

Chapter 4

Nanomolecular Diagnostics

Introduction

Clinical application of molecular technologies to elucidate, diagnose, and monitor human diseases is referred to as molecular diagnosis. It is a broader term than DNA (deoxyribonucleic acid) diagnostics and refers to the use of technologies that use DNA, RNA (ribonucleic acid), genes, or proteins as bases for diagnostic tests. The scope of the subject is much wider and includes *in vivo* imaging and diagnosis at the single-molecule level. A more detailed description of molecular diagnostics is presented elsewhere (Jain 2012a).

Because of the small dimension, most of the applications of nanobiotechnology in molecular diagnostics fall under the broad category of biochips/microarrays but are more correctly termed nanochips and nanoarrays. Nanotechnology-on-a-chip is a general description that can be applied to several methods. Some of these do not use nanotechnologies but merely have the capability to analyze nanoliter amounts of fluids.

Biochips, constructed with microelectromechanical systems on a micron scale, are related to micromanipulation, whereas nanotechnology-based chips on a nano-scale are related to nanomanipulation. Even though microarray/biochip methods employing the detection of specific biomolecular interactions are now an indispensable tool for molecular diagnostics, there are some limitations. DNA microarrays and ELISA rely on the labeling of samples with a fluorescent or radioactive tag – a highly sensitive procedure that is time-consuming and expensive.

The chemical modification and global amplification of the nucleic acid samples are achieved by polymerase chain reaction (PCR), which can introduce artifacts caused by the preferential amplification of certain sequences. Alternative label-free methods include surface plasmon resonance (SPR) and quartz crystal microbalance, which rely on mass detection. Nanotechnologies also provide label-free detection. Nanotechnology is thus being applied to overcome some of the limitations of biochip technology. This chapter focuses on the application of nanotechnologies to nucleic acid as well as protein diagnostics.

Nanodiagnostics

Nanomolecular diagnostics is the use of nanobiotechnology in molecular diagnostics and can be termed “nanodiagnostics” (Jain 2003). Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It seems quite likely that there will be numerous applications of inorganic nanostructures in biology and medicine as biomarkers. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive, and more flexible when certain nanoscale particles are put to work as tags or labels. Nanotechnology will improve the sensitivity and integration of analytical methods to yield a more coherent evaluation of life processes.

It is difficult to classify such a variety of technologies, but various nanotechnologies with potential applications in molecular diagnostics are listed in Table 4.1. Nanotechnology-on-a-chip was described in Chap. 2. Some of the other technologies will be described briefly in the following text using examples of commercial products. Applications in clinical laboratory have been reviewed elsewhere (Jain 2007).

Rationale of Nanotechnology for Molecular Diagnostics

Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It is likely that there will be numerous applications of inorganic nanostructures in biology and medicine as markers:

- Nanoscale probes would be suitable for detailed analysis of receptors, pores, and other components of living cells that are on a nanoscale.
- Nanoscale particles, used as tags or labels, increase the sensitivity, speed, and flexibility of biological tests measuring the presence or activity of selected substances.
- Nanotechnology will improve the sensitivity and integration of analytical methods to yield a more coherent evaluation of life processes.

Nanoarrays for Molecular Diagnostics

Several nanoarrays and nanobiochips are in development (Jain 2012b). Some of these will be reviewed here.

Table 4.1 Nanotechnologies with potential applications in molecular diagnostics**Nanotechnology to improve polymerase chain reaction (PCR)****Nanotechnology-on-a-chip**

Microfluidic chips for nanoliter volumes: NanoChip

Optical readout of nanoparticle labels

Nanoarrays

Protein nanoarrays

Nanotechnology-based cytogenetics

Study of chromosomes by atomic force microscopy (AFM)

Quantum dot fluorescent in situ hybridization (FISH)

Nanoscale single-molecule identification

Nanoparticle technologies

Gold particles

Nanobarcodes

Magnetic nanoparticles: ferrofluids, supramagnetic particles combined with MRI

Quantum dot technology

Nanoparticle probes

Nanowires**Nanopore technology**

Measuring length of DNA fragments in a high-throughput manner

DNA fingerprinting

Haplotyping

DNA nanomachines for molecular diagnostics**Nanoparticle-based immunoassays**

DNA-protein and nanoparticle conjugates

Nanochip-based single-molecular interaction force assays**Resonance light scattering technology****Nanosensors**

