of particle size, shape, composition, cargo, and surface properties, all of which affect how nanoparticles loaded with an anticancer agent will behave in the body. A-H show nanoparticles of different sizes, shapes, and compositions, made using PRINT (from Gratton et al. 2008).

PRINT particles are presently being designed for use as new therapies in cancer prevention, diagnosis, and treatment. The ability to control the size, composition, and shape of the particles on the nanoscale permits construction of specific materials that are ideally suited for many specialized diagnostic and drug-delivery applications.

Tissue Regeneration

There is a major need in the medical field for tissue-regeneration technologies that can contribute to potential applications such as bone, cartilage, vascular, bladder, and neural regeneration. Each year almost 500,000 patients worldwide receive hip implants while about the same number need bone reconstruction due to injuries or congenital defects. Moreover, 16 million Americans lose teeth annually and require dental implants. The projected annual market for medical implant devices in the U.S. alone is estimated to be >$20 billion and is expected to grow by 10% a year for the next decade. Unfortunately, medical implant devices have been associated with a variety of side effects, including wear, immunogenicity, inflammation, and fibrosis. Contributing to these side effects is poor tissue integration that leads to loosening of implants and mechanical damage to the surrounding host tissues.

There is a growing consensus that nanostructured implants have potential advantages over conventional materials and that the use of nano-architectures and synthetic pro-morphogens (bioactive analogs of growth factors) can dramatically enhance tissue regeneration by, for instance, mimicking natural extracellular matrix (ECM). A key design feature of these types of engineered nanoparticles appears to be their surface topography, especially with regard to surface features that affect cell attachment, growth signals, and ability to induce genes involved in cellular differentiation in cell types such as endothelial cells, vascular smooth muscle cells, chondrocytes, osteoblasts, neurons, and embryonic stem cells.

Although there is a consensus that regenerative medicine could have a major impact on the healthcare system, cost is an issue. Research must focus not only on addressing major chronic disease processes with poor clinical solutions but on high healthcare costs that can be lowered by nano-enabled materials. Thus, the major impact of nanotechnology in the field of regenerative medicine will likely be in the areas of wound healing, urinary incontinence,

osteoarthritis of major joints, diabetes, coronary artery disease and cardiac failure, stroke, Parkinson's disease, spinal cord injury, and renal failure. The competitive scenario is that while orthopedics, dental implants, and wound healing are the easiest markets to penetrate, significant advances are taking place in addressing cardiovascular disease, stem cell therapy, and spinal cord injury. These advances are made possible through research advances that are moving the field of regenerative medicine from inert polymers that mimic biomechanical properties of native tissue to the construction of bioactive materials that promote tissue self-healing.

For instance, because most ECM features naturally operate at the nanoscale level, advanced bio-inspired materials are incorporating nano-architecture features into multifunctional ECM analogs. This work necessitates understanding how cells detect biomaterials as well as understanding the downstream pathways that could lead to inflammation, fibrosis, and immune rejection. There is also is a high clinical demand for therapeutic tools that control inflammation and jumpstart ECM production by endogenous or transplanted cells. High-throughput screening (HTS) approaches can help to answer some of these questions by using cellular endpoints and nanomaterial libraries with property variations, allowing study of cellular biology responses that can assist tissue generation. HTS can also be used to detect potential adverse outcomes that result from potentially hazardous nanomaterial properties that can be redesigned to improve material safety (Nel et al. 2009; Nel et al. 2006; George et al. 2010). This aspect is described in more detail in Chapter 3.

Two major classes of multifunctional ECM analogues are emerging, namely nano-architectured materials and synthetic pro-morphogens. Nano-architectured bio-mimics require the development of 3D engineered or self-assembling scaffolds that can be achieved by nanofabrication techniques such as electrospinning and soft lithography. Functionalized nanowires, nanoparticles, and CNTs are good platforms for constructing multitasking engineered nanomaterials (ENMs). Other work is being done to engineer self-adaptive materials that modify their response properties in relation to environmental changes. Biocompatible ENM components could also be designed to be sensitive to on-demand stimuli such as chemical, electric, mechanistic, photonic, and thermal triggers. One example is a polymer substance [poly(N-isopropylacrylamide)] that undergoes a temperature-dependent transformation from hydrophobic to hydrophilic upon incubation of the coated tissue culture dishes at 20°C rather than 37°C (body temperature). This hydrophilic transformation allows primary human tissue culture cells such as myocardiocytes or corneal epithelial cells to detach from the surface as intact sheets of cells that maintain an intact ECM (Elloumi-Hannachi et al. 2010; Shimizu et al. 2009). When, for instance, this approach is used to harvest sheets of myocardial cells (Figure 7.20), the intact ECM allows the sheets to adhere to themselves, thereby forming a spontaneously contracting multilayer of cells that can be used as myocardial patches for the heart, e.g., dyskinetic post-myocardial infarct segments. Cell sheet engineering offers the promise of means to patch up damaged cornea and other tissue as well as heart tissue. Moreover, the technique can possibly also be used to grow an entire organ, such as a heart or a bladder, on a series of 3D templates.

Synthetic pro-morphogens drive tissue regeneration through the provision of biochemical cues. Examples include nanostructured biomaterials that incorporate morphogens into their structure to provide extracellular or intracellular cues in an appropriate 3D context, laying down gradients and/or timing the delivery. Examples include dendrimer systems presenting bio-ligands, nanoparticles carrying growth factor analogues, and molecularly driven nanogel gradients and nanopatches.

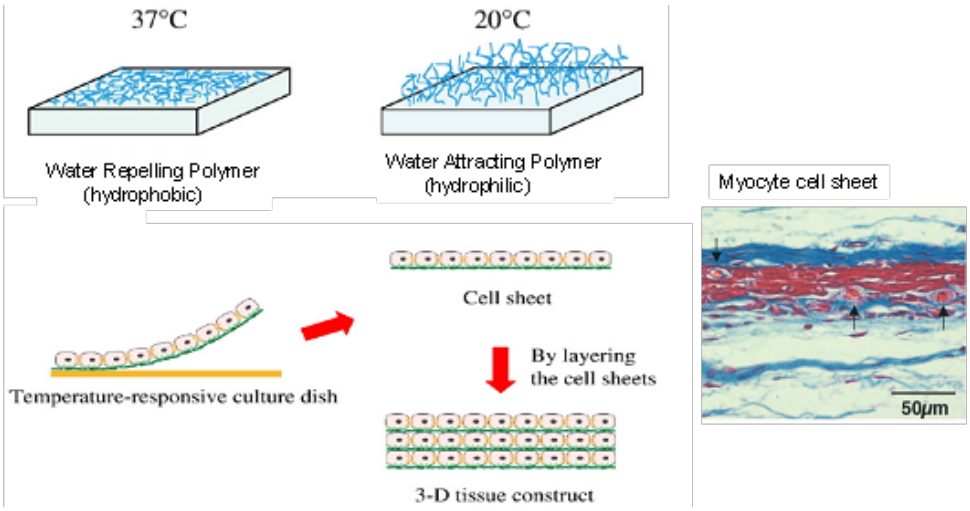

Figure 7.20. Myocardial cells sheet engineering. Cell sheet technology is based on the use of thermoresponsive polymers, poly(N-isopropylacrylamide), which are hydrophobic at 37°C, allowing primary tissue culture cells to lay down an ECM, which remains intact when the temperature of the culture dish is brought down to 20° C and the cell sheet released due to the fact that the polymer then becomes hydrophilic. Harvesting of numerous layers of myocyte cell sheets allows layering and the formation of a 3D spontaneously beating myocardial-like tissue construct that can be used for patching real-life myocardial defects as well as offering the possibility to reconstruct an entire myocardial tube (adapted from Masuda et al. 2008)

Increasingly, stem cells are being proposed as agents for cell-based therapies due to available isolation techniques, plasticity, and capacity for *ex vivo* expansion. However, to date the available technologies are hindered by a lack of reproducible control of the desired differentiation pathway, lack of control over the production volume, and the low percentage of cells reaching the target site. Nanotechnology can contribute to two types of products that enhance cell therapy, namely (1) a delivery system for cell transplantation and (2) appropriately differentiated cells. Delivery vehicles include polymeric and biocompatible devices that act as cellular reservoirs that also provide immune protection, enhance cellular survival, and provide controlled release of recruitment and growth factors at the site of carrier degradation. An example would be a material able to surround pancreatic islets during transplantation and grafting via the portal vein. Alternatively, nanomaterials can be used to construct 3D multifunctional scaffolds that provide constant mechanical stability and structural integrity comparable to the native tissue. During the culture, the cell-biomaterial compound can be provided with physicochemical cues that mimic *in vivo* tissue growth and maturation conditions. Work by the Stupp Laboratory at Northwestern University illustrates this in the context of spinal cord injury and repair (Stupp et al. 2008). As shown in Figure 7.21, peptide amphiphiles (Pas) are unique materials that self-assemble into cylindrical nanofibers that display surface peptides known to promote axon growth (Stupp et al. 2010**)**. In a mouse model of spinal cord injury, significant differences in axon regeneration were observed between untreated and those animals treated with peptide amphiphile-generated nanofiber scaffolds for axonal regeneration (Stupp et al. 2008.).

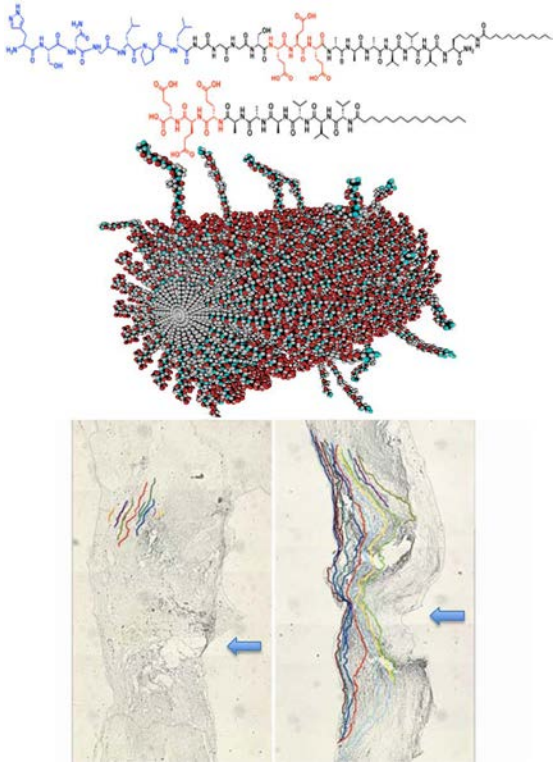

Figure 7.21. Peptide amphiphiles (PAs) and spinal cord injury. (*Top*) Two peptide PA molecules are shown. The top PA has a peptide growth factor extending from the core PA that is depicted underneath. (*Middle*) Mixing PAs in aqueous saline solution results in the self-assembly of PA nanofibers which display peptide growth factors. (*Bottom*) In a mouse model of spinal cord injury, injection of PAs at the site of injury (arrow) promotes axonal outgrowth (colored lines) and bridging of the area of injury (*right*) versus untreated mice (*left*) (courtesy of S. Stupp).

Cellular therapeutics also requires engineering and manufacturing of patient-specific rather than off-the-shelf cell products. Because culturing of cells or tissue-engineered products is still characterized by high variability and poor efficiency, economically viable tissue culture manufacturing systems need to be developed. Nanomaterials are likely to play a role in this development through their ability to provide physicochemical environmental cues for 3D culture systems and bioreactor designs.

Nanobiotechnology and Cell Biology

The nanostructures that are fundamental to life, including proteins, DNA, and lipids, operate collectively in cells that may be considered the units of complex biosystems that build from cells to tissues to organs to organisms. Efforts to analyze and engage with complex systems, such as in human medicine, are often targeted at the cellular level. For example, cancer diagnostics methods seek biomarkers on cell surfaces or secreted by cells; drugs must be delivered to certain types of cells and must negotiate cellular barriers and pathways; successful tissue engineering and implants require compatible cellular interactions and growth. Progress in nanomedicine will always be aided—or limited—by researchers' understanding of how cells operate, in exquisite detail. Similarly their ability to make smart hybrid devices for medical or for other purposes will benefit enormously from the intricate

tools that have evolved in nature to sustain complicated cellular systems. In many ways, cells lie at the heart of nanobiotechnology's promise.

From a biological perspective, cells are hierarchical organizations of diverse biomolecular constituents that enable cells to maintain homeostasis in a particular biological environment and to respond appropriately to external signals. Nanobiotechnology offers micro- and nanofabricated materials, structures, and devices to examine and engage with cellular systems on the subcellular and molecular level. Other sections in this chapter describe nanotechnology-based analytical methods that can now determine the composition of individual cells. This information can be considered in the context of increasing biological understanding of the cellular processes that the components represent. For example, many laboratories have investigated signal transduction pathways that are stimulated in a large variety of physiologically relevant cell types. By incorporating genetic, biochemical, and physical approaches, key enzymes as well as regulatory structures and mechanisms have been defined. However, the necessary spatial arrangements and regulation have been very difficult to approach because of the heterogeneity of cells and the nanoscale resolution required. Advances in microfabrication and nanofabrication have opened new opportunities to investigate complicated questions of cell biology in ways not before possible. In particular, the spatial regulation of cellular processes can be examined by engineering the chemical and physical environment at the level at which the cell responds. Lithographic methods and selective chemical modification schemes provide biocompatible surfaces that control cellular interactions on the micron and submicron scales on which cells are organized. Combined with fluorescence microscopy and other approaches of cell biology, a widely expanded toolbox has become available.

Responsive interactions of cells with surfaces are fundamental to physiology, but if dysregulated they can lead to pathological conditions such as tumor growth. Surface patterning with microcontact printing has made it possible to examine systematically the importance of the spatial dimensions over which these chemical interactions occur (Chen et al. 1998; James et al. 2008; Mrksich et al. 1996; Mrksich et al. 1997; Mrksich and Whitesides 1996). In the last several years a large number of studies have taken advantage of patterned surfaces to study spatial control of cell surface interactions and address specific questions of morphology and adhesion, dendritic branching, migration, and mechanotransduction (how cells convert mechanical signals into chemical responses). Quantitative measurements have been made of mechanical interactions between cells and microfabricated surfaces containing needle-like posts made of elastomeric PDMS (polydimethylsiloxane) (Heydarkhan-Hagvall et al. 2007; Silver et al. 1999).

Cells respond to chemical messages (e.g., antigens, growth factors, cytokines) in the environment by means of specific cell surface receptors. Binding of these molecular stimuli to receptors stimulates transmembrane and intracellular signaling events that result in a global cellular response. Spatial and temporal targeting of signaling components in the membrane on micron and submicron scales is critical for overall efficiency and regulation of the cellular response. If the environment is engineered, then the spatially regulated response of the cell to defined stimuli can be investigated by monitoring redistributions of selectively labeled components or alterations in signaling pathways. Patterned surface approaches lend themselves readily to signaling that involves specialized regions or interactions such as membrane domains formed by clustered receptors or synapses formed at a cell–cell interface. These approaches have proven valuable for examining immune system cells, which are stimulated by clustering of antigen receptors. One example is the receptor (FcεRI) for immunoglobulin E (IgE), which plays a central role in the allergic immune response.

Spatial regulation arises early in the signaling process at two stages: (1) ligand-dependent cross-linking of the IgE-FcεRI complexes required for cell activation and (2) selective targeting of signaling proteins to the region of the activated receptors. Structurally defined ligands can be used to examine structural constraints in signaling pathways. The first stage of spatial regulation is examined with a bottom-up approach of nanobiotechnology: synthesizing multivalent ligands with defined architectural features that control the manner in which the IgE-FcεRI come together when cross-linked to activate the cells. Bivalent ligands with antigenic groups on both ends and flexible spacers of poly(ethylene) glycol in the range of 10 nm cross-link the two binding sites of IgE intramolecularly. These serve as potent inhibitors of intermolecular IgE cross-linking and cell activation and provide a potential lead for allergy therapeutics. Signaling mechanisms stimulated by cross-linked IgE-FcεRI were investigated with bivalent ligands containing rigid spacers of double-stranded DNA (dsDNA), which has a persistence length of ~50 nm. These ligands stimulated low-level degranulation and also revealed a length dependence. Branched dsDNA constructions were found to be more effective because trimeric and larger cross-linking of IgE receptors causes significantly higher levels of cellular responses. Trivalent Y-DNP_3 ligands trigger robust signaling responses in two distinguishable pathways, one that depends on ligand length and another that is independent of length. This spatial differentiation provides direct support for physical coupling of proteins as a key mechanistic step in one pathway, and it also reveals branching of pathways in signaling events.

Patterned antigens have proven to be valuable for examining membrane-compartmentalized signaling. Spatial targeting of signaling components at the plasma membrane has been a subject of considerable interest, and accumulating evidence points to the participation of membrane domains. However, direct methods to investigate spatial rearrangements in the membrane and targeting of components were initially limited by typical heterogeneity of the cells and stimuli, as well as by the diffraction limit of light. Surfaces were patterned with lipid bilayers containing receptor-specific ligands using a parylene lift-off method. These microfabricated substrates allowed observation by fluorescence microscopy of labeled cellular components co-redistributing with the receptors clusters in the same pattern under physiological conditions. Substrates with patterned lipid bilayers lend themselves to other optical measurements. For example, fluorescence photobleaching recovery can been used to evaluate the dynamic nature of the proteins concentrating in the regions of patterned ligands and clustered IgE-FcεRI (Figure 7.22).

Patterned substrates also reveal selective targeting of higher-order cellular structures to clustered, activated receptors or elsewhere. One study showed that cell surface integrins preferentially bind to the silicon surface, whereas particular cytoskeleton binding proteins (e.g., paxillin) cluster with the receptors, independent of the integrins. Complementary biochemical studies with siRNA showed that paxillin participates in signaling initiated by the clustered receptors. Other studies monitored stimulated membrane trafficking and revealed that recycling endosomes are targeted to clustered receptors, whereas secretory lysosomes are targeted elsewhere.

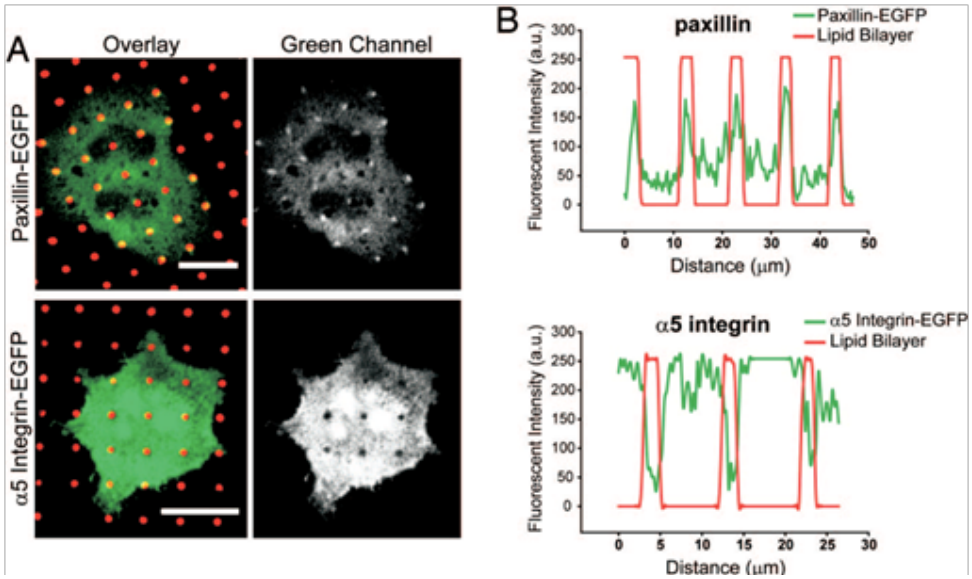

Figure 7.22. Paxillin co-redistributes with clustered IgE-FcRI and patterned lipid bilayers, but integrins avoid these regions. Confocal micrographs show cells expressing paxillin-EGFP or integrin α5-EGFP (green) after incubation with patterned lipid bilayers (red dots) containing receptor-specific ligands. (*A*) Paxillin is recruited with IgE-FcεRI in the regions of patterned ligands, and α5-integrin is excluded (scale bars: 20 μm). (*B*) Intensity line profiles from images in A confirm that paxillin accumulation and integrin exclusion correspond exactly to where the patterned bilayers are localized (from Torres et al. 2008).

Nanoapertures provide the means to examine plasma membrane dynamics with single-molecule resolution. Although surfaces patterned with micron-size features have proven valuable in providing new information about spatial regulation of cell signaling that occurs proximal to the plasma membrane, ultimate elucidation of cellular homeostasis and stimulated responses requires investigations of molecular dynamics and interactions that occur on the length scale of 100 nm. Although it has been possible to pattern these features as small as 50 nm, visualization by fluorescence microscopy with live cells has not been practical below 600 nm. New super-resolution fluorescence methods, including PALM, STORM, and STED, are beginning to overcome these limitations. Fabrication techniques, including zero-mode waveguides (ZMWs), can also overcome the diffraction limit of light. ZMWs were initially used to examine diffusion of fluorescent lipid probes on model membranes and single-enzyme turnover of fluorescent substrates with micromolar concentrations. Subsequent studies showed that the plasma membrane of live cells can be probed with high sensitivity with these optical nanostructures. Characterization of mast cell interactions with ZMW using a combination of fluorescence and electron microscopy showed that plasma membranes from live cells penetrate these nanostructures in a cytoskeleton-dependent manner. This positioning of the plasma membrane into the apertures allows high-resolution examination of fluorescently labeled molecules with fluorescence correlation spectroscopy. These optical nanostructures offer the unique advantage of studying single-molecule dynamics in the plasma membrane, or within close proximity, at physiologically relevant molecular concentrations.