Cantilever arrays

Living spores as nanodetectors

Nanopore nanosensors

Quartz nanobalance DNA sensor

PEBBLE (probes encapsulated by biologically localized embedding) nanosensors

Nanosensor glucose monitor

Photostimulated luminescence in nanoparticles

Optical biosensors: for example, surface plasmon resonance technology

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Nanofluidic/Nanoarray Devices to Detect a Single Molecule of DNA

One of the more promising uses of nanofluidic devices is isolation and analysis of individual biomolecules, such as DNA, which could lead to new detection schemes for cancer. One of these devices entails first constructing silicon nanowires on a substrate, or chip, using standard photolithographic and etching techniques,

followed by a chemical oxidation step that converts the nanowires into hollow nanotubes (Fan et al. 2005). Using this process, the investigators can reliably create nanotubes with diameters as small as 10 nm, though devices used for biomolecule isolation contain nanotubes with a diameter of 50 nm. To trap DNA molecules requires a device consisting of a silicon nanotube connecting two parallel microfluidic channels. Electrodes provide a source of current used to drive DNA into the nanotubes. Each time a single DNA molecule moves into the nanotube, the electrical current changes suddenly. The current returns to its baseline value when the DNA molecule exits the nanotube. On average, a DNA molecule remains within the nanotube for about 7.5 ms, which should be sufficient to make a variety of analytical measurements that could reveal cancer-associated mutations. The investigators are now adding optical and electrical circuitry to probe the trapped DNA molecules.

The nanoAnalyzer System (BioNano Genomics) is designed to enable direct visualization and linear analysis of multi-megabase genomic DNA at the single-molecule level with high feature resolution in massive parallel fashion. The platform is also anticipated to significantly reduce the cost and time needed for the extensive data and integrative analyses that have hindered widespread use of whole genome studies to date. It is expected to have broad application in systems biology, personalized medicine, pathogen detection, drug development, and clinical research.

Protein Nanoarrays

Protein microarrays provide a powerful tool for the study of protein function. However, they are not widely used, in part because of the challenges in producing proteins to spot on the arrays. Protein microarrays can be generated by printing complementary DNAs onto glass slides and then translating target proteins with mammalian reticulocyte lysate. Epitope tags fused to the proteins allowed them to be immobilized in situ. This obviates the need to purify proteins, avoided protein stability problems during storage, and captured sufficient protein for functional studies. This technology has been used to map pairwise interactions among 29 human DNA replication initiation proteins, recapitulate the regulation of Cdt1 binding to select replication proteins, and map its geminin-binding domain.

NanoArray Assay SystemTM (NanoInk) enables detection, identification, and quantitation of clinically relevant, low abundance proteins from a wide variety of sample types for applications such as biomarker analysis, translational medicine, and toxicology. NanoInk assays consume much smaller sample and reagent volumes than do traditional ELISA and bead-based assays, generating more proteomic data with less starting material and lowering assay costs for tests that are typically expensive to run.

Protein Nanobiochip

Nanotechnology Group of the NEC Corporation has developed a prototype protein analysis technology that can analyze samples about 20 times faster than conventional techniques. This technology can complete an analysis of a blood sample in about 60–70 min, compared to a day or so required for such analysis by conventional methods.

Biomarker proteins as early warning signs for diseases such as cancer can be identified for diagnostic purposes by finding their isoelectric points and their molecular weights. Isoelectric points are chemical features that refer to the electrical state of a molecule when it has no net charge. Conventional protein chips use a gel across which an electric current is applied to find the targeted protein's isoelectric points. In the new process, instead of being filtered through a block of gel, the protein molecules are separated by their isoelectric points by a capillary action as the proteins flow in a solution along channels in the chip. A test chip by NEC measures 21 mm² and contained four sets of tiny channels in which the capillary action takes place. The protein molecules are then dried and irradiated by a laser. Their molecular weights are then measured by a mass spectrometer. The laser helps the proteins leave the chip, and the mass spectrometer is used to judge the molecular weights of the protein molecules in the samples by measuring how early they reach a detector. In the mass spectrometer, light molecules fly faster than heavy ones in an electric field. The mass spectrometer judges the weight of the molecules by monitoring the timing of when each molecule reaches a detector. In addition to being faster than techniques that use gel blocks, the new method also needs blood samples of about 1 μ L compared to about 20 μ L or more that are needed using gel-based techniques. The company should commercialize the technology, and the technique could be used for health checks that might cost as little as \$100. The current status of this product is not known.