7.3 GOALS, BARRIERS, AND SOLUTIONS FOR THE NEXT 5–10 YEARS

While the science and engineering of nanobiosystems are some of the most exciting, challenging, and rapidly growing sectors of nanotechnology, society continues to face daunting challenges in biomedicine and healthcare delivery. Over the next 5–10 years, nanotechnology will play a significant role in overcoming these challenges. Several specific goals are described below, the current barriers to success are discussed, and then solutions are proposed where nanotechnology can overcome the barriers and reach these goals.

Biomarkers

Successful treatment of human disease requires accurate diagnosis and effective treatments. Successful realization of personalized care requires that markers exist both for the disease and for identifying subsets of patients who will respond to specific treatments. Thus, improved diagnostics are needed to detect disease as early as possible, even to the point of detecting single defective cells or biomarkers that predict the future onset of a disease. Currently, there is insufficient knowledge of disease-specific biomarkers; further, the sensitivity of current diagnostic tests may not allow for the detection and quantification of the most informative low-abundance biomarkers. Finally, it will likely be the case that a panel of biomarkers is most accurate with regard to disease detection, and diagnostic platforms are needed that can detect multiple targets rapidly, cheaply, and at the point of care. Advances in nanotechnology will provide both *in vitro* and *in vivo* diagnostics using nanoscale materials that are capable of specifically binding with target biomarkers and amplifying the detection response for identification and quantification. Importantly, adapting these technologies so that they can be made to identify low-abundant unknown biomarkers, and then identify which ones are specific to a given disease state, is possible with exquisitely sensitive nanotechnologies coupled with imaging modalities that relay morphological, structural, and functional features of disease; devices that use multiplexing for *in vitro* diagnoses; and implantable devices that monitor analytes and therapies.

In vitro Diagnostics

Improved diagnostics that achieve PCR-like sensitivity through the use of nanoscale operations that assess specific disease biomarkers at points of care are a goal for future development. However, the development of such platforms is hindered by insufficient knowledge about specific and non-specific biological interactions, inadequate affinity agents and amplification methods, and the lack of appropriate and validated biomarkers. To overcome these barriers, it will necessary to achieve high sensitivity and specificity in nanoscale processes, coupled with a more robust knowledge of the bio–nano interface affecting nanomaterial probes and target analytes. The surface functionality and modification processes of nanomaterials will be tailored to better resist non-specific biomolecular interactions and foster specific ones. Finally, sample processing and autocatalytic nanotechnologies will provide unprecedented purification and signal generation upon binding to target analytes. Taken together, advances in nanotechnology and a more thorough understanding of the bio-nano interface will provide robust, multiplexed diagnostic assays where specific signal(s) generated from only a few target molecules can be readily distinguished from background noise.

Nanotherapeutics

The pharmaceuticals industry is a sector seeking radical innovation. A significant goal for nanotechnology over the next 5–10 years is developing nanoscale pharmaceuticals that offer

new therapeutic approaches. Significant barriers exist with regard to both the scientific and the business aspects of these endeavors. Development and translation of nanotherapeutics to patient use are expensive, time-consuming, dependent upon the approval of regulatory agencies, and require appropriate reimbursement. Over the next decade, significant financial input from pharmaceutical companies and the Federal government is expected to more fully realize the promise of developing nanotherapeutics, especially at a time when venture capital financing of small businesses has diminished. In addition, regulatory agencies are realizing that novel approaches are needed with respect to nanotherapeutic testing in humans and human clinical trial design. With regard to product development, nanotherapeutics are facing significant challenges with regard to demonstrating favorable pharmacokinetics, biodistribution profiles, targeted drug delivery, tissue penetration, drug release, and the ability to provide an image signature of location and efficacy. Each of these challenges is being met head-on with development of novel methods of nanoparticle fabrication, increasingly sophisticated nanoparticle architectures, a better understanding of the bio-nano interface, and better ways of developing hierarchical nanotherapeutics.

Nanotechnology and Stem Cells

Stem cells interact with their physical and chemical environments, and these interactions dictate stem cell maintenance and differentiation. At present, insufficient knowledge of stem cell regulation, including the biology of the multi-potent state and underlying biological regulation mechanisms, are limiting the directed manipulation of stem cells. Nanotechnology can be harnessed to more thoroughly understand the complex stem cell-environment interactions in order to more formally monitor stem cell responses and then to systematically understand those features which dictate stem cell differentiation and phenotype. Nanoarray technology, nanoparticles used to deliver differentiating signals to stem cells, and a robust understanding of the bio-nano interface will provide ways of building and manipulating appropriate scaffolds for directing stem cell fate.

Tissue Engineering

Engineering functional tissues that repair or replace damaged tissues and organs holds significant promise for treating some of the most devastating and debilitating human neurological and musculoskeletal diseases. Currently, there exist no off-the-shelf technologies or means to rapidly and reproducibly build appropriate tissue architectures. Nano-architectures and synthetic pro-morphogens (bio-active analogues of growth factors) that recapitulate natural extracellular matrix and provide growth signals can be fabricated using a number of techniques, including (1) electrospinning and molecular printing strategies (e.g., dip pen nanolithography, contact printing) to develop 3D engineered or self-assembled scaffolds; (2) functionalized nanoparticles, nanowires, and carbon nanotubes to build composite multi-tasking engineered nanomaterials; and (3) dendrimers presenting bio-ligands or nanobeads carrying growth factor analogues. Such materials and technologies will permit rapid growth in the field of tissue regeneration and repair.

Economics of Nanomedicine

The goal of nanomedicine is to reduce costs, provide more accurate and earlier diagnoses, deliver effective and personalized care, and simplify and standardize healthcare delivery. This is especially true with regard to chronic diseases (e.g., cardiovascular disease and diabetes) that have high healthcare costs. However, the development of nanotechnologies is expensive, there is competition from generic companies, the environment for reimbursement is difficult, regulatory agencies have not previously focused on complex and often

multidisciplinary drugs and/or devices, and the funding environment is quite restricted. Ultimately, nanotechnology can deliver low-cost, accurate, quantitative, reliable, and accessible materials for diagnosis and treatment, and these cost savings will emerge through innovative plans to fund, regulate, and commercialize nanotechnologies. It is difficult to predict the overall economic impact of the nanobiomaterials discussed in this report and of the others that will be translated into use in the medical arena over the next 5–10 years. Many estimates exist for the overall market size of nanotechnologies. One recent review (Hobson 2009) cited three different sources and estimated that the overall market size would be between $2.6 and 2.95 trillion by 2014 or 2015. NSF has estimated a market size of $1 trillion by 2015, $180 billion of which will be attributed to nanopharmaceuticals (Hobson 2009). Regardless of the estimate, and despite the challenges to nanobiosystems development noted throughout this chapter, nanotechnologies will most certainly have a substantial impact in health and medicine over the next 5–10 years.

7.4 SCIENTIFIC AND TECHNOLOGICAL INFRASTRUCTURE NEEDS

In order to fully realize the promise of nanotechnology in biomedicine, significant improvements in scientific and technological infrastructure are needed. Investment in innovative nanotechnological research is imperative. Centers of excellence have proven to be very effective tools for bringing together many disciplines within medicine, science, and engineering to tackle important problems in the field. A significant emphasis should be on establishing such centers on a disease-by-disease basis. This will require increased and targeted funding approaches, but importantly, such centers will centralize and galvanize new opportunities for commercialization and business development in the nanomedicine arena. An infrastructure for doing clinical trials based upon the pipeline of new diagnostic and therapeutic tools emerging from the field must be established. The U.S. Food and Drug Administration (FDA) must build its own internal infrastructure to handle the evaluation of these important innovations in a timely, efficient, and safe manner. In addition, thought must be given to how the adoption of these technologies by physicians can be facilitated through targeted education, participation in the clinical trials, and ultimately, reimbursement policies.

Investment and innovation in education is required from the K–12 levels up through physicians and professors to enhance matriculation to careers in science and math fields and to improve the adoption of emerging nanotechnologies in the clinic. Enhanced communication is required between those on the front lines of academic nanomedicine research, clinicians, and individuals in the pharmaceutical and biotechnology industries. Focused understanding of the most significant problems facing patients, and then focused energies on developing collaborative solutions to key problems will certainly facilitate and expedite translation of the most promising technologies.

In order to fully realize the potential of nanotechnology in biomedicine, reliable production of high-quality nanomaterials and their characterization is imperative. This will enable reproducible fabrication of high-quality materials platforms and answering of directed questions with regard to their use in *in vitro* diagnostics, as therapeutics, as tissue scaffolds, and as materials for directing stem cell fate, among others. Furthermore, broad access to new tools for characterizing such materials and their interactions with biological systems is needed to transform what has been heretofore an empiric exercise into a predictive one. Establishing scientific consensus as to the array of *in vitro* and *in vivo* tests needed for nanomaterials would provide scientists with a framework for evaluating novel agents.

7.5 R&D INVESTMENT AND IMPLEMENTATION STRATEGIES

The challenges described above demonstrate that substantial investment in nanobiomedicine is required; however, the return on investment will be substantial with regard to jobs created, the U.S. competitive position in the global economy, and improvements in human health that directly result from nanotechnology. Often small start-up companies are the initial entities making strides toward product development. One way to substantially enhance translation of their efforts to commercial success would be a system of financial incentives available to both the start-up company and to large biotechnology and pharmaceutical companies who choose to invest in them. Another way, with a more direct impact on small start-up success, would be to increase government spending on small start-up nanotechnology companies in order to facilitate their ability to attract more substantial investment from the private sector.

Next, it is critical to identify and address the most substantial problems in biomedicine. Identifying key issues is not a straightforward process. Nanoscience research is often done by chemists, materials scientists, and the like, who undertake technology development based upon the known novel properties of nanostructures; however, they may not focus on specific applications in biomedicine. Increasing the communication between practicing health professionals and pharmaceutical industry experts, for example, with those in academia may be a direct way to augment nanotechnology development efforts in all areas of biomedicine in order to facilitate early commercial and clinical interest and, therefore, enhance the likelihood of translation. As evidence continues to be generated and nanotechnology moves from an empiric science to one based upon well-characterized and predictable nanosystems, it will be imperative to broadly disseminate this information so as to avoid redundancy in R&D and more clearly focus on developing nanomaterials and systems for the next generation of *in vitro* diagnostics, nanotherapeutics, imaging agents and technologies, combination theranostics, and materials to manipulate stem cells and regenerate human tissue.

7.6 CONCLUSIONS AND PRIORITIES

Over the last ten years there have been substantial developments in nanobiosystems. Currently, nanotechnology-enabled diagnostic, therapeutic, and imaging agents are being used in patients; these agents are being closely scrutinized with regard to the benefits that they provide in relation to conventional technologies and patient health outcomes. Furthermore, substantial advances have been made for developing nanomaterials that can manipulate stem cells and engineer cells and tissues. Finally, research is honing in on the bio-nano interface and how changes in nanomaterials and nanoengineered surfaces result in specific and nonspecific bio-nano interactions.

Building upon these advances, much work needs to be done over the next 5 to 10 years to more fully realize the promise of nanobiosystems. First, *in vitro* diagnostic capabilities need to be adapted to not only detect vanishingly small quantities of known target biomarkers, but also to identify new biomarkers specific for given diseases. Biomarker discovery efforts may well reveal multiple biomarkers, and nanotechnologies are needed that can robustly perform multiplexed detection. For therapeutics, novel methods are required to efficiently target nanoparticles to specific cells and tissues harboring disease. These nanostructures should be capable of carrying multifunctional cargo to enable drug delivery (e.g., small molecules or nucleic acid therapeutics), target-specificity, tissue penetration, and the capability for imaging both location and molecular mechanism of action. In the special case of RNAi, nanoparticle delivery strategies may well be absolutely required to realize the massive

potential of this entire class of therapeutic molecules. Next, the bio-nano interface needs to be more fully explored to develop nanomaterials that both drive stem cell fate and regenerate tissues. In addition, a more complete understanding of the bio-nano interface is necessary to begin to predict the *in vivo* behavior of nanomaterials so as to more rationally develop nanomaterials as diagnostic, therapeutic, imaging, and theranostic agents, and also to predict toxicity.

Collectively, these advances are possible with continued and sustained investment in nanotechnology and nanobiosystems over the next decade. Innovative mechanisms are needed to provide funding for research laboratories and small start-up companies. Because advances in nanobiomedicine have largely been the result of robust collaborations that incorporate the expertise of individuals skilled in nanomaterial synthesis and characterization, biologists, engineers, and medical doctors, such collaborations should be encouraged. Finally, the value of the advances of the past 10 years, and the promise that nanotechnology has for the future of biosystems, medicine, and health care, should not be underestimated.

7.7 BROADER IMPLICATIONS FOR SOCIETY

Society stands to reap substantial benefits from advances in nanobiotechnology. New nanotechnologies already are in the clinic and benefitting patients. The future will provide ever smarter and more advanced nanotechnologies that identify disease-specific biomarker profiles to enable early detection and monitoring of disease. Nanoparticle therapeutics will be tailored to individual patient profiles, realizing the promise of personalized medicine, targeting diseased tissue, and eradicating disease without harming normal nearby cells and tissue. Furthermore, nanomaterials hold the keys to treating some of the most debilitating and devastating human diseases through stem cell and tissue engineering approaches. Overall, continued investment in nanobiotechnology R&D is expected to dramatically improve health care, create jobs, help build a new high-tech industrial base, and drive a new era of U.S. prosperity.

7.8 EXAMPLES OF ACHIEVEMENTS AND PARADIGM SHIFTS

7.8.1 National Cancer Institute: Alliance for Nanotechnology in Cancer

Contact person: Piotr Grodzinski, National Institutes of Health/National Cancer Institute

Although the past fifty years have seen enormous advances in our understanding of the mechanisms and biology of cancer occurrence and spread, the standard regime for cancer treatment and management—surgical resection followed by chemo- and/or radiotherapy and periodic screenings to search for recurrence or metastasis—is largely the same now as it was in 1960. Although highly heterogeneous in its causes and characteristics, cancer is a disease of cells, typically localized to specific tissues or organs in its early stages; however, its treatment and management have been to a large extent highly invasive, extended across the whole body, and uniform across patient populations. These blunt-instrument approaches to the disease result in treatments with a burden of side effects that can limit or even terminate treatment, screening procedures that are high in false positives, and unnecessary costs and suffering, yet they are unable to detect the most dangerous and aggressive cancers early enough for effective intervention. The ability of nanotechnology to improve this situation is already registering, through nanoformulations that use tumor-specific drug delivery to localize treatments and improve the therapeutic index of highly toxic chemotherapeutic agents, and the use of nanomaterials with novel physical (optical, magnetic, and mechanical)

properties that enable more sensitive measurement of cancer biomarkers and more reliable and specific *in vivo* imaging for cancer detection. The combination of advanced imaging with traditional surgical techniques for intraoperative guidance enables more successful resection of cancerous growths, which is still the most effective cure available for many cancers. These new technologies are beginning to shift cancer care to more targeted, less destructive treatment strategies that recognize and act only on cancerous cells, coupled with multiplexed, multimodal imaging and screening technologies that can detect, stratify, and monitor disease accurately and in real time.

Solid tumors are typically supported by a rapidly grown vasculature that is porous and lacking in sufficient lymphatic drainage. The fenestrations in this vasculature allow nanoscale materials (50–250 nm) to extravasate into the tumor, and the inadequate drainage allows these materials to accumulate in the tumor. This enhanced permeability and retention effect leads to higher tumor loading by large-molecular-weight therapeutics and nanoparticles than by small molecules. Increased loading of imaging agents and therapeutics in tumors reduces systemic distribution, reducing off-target effects of drugs and background signal in imaging. The large surface area and encapsulated volume of nanoparticles also enables delivery of multiple cargoes for combined imaging and drug delivery. Surface attachment of cancer cell-specific ligands, peptides, and antibodies leads to increased cellular internalization of cargo loaded into tumors. Intracellular delivery of drugs results in higher therapeutic efficacy and provides access to a greater variety of drug targets, including genes for silencing by RNA interference therapy. Early evidence also indicates that the endocytosis-mediated internalization of nanomaterials can evade efflux pump mechanisms of drug resistance in cancer cells.

As the use of nanocarriers to deliver already approved imaging agents and therapeutics is being clinically established, two further advances in nanoparticle-mediated treatment of cancer present themselves. The first is the exploitation of novel nanomaterial properties for therapeutic and/or diagnostic effect. Examples include the use of externally applied alternating magnetic fields to heat magnetic nanoparticles at a cancer site, resulting in hyperthermia and cell death, and the exploitation of the photothermal properties of gold nanoparticles/rods/spheres to detect and destroy cancerous tissue by exciting fluorescence or heating using infrared radiation. These physically mediated, localized cancer therapies promise low-side-effect profiles and imperviousness to cellular mechanisms of therapy resistance. The second advance is the introduction of functional nanosystems. These systems include particles and devices that release therapeutics in response to biochemical signals detected in the tumor or blood, while also reporting on the chemical and physical tumor microenvironment, providing therapy and monitoring in one package. An example would be a collective of magnetic nanoparticles with environmentally mediated aggregation, in which both drug release and MRI contrast are controlled by aggregation state. The ability to decorate the surface of nanoparticles with multiple reporter molecules and to load multiple nanoparticles into a single device will also enable multimodal and multiplexed molecular imaging and more informative monitoring. Such functional molecular imaging can act as optical biopsies, with tumors being continuously typed and staged.

The development and validation of targeting moieties directed at both cancer cell surfaces and tumor vasculature and microenvironment is necessary to increase the efficacy of the cancer nanotechnology strategies outlined above. Further enhancement of intratumoral and intracellular delivery will also result from the careful choice of nanocarrier shape in addition to size and rational design of nanocarrier properties. Well-designed nanocarriers can control drug release by time, response to internal environment (e.g., pH, presence of cancer specific enzymes or receptors) or response to external stimuli (e.g., radiation, ultrasound).

Improvements in cancer detection, therapy, and monitoring will be amplified by advances in *in vitro* assay technology. More rapid and sensitive assays will enable identification of and validation of new cancer biomarkers, including prognostic markers of tumor metabolism, growth, and dormancy as well as type and stage. Many of these technologies will rely on both the innate characteristics of nanomaterials and their large surface area–to-volume ratio to increase assay sensitivity. Examples include the magnetic nanoparticle-based magnetoresistive sensors, nanoscale cantilever resonance-based molecular detectors, and signal amplification through surface functionalization. Non-protein-based biomarkers will be essential for the development of cheap, reliable *in vitro* assays. Low-cost genomic and proteomic profiling of tumor types will also be crucial to choosing optimal care strategies for individual patients. Microfluidics will be a backbone technology for many of these advances, especially cancer cell detection strategies.

The use of nanotechnology for cancer prevention is currently an underdeveloped area. Nanoformulations of chemopreventives are currently under investigation, as are methods to identify biomarker profiles for at-risk patients. More ambitious research will incorporate the cancer detection technologies discussed above into the standard of care for high-risk populations, and even integrate treatment delivery for triggered release upon disease discovery.

7.8.2 Perspective on Nanobiotechnology and Synthetic Biology

Contact person: Carl Batt, Cornell University

The history of biotechnology, dating back to ancient times, has been highlighted by development of processes, identification of causative microorganisms, and in recent years, deliberate improvement of these microorganisms. Although the Egyptians more than five-thousand years ago did not comprehend the microbiological basis for the conversion of bread into beer, modern biotechnology has given rise to processes for producing the rarest of biomolecules for treating complex diseases. Genetic engineering has been used to deliberately improve single and multiple traits important for the individual products; however, genetic engineering is limited by the host background and the competing metabolic activities within the host cell. In the next 10 years significant advances will be made in synthetic biology, and these will be enhanced by nanotechnology-derived tools.

Over the past 50 years, advances in the broad area of biotechnology have been supported by the products of nanotechnology. Progress in nucleic acid sequencing and synthesis has enabled new applications, which in turn has driven demand. Improved speed, accuracy, and reduced cost of reading and creating nucleic acid sequences (Figure 7.23) have been realized through nanotechnology-based components. Deconstruction of complex biological systems and then reconstruction of them will at some point largely be replaced by *de novo* approaches that rely upon our knowledge base and the ability to predict outcomes, where components are created without reference to existing systems. Synthetic biology is in essence an effort to totally create complex biological systems from scratch. To accomplish this, a concerted effort is being mounted that will drive the field of nanobiotechnology more than previously witnessed, when the technology was being used more to understand than to create.