Fullerene Photodetectors for Chemiluminescence Detection on Microfluidic Chip

Solution-processed thin-film organic photodiodes have been used for microscale chemiluminescence (Wang et al. 2007). The active layer of the photodiodes comprised a blend of the conjugated polymer poly(3-hexylthiophene) and a soluble derivative of fullerene C60. The devices had an active area of 1 \times 1 mm and a broadband response from 350 to 700 nm, with an external quantum efficiency of more than 50 % between 450 and 550 nm. The photodiodes have a simple layered structure that allows integration with planar chip-based systems. To evaluate the suitability of the organic devices as integrated detectors for microscale chemiluminescence, a peroxyoxalate-based chemiluminescence reaction (PO-CL) was monitored

within a poly(dimethyl-siloxane) (PDMS) microfluidic device. Quantitation of hydrogen peroxide indicated excellent linearity and yielded a detection limit of 10 microM, comparable with previously reported results using micromachined silicon microfluidic chips with integrated silicon photodiodes. The combination of organic photodiodes with PDMS microfluidic chips offers a means of creating compact, sensitive, and potentially low-cost microscale CL devices with wide-ranging applications in chemical and biological analysis and clinical diagnostics.

AFM for Molecular Diagnostics

Nanofountain AFM Probe

Nanofountain AFM probe (NFP) has been used for nanofabrication of protein dot and line patterns (Loh et al. 2008). Biomolecules are continuously fed in solution through an integrated microfluidic system and deposited directly onto a substrate. Deposition is controlled by application of an electric potential of appropriate sign and magnitude between the probe reservoir and substrate. Submicron dot and line molecular patterns were generated with resolution that depended on the magnitude of the applied voltage, dwell time, and writing speed. By using an energetic argument and a Kelvin condensation model, the quasi-equilibrium liquid-air interface at the probe tip was determined. The analysis revealed the origin of the need for electric fields in achieving protein transport to the substrate and confirmed experimental observations suggesting that pattern resolution is controlled by tip sharpness and not overall probe aperture. As such, the NFP combines the high-resolution of dip-pen nanolithography with the efficient continuous liquid feeding of micropipettes while enabling scalability to 1D and 2D probe arrays for high throughput.

AFM for Immobilization of Biomolecules in High-Density Microarrays

Nanoscale resolution is an important step in the preparation of nanoarrays and placement of probe biomolecules. A flexible procedure has been described for simultaneous spatially controlled immobilization of functional biomolecules with submicrometer resolution by molecular ink lithography using AFM (Breitenstein et al. 2010). Bottom-up fabrication of surface bound nanostructures enables the immobilization of different types of biomolecules. The method works on transparent as well as on opaque substrates. The spatial resolution is better than 400 nm and is limited only by the AFM's positional accuracy after repeated z-cycles since all steps are performed in situ without moving the supporting surface. The principle is demonstrated by hybridization to different immobilized DNA oligomers and was validated by fluorescence microscopy. This method not only enables deposition of DNA at submicrometer resolution but also proteins and other molecules of biological relevance that can be coupled to biotin.

AFM for Nanodissection of Chromosomes

Chromosomal dissection provides a direct advance for isolating DNA from cytogenetically recognizable region to generate genetic probes for fluorescence in situ hybridization (FISH), a technique that became very common in cytogenetics and molecular genetics research and diagnostics. Several methods for microdissection (glass needle or a laser beam) are available to obtain specific probes from metaphase chromosomes. There are limitations of the conventional methods of dissection because a large number of chromosomes are needed for the production of a probe. Moreover, these methods are not suitable for single chromosome analysis, because of the relatively large size of the microneedles. New dissection techniques are required for advanced research on chromosomes at the nanoscale level. Both AFM and scanning near-field optical microscopy (SNOM) have been used to obtain local information from G-bands and chromosomal probes.

AFM has been used as a tool for nanomanipulation of single chromosomes to generate individual cell-specific genetic probes. Molecular and nanomanipulation techniques have been combined to enable both nanodissection and amplification of chromosomal and chromatidial DNA (Di Bucchianico et al. 2011). Cross-sectional analysis of the dissected chromosomes reveals 20- and 40-nm-deep cuts. Isolated single chromosomal regions can be directly amplified and labeled by the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) and subsequently hybridized to chromosomes and interphasic nuclei. FISH, performed with the DOP-PCR products as test probes, has been tested successfully in avian microchromosomes and interphasic nuclei. Chromosome nanolithography, with a resolution beyond the limit of light microscopy, could be useful for the construction of chromosome band libraries and for molecular cytogenetic mapping in investigation of genetic diseases.