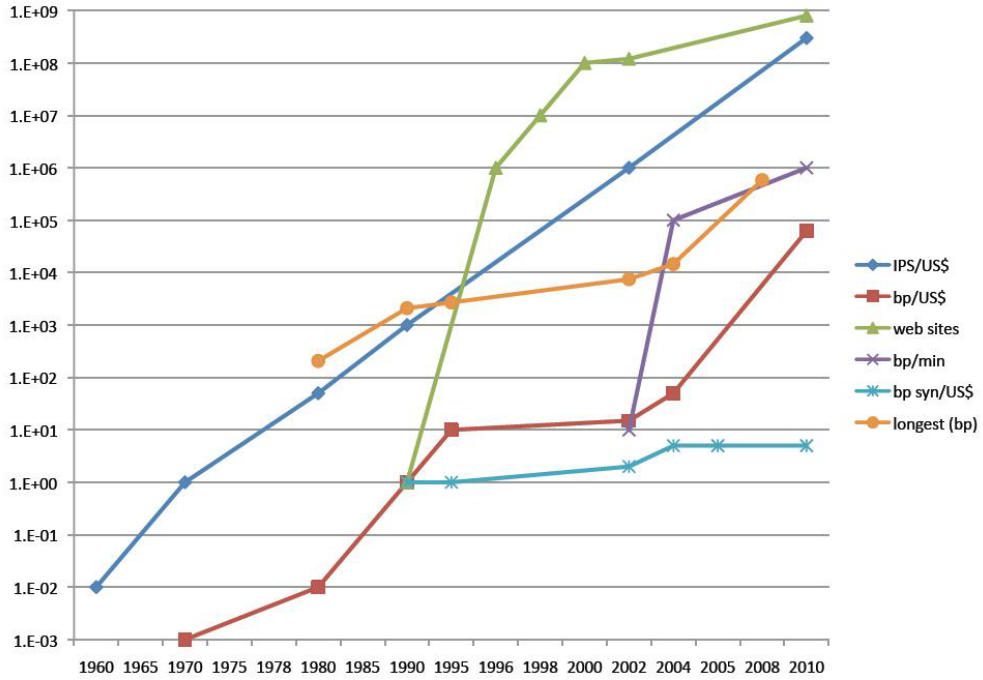

Figure 7.23. The change in lengths of time for advances in DNA synthesis, sequencing costs, speed and gene synthesis as compared to advances in computing power and numbers of web sites. Instructions per second (IPS/US$), base pairs sequenced (BP/US$), base pair synthesized (BP syn/US$), sequencing speed (BP/min) and reported longest single DNA sequence (longest BP) are from reported values and or estimates derived from reported values (Shendure et al. 2004).

The reduction of costs of DNA synthesis and sequencing parallel those seen with Moore's Law demonstrating the power of the demand for better tools for biotechnology (Figure 7.24). The rate of improvement in both DNA synthesis and sequencing has perhaps exceeded that predicted by Moore. The rationale in part due to the more direct linkage between speed, cost and ultimate benefits that can arise from an enhance ability to manipulate nucleic acid.

Nanotechnology has enabled significant improvements in both nucleic acid sequencing and synthesis. In the field of nucleic acid sequencing, nanometer-scale electrophoresis platforms allow for separation of nucleic acid fragments with single-base-pair resolution and enhanced sensitivity by reducing the focal volume of the eluate. Technologies that have advanced beyond the industry-wide Sanger (chain-termination) sequencing coupled to capillary electrophoresis separation format have been enabled by nanotechnology. For example, advances in nucleic acid sequencing have been realized by moving from a relatively linear singular format to a massively parallel platform. The parallel sequencing format has been achieved using nanoscale beads and microfluidic handling that facilitate the reading of massive numbers of DNA molecules at the same time.

In a similar fashion, nucleic acid synthesis based upon well-refined phosphoramidite chemistry was similarly advanced using nanotechnology, and the formulation of highly controlled solid-state supports has pushed the limits of the length of sequences that can be made, along with cost reductions. The relatively lower cost of DNA synthesis is primarily a matter of the market and the feasible commercial costs of set up, shipping, and handling. However, the most dramatic increase is observed for the length of DNA sequences that can be synthesized and assembled. Now advanced methods of chemical synthesis of DNA, coupled to facilitated assembly, provides a means to build long stretches of nucleic acid.

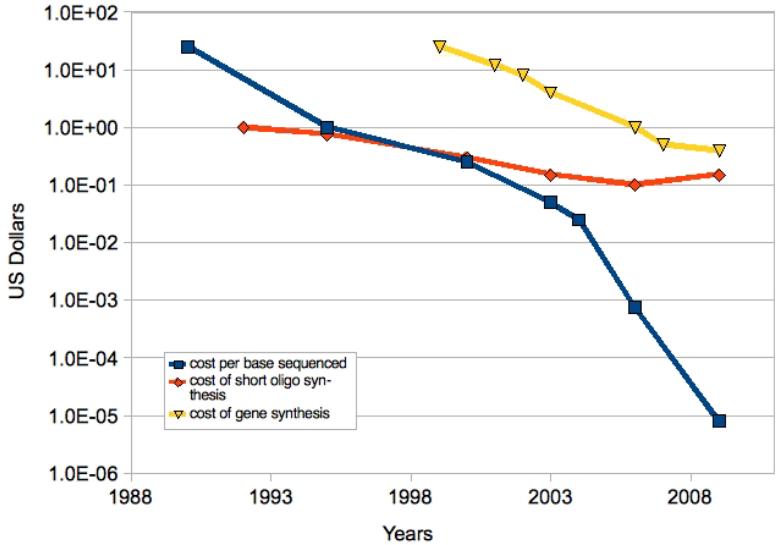

Figure 7.24. Reduction in cost of DNA synthesis, 1990–2006 (from R. Carlson, http://www.synthesis.cc).

Combined advances in nucleic acid sequencing and synthesis have created a robust platform for biotechnology and the engineering of biological systems to create an array of products. Entire microorganisms have been recreated: a first test of a relatively simple one, *Mycoplasma,* was reported by Gibson et al. in 2008. Over the next 10 years the ability to synthesize longer and longer DNA sequences will be enhanced, and routine genome construction will begin with a design and end with a complete genome. While advances in genome synthesis will continue, use of genomes to prime the creation of new organisms will have significant challenges, as outlined below.

Synthetic biology is a view of biological systems from a *de novo* design perspective, incorporating knowledge gathered over the past 100 years to build potentially better systems. In the next 10 years, the available tools and advances driven by the demands of the entire biotechnology field will coalesce with nanotechnology, pushing the emerging field of synthetic biology forward.

Initial demonstrations of bottom-up construction of viruses have evolved into the *de novo* creation of simple single-cell organisms. Nanotechnology offers the tools and processes to further create hybrid biological systems that do not rely solely on natural biomolecules (nucleic acids, proteins, lipids, carbohydrates) but incorporate non-biological molecules, including semiconductor materials. Self-replicating hybrid biological systems that include what are not traditionally thought of as biological materials are already on exhibition in nature. Diatoms and other similar organisms incorporate silicon onto protein templates to create rigid exostructures. This biomineralization process suggests that synthetic biology extended to include semiconductor-based structures could be replicated through harnessing those biomineralization mechanisms used by diatoms and encoded in their genomes. The expansion of the materials palette compatible with biological systems is already exemplified by the development of components for the incorporation of unnatural amino acids (Noren et al. 1989). This expanded amino acid repertoire enables a number of post-translational chemistries to be used, further increasing the spectrum of functionality in biological systems. For example the incorporation of amino acid analogs with an azide moiety could be used to carry out a subsequent "click" reaction (Gauchet et al. 2006); the most simplistic application would be to immobilize a protein to a surface, but more elaborate schemes of mimicking various post-translational modifications (glycosylation) could be imagined. For

nanotechnology to advance and be a vital part of synthetic biology, it will be necessary to overcome challenges that exist in interfacing the various elements and ensuring that processes are compatible, especially with regard to the relative fragility of biomolecules. Nevertheless, the resiliency of biomolecules is surprising, and life in extreme environments (pH, temperature, ionic strength) suggests routes to obtain a set of biological components compatible with the nanotechnology tools and processes.

The biggest challenges in the future relate to the *de novo* design of systems and an ability to accurately predict the performance of these synthetic systems. Mimicking biological systems is not pushing the limits of potential and imagination. From the standpoint of *de novo* constructed systems, the most immediate challenge is "booting up the system" where the only inputs are nucleic acids. These systems have developed over eons of evolution, and the generally recognized path starts with catalytic nucleic acid molecules. There is a potential analog in booting up the system; nanotechnology may provide a basis through the design and fabrication of devices that have the precision to orient parts, much as RNA polymerase helps to transcribe the DNA sequences and ribosomes help to translate this information into proteins. The debate about mechanosynthesis and its ultimate feasibility and/or utility will continue (Baum 2003).

In essence, the future of synthetic biology will take a chapter from evolution but replace the laws of natural selection with ones that are derived from theoretical models. Since these models will be constructed and validated based upon observations made over the past 100 or more years, there will still be elements of evolution, but the process will be replaced by a few rounds of computation.

7.8.3 Paradigm Changes in Use of Nanoscale Sensors to Monitor Human Health/Behavior

Contact person: Harry Tibbals, University of Texas Southwestern Medical Center

Nanotechnology-based sensors for measurements in the human body have advanced significantly since 2000. In addition to incremental improvements in the technologies and types of applications envisioned ten years ago, a number of developments have created paradigm shifts in methods and approaches. Significant advances for *in vivo* biomedical nanosensors have been achieved in the following areas: physical and physiological sensors, imaging, biomarkers and diagnostics, and integrated nanosensor systems (Tibbals 2010).Measurement capability for biological physiology has improved in the past decade by application of nanotechnology in blood pressure, tissue pressures, pH, and electrophysiological monitoring of brain activity. Tables 7.2 and 7.3 and Figure 7.25 give selected examples of key nanotechnology-based advances in medicine since the year 2000.

Table 7.2. Paradigm changes in use of nanoscale sensors for monitoring human health and behavior

Pre-2000	2000-2010	2010-2020	2020-2030	Medical Applications
Pulse oximetry				
Attached, wired, 1-2 cm^3	Attached wired <1 cm^3	Adhered, wearable, wireless reporting 0.1 cm^3	Implanted, embedded, wireless, remote 0.001 cm^3	Cardiovascular, ICU, intraoperative surgical monitoring
Accelerometry				
External, attached, wired, 1-2 cm^3	Attached wired <1 cm^3	Adhered, implanted, wireless reporting .001 cm^3	Implanted, embedded, wireless, remote < 0.001 cm^3	Home monitoring, post-operative, geriatrics, orthopedics, neurology (gait analysis), cardiovascular

Pre-2000	2000-2010	2010-2020	2020-2030	Medical Applications
Pressure				
External, attached, wired, 2-4 cm^3	Embedded wireless <1 cm^3	Implanted Wireless 0.001 cm^3	Implanted, ingestible wireless, remote < 0.001 cm^3	Ob-Gyn (uterine), intra- & post-operative monitoring, gastro-, intestinal, orthopedics, prosthetics, cardiovascular
Humidity				
External, attached, wired, 3-4 cm^3	Attached wired <2 cm^3	Wireless/remote optical sensing 0.001 cm^3	Implanted, ingestible wireless, remote < 0.001 cm^3	Ob-Gyn (uterine), intra- & post-operative monitoring, gastro-, intestinal, urology, orthopedics, cardiovascular,
Galvanic potential (skin)				
External, attached, wired, 3-4 cm^3	Attached wired <2 cm^3	Wireless/remote optical sensing 0.001 cm^3	Implanted, ingestible wireless, remote < 0.001 cm^3	Ob-Gyn (uterine), intra- & post-operative monitoring, gastro-, intestinal, urology, orthopedics, cardiovascular,
Impedance (internal)				
External, attached, wired, 3-4 cm^3	Attached wired <2 cm^3	Wireless/remote optical sensing 0.001 cm^3	Implanted, ingestible wireless, remote < 0.001 cm^3	Ob-Gyn (uterine), intra- & post-operative monitoring, gastro-, intestinal, urology, orthopedics, cardiovascular
pH				
External, attached wired 3-4 cm^3	Attachable wireless <1 cm^3	Wireless, implantable ingestible 0.001 cm^3	Implantable, ingestible wireless, remote < 0.001 cm^3	Gastrointestinal, intra- & post-operative monitoring, urology, wound healing
Glucose sensors				
External sampling	Sampling through skin with nano-needle arrays, implantable MEMS	Long-term implantable MEMS/NEMS with wireless monitoring and feedback for insulin delivery via wearable micropump	Incorporation of nanosensors in integrated artificial pancreas and/or encapsulated live-cell bioreactor	Diabetes, Endocrinology, Immunology
EMG: electromagnetoencephalography				
External, room-sized (SQUID)	External, cabinet (SQUID)	Attachable Wireless 1,000 cm^3 (MEMS)	Wearable Wireless <1000 cm^3 (NEMS)	Neurology, Neurosurgery (with magnetic brain induction) Prosthetics (brain interface)
Fluorescence: nanodots for biomarkers				
External (in cell cultures and lab samples)	Internal: IV, injectable; (detection with fiberoptic probes, endoscopy) (cancer)	Internal: IV, Injectable, (detection with wireless spectroscopic probes) (other diseases)	Internal, IV, ingestible Injectable, ingestible, (detection with wireless spectroscopic probes, external IR through-skin monitors) (wide variety of diagnostics)	Cancer, infectious diseases, inflammatory, degenerative, genetic diseases
Ultrasound and opto-acoustic-enhancement nanoparticles				
External (in cell cultures and lab samples)	Internal: IV, injectable; (detection with fiberoptic probes, endoscopy) (cancer)	Internal: IV, Injectable, (detection with wireless spectroscopic probes) (other diseases)	Internal, IV, ingestible injectable, (detection w/ wireless spectroscopic probes, external IR through-skin monitors) (wide diagnostic variety)	Cancer, infectious diseases, inflammatory, degenerative, genetic diseases
FTIR and Raman				
External (in cell cultures and lab samples)	External (single-cell detection)	Attachable, implantable MEMS for internal single-cell wireless monitoring	Attachable, implantable injectable, ingestible, NEMS for internal single-cell resolution screening, monitoring	Cancer, infectious diseases, inflammatory, degenerative, genetic diseases
MS: mass spectrometry				

Pre-2000	2000-2010	2010-2020	2020-2030	Medical Applications
External (cell cultures, extracts, samples)	1st single-molecule MS, external, lab (experimental proof-of-concept)	Insertable, attachable single-molecule MS for monitoring	Implantable « « Etc.	Cancer, infectious diseases, inflammatory, degenerative, genetic diseases
Nanosensors in tissue scaffolding				
Concept	Experimental guidance of stem cell and autologous cell growth	Proof-of-concept; 1st prototypes; clinical trials; 1st approved uses	Use in medical practice; on-demand generation of live-cell tissue scaffolds	Surgery, nerve, brain, and bone regeneration, joint and cartilage, heart, burns, wounds
Nanosensors in neurosensory prosthetics				
Concept, wireless operation	Experimental prototypes for improved retinal prostheses and cochlear implants with nanosensors	Reduction in size, power requirements; use of power harvesting via nanogenerators	Nano-surface engineering of sensor interfaces for improved bio-compatibility, longer lifetime; live-cell integration	Vision, hearing
Integrated nanodevices for targeted therapeutics				
Concept, theranostic targeted nanoparticles for drug and RF therapy delivery	Development of a number of specific targeted NPs for imaging, thermo- and radio-therapy, drug delivery	Platforms for customizable targeted nano-therapeutics based on individual genotypes and phenotypes	Implantable, integrated nanosystems for monitoring and responding to pathological conditions	Infectious diseases, cancer, degenerative diseases, chronic metabolic conditions, traumatic injury, stress-induced conditions

Table 7.3. Advances in medical nanotechnology for measurements in the human body

Technology	Application Area	Qualitative/ Quantitative Advances	Stage of Development				
			Proof of concept	Proto-type	Pre-clinical	Clinical trials	Appli-cation
MEMS pressure monitoring:	Blood pressure, intra-arterial (cardioMEMs)	Wireless, min-invasive, continuous, x3 accuracy	2001	2002	2003	2005	2006
MEMS gastric and esophageal Monitoring	Gastric acid reflux, pre-cancer: pH, Impedance	Wireless, min-invasive, x5 sensitivity	Bravo: 2002; UTA: 2004	Bravo: 2003 UTA: 2005	Bravo: 2003; UTA: 2007	Bravo: 2005	Bravo: 2007
MEG: MEMS Magneto-encephalography	Brain monitor; prosthetics	Min-invasive; sensitivity = x100 to x1000	NIST, Bell Labs: 2003-4	2005	2006-2007		
Gold nanodots and nanorods	Diagnostic imaging: x-ray and ultrasound enhancement	x1000 in intensity, contrast, selectivity	2000-2005	2003-2006	2004-2008	2004-2009	2005-
Si, ceramic dye–doped Nanoparticles	Detection of biomarkers, *in vivo* diagnostics	Selectivity, stability, sensitivity = x100 - 1000	2001-2005	2003-2006	2005-2008		
MRI nano-engineered particles	Contrast enhancement for MRI imaging	Contrast enhancements = x50	2005-2006	2006-2007	2007-2009		
Nanostructures for surface-enhanced Raman spectra	Detection of biomarkers on skin, in tissue, in breath, body fluids	Detection sensitivity increase of $x10^{14}$-10^{15}	2000-2005	2003-2006	2005-2009		
Label-free nano-engineered lab-on-chip arrays based on CNTs, Si nanowires, BioFETs, etc.	Detection of DNA, proteins, biomarkers, for early diagnosis of cancer, degenerative diseases, personalized medicine	Reductions of 100X in detection time, 20X in detection costs, 100X in device costs	2001-2005	2004-2007	2006-2008	2009-2010	2010
Nanotech for DNA sequencing	Personalized medicine; targeted therapeutics	400 million base pairs per 40 min.; entire human genome in 15 minutes (est. by 2013); Following Moore's law: doubling capacity every 18 months	2000	2001-2005	2005-2009	2005-2009	2009-2010-
Wearable/ implantable medical sensors with wireless communication	Monitoring for post treatment, preventative care, health maintenance, remote checkup and disease management (e.g., diabetes, cardiovascular)	Following Moore's law	2002-2005	2003-2006	2005-2009	2007-2010	2010-

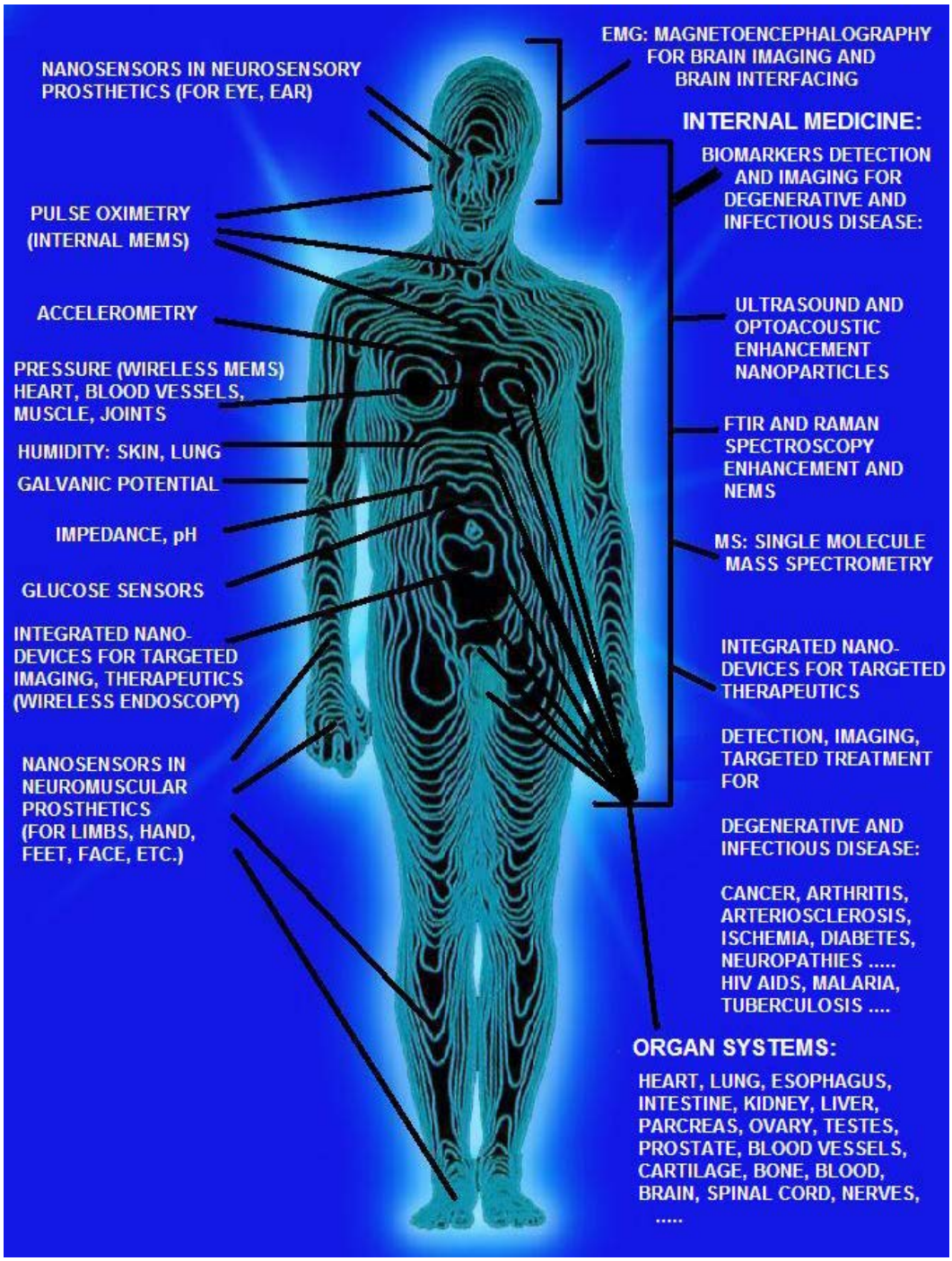

Figure 7.25. Sensors for monitoring human health and behavior; see Tables 7.2 and 7.3 for details and time frame for application (adapted from http://www.csm.ornl.gov/SC98/viz/viz4.html).