Nanoparticles for Molecular Diagnostics

Gold Nanoparticles

Bits of DNA and Raman-active dyes can be attached onto gold nanoparticles, which assemble onto a sensor surface only in the presence of a complementary target. If a patterned sensor surface of multiple DNA strands is used, the technique can detect millions of different DNA sequences simultaneously. The current non-optimized detection limit of this method is 20 femtomolars. Gold nanoparticles are particularly good labels for sensors because a variety of analytical techniques can be used to detect them, including optical absorption, fluorescence, Raman scattering, atomic or magnetic force, and electrical conductivity. Gold nanoparticles and Raman spectroscopy have been used to detect bacteria and viruses. This approach could replace PCR and fluorescent tags commonly used today. The detection system can also be used on biochips dotted with DNA. If the targeted disease exists in the sample, its DNA will bind onto the complementary strands of DNA on the chip and

gold nanoparticle. The chip is treated with silver-based solution, which coats the nanoparticles. When exposed to a light scanner, the coating enhances the signal enough to detect minute amounts of DNA. Since the Raman band is narrower than the fluorescent band, it allows more dyes to detect more targets quickly. If the sequence of interest is present in the sample, it will bind to the DNA and cause the solution to change color. Labeling oligonucleotide targets with gold nanoparticle rather than fluorophore probes substantially alters the melting profiles of the targets from an array substrate. Nanoparticle-based DNA detection systems are more sensitive and specific than current genomic detection systems.

QDs for Molecular Diagnostics

There is considerable interest in the use of QDs as inorganic fluorophores, owing to the fact that they offer significant advantages over conventionally used fluorescent markers. For example, QDs have fairly broad excitation spectra – from ultraviolet to red – that can be tuned depending on their size and composition. At the same time, QDs have narrow emission spectra, making it possible to resolve the emissions of different nanoparticles simultaneously and with minimal overlap. Finally, QDs are highly resistant to degradation, and their fluorescence is remarkably stable. Advantages of QD technology are:

- Simple excitation – lasers are not required
- Simple instrumentation
- Availability of red/infrared colors enables whole-blood assays
- High sensitivity

QDs have been used as possible alternatives to the dyes for tagging viruses and cancer cells. A major challenge is that QDs have an oily surface, whereas the cellular environment is water-based. Attempts are being made to modify the surface chemistry of QDs so that they interact with water-friendly molecules like proteins and DNA. The current goal is to develop QDs that can target a disease site and light it up. This can someday lead to an integrated system that will also use the QDs to diagnose as well to deliver drug therapies to the disease site. QDs can be designed to emit light at any wavelength from the infrared to visible to ultraviolet. This enables the use of a large number of colors and thus multiplexed assays can be performed. Potential applications of QDs in molecular diagnostics can be summarized as follows:

- Cancer
- Genotyping
- Whole-blood assays
- Multiplexed diagnostics
- DNA mapping
- Immunoassays and antibody tagging
- Detection of pathogenic microorganisms

Quantum Dots for Detection of Pathogenic Microorganisms

QDs have been used as fluorescent labels in immunoassays for quantitative detection of foodborne pathogenic bacteria such as *Salmonella typhimurium*. QDs coated with streptavidin are added to react with biotin on the secondary antibody. Measurement of the intensity of fluorescence produced by QDs provides a quantitative method for microbial detection. QDs can be used for ultrasensitive viral detection of a small number of microorganisms.

Bioconjugated QDs for Multiplexed Profiling of Biomarkers

Bioconjugated QDs provide a new class of biological labels for evaluating biomarkers on intact cells and tissue specimens. In particular, the use of multicolor QD probes in immunohistochemistry is considered one of the most important and clinically relevant applications. At present, however, clinical applications of QD-based immunohistochemistry have achieved only limited success. A major bottleneck is the lack of robust protocols to define the key parameters and steps. Preliminary results and detailed protocols for QD-antibody conjugation, tissue specimen preparation, multicolor QD staining, image processing, and biomarker quantification have been published (Xing et al. 2007). The results demonstrate that bioconjugated QDs can be used for multiplexed profiling of biomarkers and ultimately for correlation with disease progression and response to therapy. In general, QD bioconjugation is completed within 1 day, and multiplexed molecular profiling takes 1–3 days depending on the number of biomarkers and QD probes used.