A number of research directions can be successfully addressed by 2020:

- Many of the medical technologies for monitoring the human body are just now at the "proof-of-concept" stage. The next ten years will see them move into prototyping of devices and systems for medical application, followed by clinical trials. Those that pass tests of safety and effectiveness will rapidly appear in medical practice.

- We will continue to see convergent integration of sensors, communications, and therapeutics into systems that detect, monitor, and apply therapies in response to changes in health status of individuals and populations.
- Several new areas of smart integrated nanosensors and nanomaterials are especially exciting for their future potential to regenerate tissue affected by brain and nerve injury, cardiovascular ischemia, and degenerative diseases such as Alzheimer's disease and ALS. One is the application of protein nanoscience to the epigenetic reprogramming of cells. Cellular-level nanotechnology is being applied to create stem cells from a person's own mature cells, eliminating problems associated with immune rejection, embryonic stem cells, and genetic engineering of cells for medical treatments. Smart nano-implant nanotechnology will be applied to the guidance of cell growth, differentiation, and migration; this will be important in making stem cell therapy more successful than it is currently, by providing nano-engineered tissue scaffolding to support stem cell implants.
- Another area that will develop in the next decade in increased use of sophisticated nanosensors to guide surgery and medical interventions such as tissue transplants, including the generation of live tissue scaffold implants on demand in the operating room. These custom implants will be generated in real time in response to sensor evaluation of tissue injuries, immunologies, and DNA matching, by three-dimensional tissue printing with nano-engineering rapid prototyping systems that assemble implants with biopolymers and cells to fit the shape, size, and structural requirements of a specific surgical operation while it is taking place.
- Another area is the increase in use of low-cost wearable and implantable intelligent nanosensors to give early warning of incipient health conditions, to monitor the progress of treatment, and to apply therapies by nano-activation of energy or chemical medications in the body, either automatically in response to embedded control systems, or on command from healthcare providers who can monitor the patient remotely. These will be used increasingly for remote examination and evaluation of patients by healthcare providers, and for organization of collaborative and consultative care (Tibbals 2010).
- The exponentially growing development of nanotechnology-based biosensor capabilities is contributing to more effective and lower-cost approaches to health care. These approaches are needed to deal with economic, demographic, environmental, and epidemiological challenges. Nanosensor capabilities for biomedicine are a potentially disruptive technological force that will realign the tools and delivery modes of healthcare—a realignment that will represent a strategic approach to changing healthcare needs. Whether this challenge is met will depend on continued commitment to basic research and development as well as further work to move demonstrated new technical capabilities into practical medical application.

7.9 INTERNATIONAL PERSPECTIVES FROM SITE VISITS ABROAD

7.9.1 United States-European Union Workshop (Hamburg, Germany)

Panel members/discussants

Chrit Moonen (co-chair), CNRS/University Bordeaux 2, France
Andre Nel (co-chair), University of California, Los Angeles, U
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Günter Gauglitz, University of Tübingen, Germany
Milos Nesladek, Hasselt University, Belgium
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Contributors: *Chad Mirkin, Northwestern University; C. Shad Thaxton, Northwestern University*

This summary builds largely on the abstract by Chad Mirkin, Andre Nel, and C. Shad Thaxton of the March 9–10, 2010, workshop held in Evanston, Illinois, USA. The group working on the Nanobiosystems, Medicine, and Health topic at the Hamburg meeting fully agreed with the outcome of the Evanston meeting (Part 1 below). Most of the discussion in Hamburg then concentrated on the very important role of regulation. This is summarized in Part 2.

Part 1: Abstract of the Evanston Meeting

Nanostructures are now being used in the development of powerful tools for manipulating and studying cellular systems; ultrasensitive *in vitro* diagnostics that can track indications of disease at very early stages; *in vivo* imaging agents that provide better contrast and more effective targeting compared to molecular systems; and novel therapeutics for debilitating diseases such as cancer, heart disease, and regenerative medicine. These advances are empowering scientists and clinicians, and in the process they are changing the lives of citizens in very profound and meaningful ways. While the science and engineering of nanobiosystems is one of the most exciting, challenging, and rapidly growing sectors of nanotechnology, society continues to be faced with daunting challenges in biomedicine and healthcare delivery. Significant challenges that need to be addressed over the next decade include:

- Identification of the molecular and cellular origin of disease processes such as cancer, cardiovascular disease, diabetes mellitus, aging, and tissue/organ regeneration
- Health monitoring and early detection of disease through the identification of novel biomarkers, diagnostic tool kits, and advanced imaging technologies
- Establishment of scientific bases for regenerative medicine, biocompatible prosthetics, and stem cell engineering
- Development of a quantitative understanding of how biological nanosystems and subcellular constituents work in synchrony to establish systems biology that can be interrogated at the nanoscale level
- Synthesis of new devices or systems that provide targeted and on-demand drug delivery that also provides an imaging component
- Fostering an understanding of the tenets of nanoscience in the biomedical community, with the potential to accelerate bench-to-bedside implementation of new technologies
- Realization of personalized medicine and point-of-care (POC) healthcare delivery that is rapid, efficient, and cost-effective

In order to address these challenges through translational advances in biomedical nanotechnology, significant investment is imperative in biomedical nanotechnology research, small businesses aiming to commercialize nanotechnologies, and science and math education at all grade levels. Furthermore, regulatory changes are needed with regard to more rapid evaluation of new technologies for early approval and safe delivery to patients by educated healthcare providers. Nanomedicine has raced forward at a staggering pace with significant investment and focused collaborative energy. As a result, some paradigm shifts are now becoming reality; these include approaching cancer from a holistic perspective based upon early diagnosis and treatment, as well as personalized medicine and cancer prevention. The overall impact of nanotechnology on biomedicine will be multifaceted and consist of advanced screening, diagnosis and staging, personalized treatment and monitoring, and follow-up. Rapid success depends upon focused collaborative energies, future investments, and wide spread education about the realities and promise of nanomedicine.

Part 2: Regulatory Aspects of Nanomedicine and Nanobiosystems Development

It is evident that regulatory issues have a large impact on the translation to the clinic, on the impact of nanotechnologies on health, and on the creation of companies. There is a need for improved EU-U.S. collaboration on issues important for regulatory bodies, such as reproducibility, good manufacturing processes, bio-distribution, toxicity, immunogenicity, etc. Scientists working in medical nanotechnologies should be involved with regulatory agencies at an early stage. A major objective is the creation of a streamlined, effective, regulatory pathway. A secondary objective is the integration of research funding approaches to underpin facilitated regulatory approval. A third objective is to address the funding gap "from science to market" in view of the regulatory issues.

7.9.2 United States-Japan-Korea-Taiwan Workshop (Tokyo/Tsukuba, Japan)

Panel members/discussants

Kazunori Kataoka (co-chair), University of Tokyo, Japan
Chad A. Mirkin (co-chair), Northwestern University, United States
Andre Nel, University of California, Los Angeles, United States
Yoshinobu Baba, Nagoya University, Japan
Chi-Hung Lin, National Yang-Ming University, Taiwan
Teruo Okano, Tokyo Women's University, Japan
Joon Won Park, Pohang University of Science and Technology, Korea
Yuji Miyahara, National Institute for Materials Science (NIMS), Japan

Changes in the Vision of Nanotechnology in the Last Ten Years

Nanotechnology has definitely accelerated integration of established scientific fields, thereby accelerating the breaking down of boundaries between classic disciplines. Eventually, this culminated in establishing the new interdisciplinary science of nanotechnology and its associated biology branch known as "nanobiotechnology."

The visions emerging from nanobiotechnology over the last ten years have progressively focused on the establishment of nanomedicine, including drug delivery systems, regenerative medicine, molecular imaging, and diagnostic devices. Nanomedicine enables minimally invasive or noninvasive diagnosis and targeted therapy by breakthroughs in sensing, processing, and operating modalities, as shown in Figure 7.26.

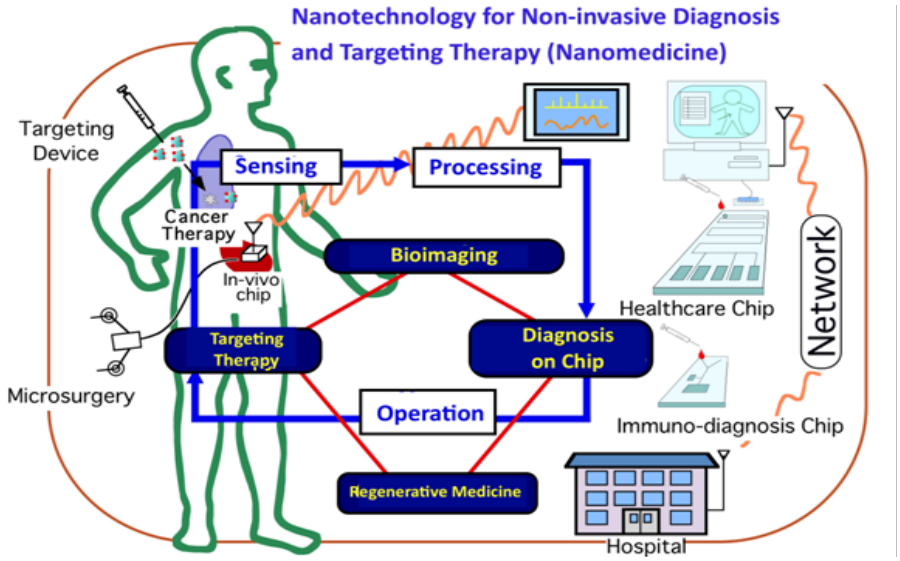

Figure 7.26. Vision for nanotechnology in medicine (courtesy of The University of Tokyo Center for NanoBio Integration, http://park.itc.u-tokyo.ac.jp/CNBI/e/index.html).

In addition, there is significant research training for blending the above capabilities into single-platform diagnostic and therapeutic nanobiodevices (nanotheranostics).

Vision of Nanobiotechnology for the Next Ten Years

Five specific topics were featured in the panel discussion to delineate visions of what may happen in nanobiotechnology over the next ten years:

- Ultra-sensitive, highly specific, low-invasive, and reliable (robust) detection technologies for diagnosis, prognosis, and prevention
- Multifunctional nanobiodevices for the integration of diagnosis and therapy
- Accelerating the translation of nanobiotechnology-based methods and devices to points of clinical care
- Innovation in nanophysiology through intravital and nanoscaled observation of operations in living organisms through the use of multifunctional nanodevices
- Nanobiotechnology applications to ensure the implementation of environmentally friendly nanofabrication processes, and detection and monitoring of harmful chemicals and organisms

Main Scientific/Engineering Advancements and Technological Impacts of Nanobiotechnology in the Last Ten Years

The panel selected the following major advancements in the field of nanobiotechnology that have had high impact over the last 10 years:

- Development of nanocarrier systems for cancer chemotherapy premised on the EPR effect has yielded new cost efficient therapeutics with higher efficacy and lower side effects.
- Cell sheet engineering for tissue repair and regeneration has been implemented to treat cardiac failure and diseases of the cornea, with additional potential to be used for tissue repair after the surgery of esophageal epithelial cancer. In these three areas, cell sheet engineering therapy has already started to treat human patients.

- Ultrasensitive and highly specific biodetection systems by integrated bottom-up and top-down nanotechnologies led to significant impact in terms of protein biomarker detection and clinical diagnosis.

Goals for the Next 5–10 years: Opportunities, Barriers, and Solutions.

The panel discussed the following seven goals in terms of opportunities, barriers, and solutions.

Goal 1: Development of multifunctional nanobiodevices for single-platform bioimaging and targeting therapy (theranostic nanodevices)

Barriers: A major challenge is in the development of nanocarriers with high extravasation potential through transcytosis. This may enable transport of probes and drugs through the blood-brain barrier, and thus opens the door to nanotechnology-enabled diagnosis and therapy of intractable brain diseases, including Alzheimer's disease and Parkinson's disease.

Solutions: Recent progress in phage display technology has highlighted the potential to use a variety of peptide sequences to facilitate transcytosis in endothelial cells. Coupling or combining these peptides to nanocarriers is a promising approach to crossing the blood-brain barrier.

Goal 2: Development of ultrasensitive, highly specific, low-invasive, and reliable (robust) multiplexed detection technologies

This will enable diagnosis through the ability to detect disease processes at their inception, e.g., a single diseased cell or pathophysiology at the molecular level. This methodology will allow early cancer detection.

Barriers: Development of systems with the sensitivity of PCR techniques but without their complexity. The system should detect a variety of analytes, including nucleic acids, proteins, small molecules, metal ions, and should be capable of multiplexing and amenable to point-of-care use.

Solutions: Bar-code technology, lab-on-a-chip diagnostics. This requires close collaboration between the medical and nanotechnology communities. It will be important to demonstrate the utility of this approach as a key breakthrough in the major disease target to demonstrate the clinical utility of smart nanosensing devices.

Goal 3: Remote disease monitoring through the ability of nanotechnology to provide online sensing and information relay

Barriers: Relative shortage of healthcare personnel and POC delivery in underserved and rural communities. Safety and regulation issues.

Solutions: Nanosensor devices with portability. Establish regulatory policy and educate people in government regulatory agencies.

Goal 4: Construction of tissue growth facilitating structured cell sheets for organ repair and replacement

Barriers: Lack of organ donors and the availability of off-the-shelf tissue and organ resources.

Solutions: Use of nano-architectures, synthetic morphogens, and extracellular matrix mimics to induce cellular growth and reconstitution of tissues and organs. Preservation technology.

Goal 5: Application of biological nanostructures (i.e., nucleic acids, proteins) in bioelectronics and environmentally friendly nanofabrication processes

Barriers: Creating viable strategies for fabricating and integrating.

Solutions: Research on interfacing biological structures with inorganic materials. Creating design rules for system-wide construction.

Goal 6: Single-cell interventions and diagnostics, including stem cell growth and differentiation and cellular-level genomics and proteomics

Barriers: Sensitivity, transfection, and limited examples requiring this capability.

Solutions: Develop nano-bio tools with high sensitivity and more examples and applications.

Goal 7: Stem cell differentiation and site-specific delivery by devices that allow protective delivery and can serve as growth templates that include nano-architectured surfaces

Barriers: Understanding chemical and physical stimulation to cell differentiation. Understanding signal pathways.

Solutions: Nanostructured interfaces, 3D nano-architectures, and synthetic morphogens.

R&D Investment and Implementation Strategies

Implementation of a strategy that facilitates multidisciplinary research through the creation of open innovation centers for "nano-bio" equipped with cutting-edge instruments and animal facilities. Particularly, R&D investment to establish nanomedicine centers in medical schools and hospitals with the ability to bring nanotechnology-based therapeutics, diagnostics, devices, and systems to the clinic.

Emerging Topics and Priorities for Future Nanoscale Science Research and Education

The panel reached the consensus that priority should be given to research for the understanding of "life" at the single molecular and cellular level through nanotechnology. This technology should also be applied to *in vivo* nanoscale biodevices that can perform smart functions through the combination of molecular self-assembly and nanofabrication processes. These nanodevices will also be helpful to establish the new scientific fields of "nano-physiology" and nano-systems biology that can be used for theranostics approaches in nanomedicine.

The Impact of Nanotechnology R&D on Society

Significant progress in nanomedicine could allow us to cure cancers at an early stage and therefore enhance human longevity. The combination of nanomedicine and information technology enables diagnosis and therapy with high quality of life regardless of age, wealth, country, and occupation, contributing to continuous development in human welfare. Furthermore, understanding the behavior of individual cells deepens our understanding of life on earth, with the potential to induce a paradigm shift in the entire area associated with the biosphere, including health science and medical practice.

7.9.3 United States-Australia-China-India-Saudi Arabia-Singapore Workshop (Singapore)

Panel members/discussants

Yi Yan Yang (co-chair), Institute of Bioengineering and Nanotechnology, Singapore
Chad Mirkin (co-chair), Northwestern University, United States

The panel made the following main points in answering the questions related to health and nanobiotechnology in a broad context. They focused primarily on the future.

How has the vision of nanotechnology changed in the last 10 years?

- Transition from hype to realization (diagnostics, *in vivo* imaging, and therapeutics) – FDA-cleared systems and first human nanodrug trials
- Realization that nanotherapeutics will be a new drug-delivery platform
- Transition from individual components to more sophisticated multifunctional systems

Where is the field likely to go?

- Imaging live systems with molecular resolution on the time scale of chemical reactions and biological processes
- Noninvasive pathology
- Biological drug (peptides, proteins, nucleic acids) and cell delivery systems
- Overcoming and preventing drug resistance
- Preventing, reversing, or eliminating metastatic cancer
- Atomic resolution of integral membrane proteins with a view to identifying drug targets.
- Imbedded and pervasive, true point-of-care molecular diagnostic systems.

What are the main scientific/engineering advances & technological impacts in the last decade?

- FDA cleared nano diagnostic systems
- First human trials with siRNA nanodelivery systems
- Order of magnitude improved resolution in *in vivo* imaging enabled by better tools assisted by nanoparticle contrast agents.

What are the goals for the next 5-10 years: Opportunities, barriers, solutions?

- Imaging live systems with molecular resolution on the time scale of chemical reactions and biological processes
  - *Barrier:* Resolution, background, and timescale
  - *Solution:* Financial resource; understanding of contributions to background; probe sensitivity, integration of imaging modalities; overcoming computation and modeling deficiencies.
- Noninvasive Pathology
  - *Barrier:* Lack of tools and insufficient biomarker data base
  - *Solution:* Suite of *in vitro* molecular diagnostic and *in vivo* imaging tools, combined with an aggressive approach to identifying and validating molecular and structural markers for specific pathology and pathophysiology. New nanoscale contrast agents with enhanced sensitivity and specificity for disease states. Resolving sampling statistical issues.

- Biological drug (peptides, proteins, nucleic acids) and cell delivery systems
  - *Barrier:* Drug and cell instability, environment compatibility, effective targeting schemes that overcome drug resistance.
  - *Solution:* Nanomaterials that encapsulate, stabilize, deliver, and release therapeutic agents at the point of disease.
- Overcoming and preventing drug resistance
  - *Barrier:* Drug and cell instability, environment incompatibility.
  - Solution: Nanoscale dual drug/gene regulation systems; stealth delivery and entry that bypasses drug resistant mechanisms. Contemporaneous multidrug and adjuvant delivery.
- Preventing, reversing, or eliminating metastatic cancer
  - *Barrier:* Lack of understanding and the inability to effectively intervene in metastatic pathways. Surreptitious nature of metastatic disease.
  - *Solution:* Delivery of biological signal that stops the budding of metastatic cancer cells; nanoparticle delivery systems; encapsulation metastatic cells
- Atomic resolution of integral membrane proteins with a view to identifying drug targets.
  - *Barrier:* Lack of understanding of nucleation and crystallization phenomena of integral membrane proteins. Tracking protein conformational changes in a membrane environment.
  - *Solution:* Better EM and related high resolution imaging methods; amphiphilic nanostructures that mimic membrane structure and dynamics.
- Embedded and pervasive, true point-of-care molecular diagnostic systems.
  - *Barrier:* Increasing sensitivity and specificity; Molecular structural and functional imaging; Molecular monitoring of response to therapy; Non-invasive imaging and minimally invasive imaging; Automation (sample prep, imaging and identification); Molecular single cell imaging and tomography; Single cell molecule extraction; Multi-analyte extraction (DNA, RNA, Protein, metabolites) from same few cells
  - *Solution:* Multiplexed nano-enabled multifunctional platforms

What is the impact of nanotechnology R&D on society?

- Transformative
- Economy building
- Improves quality of life
- Addresses global challenges such as personalized medicine, food security, climate change, water security, and sustainability.

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