Imaging of Living Tissue with QDs

Tiny blood vessels, viewed beneath a mouse's skin with multiphoton microscopy appear so bright and vivid in high-resolution images that researchers can see the vessel walls ripple with each heartbeat. Capillaries, hundreds of microns below the skin of living mice, can be illuminated in an unprecedented detail using QDs circulating through the blood as fluorescent imaging labels. Although there are easier ways to take a mouse's pulse, this level of resolution with high signal-to-noise ratio illustrates how useful multiphoton microscopy with QDs can become in biological research for tracking cells and visualizing tissue structures deep inside living animals. Monitoring of vascular changes in malignant tumors is a potential application. This approach will pave the way for many new noninvasive in vivo imaging methods using QDs.

Carbohydrate-encapsulated QD can be used for medical imaging. Certain carbohydrates, especially those included on tumor glycoproteins, are known to have affinity for certain cell types, and this can be exploited for medical imaging. Conjugating luminescent QDs with target-specific glycans permits efficient imaging of the tissue to which the glycans bind with high affinity. Accurate imaging of primary and metastatic tumors is of primary importance in disease management. Second-generation QDs contain the glycan ligands and PEG of varying chain lengths. PEG modification produces QDs that maintain high luminescence while reducing nonspecific cell binding.

Procedures have been developed for using QDs to label live cells and to demonstrate their use for long-term multicolor imaging. Two approaches are endocytic uptake of QDs and selective labeling of cell-surface proteins with QDs conjugated to antibodies, which should permit the simultaneous study of multiple cells over long periods of time as they proceed through growth and development. Use of avidin permits stable conjugation of the QDs to ligands, antibodies, or other molecules that can be biotinylated, whereas the use of proteins fused to a positively charged peptide or oligohistidine peptide obviates the need for biotinylating the target molecule. Specific labeling of both intracellular and cell-surface proteins can be achieved by bioconjugation of QDs. For generalized cellular labeling, QDs not conjugated to a specific biomolecule may be used.

Fluorescent semiconductor QDs hold great potential for molecular imaging *in vivo*. However, the utility of existing QDs for *in vivo* imaging is limited because they require excitation from external illumination sources to fluoresce, which results in a strong autofluorescence background and a paucity of excitation light at nonsuperficial locations. QD conjugates that luminesce by bioluminescence resonance energy transfer in the absence of external excitation have been prepared by coupling carboxylate-presenting QDs to a mutant of the bioluminescent protein *Renilla reniformis* luciferase (So et al. 2006). The conjugates emit long-wavelength (from red to near infrared) bioluminescent light in cells and in animals, even in deep tissues, and are suitable for multiplexed *in vivo* imaging. Compared with existing QDs, self-illuminating QD conjugates have greatly enhanced sensitivity in small-animal imaging, with an *in vivo* signal-to-background ratio of $>10^3$ for 5 pmol of conjugate.

Several advances have recently been made using QDs for live cell and *in vivo* imaging, in which QD-labeled molecules can be tracked and visualized in 3D. QDs have been investigated for their use for multiplex immunohistochemistry and *in situ* hybridization which, when combined with multispectral imaging, has enabled quantitation and co-localization of gene expression in clinical tissue specimens (Byers and Hitchman 2011).

Use of Nanocrystals in Immunohistochemistry

A method has been described for simple convenient preparation of bright, negatively or positively charged, water-soluble CdSe/ZnS core/shell nanocrystals (NCs) and their stabilization in aqueous solution (Sukhanova et al. 2004). Single NCs can be detected using a standard epifluorescent microscope, ensuring a detection limit of one molecule coupled with an NC. NC-antibody (Ab) conjugates were tested in dot blots and exhibited retention of binding capacity within several nanogram of antigen detected. The authors further demonstrated the advantages of NC-Ab conjugates in the immunofluorescent detection and 3D confocal analysis of p-glycoprotein (p-gp), one of the main mediators of the multidrug resistance phenotype. The labeling of p-gp with NC-Ab conjugates was highly specific. Finally, the authors demonstrated

the applicability of NC-Abs conjugates obtained by the method described to specific detection of antigens in paraffin-embedded formaldehyde-fixed cancer tissue specimens, using immunostaining of cytokeratin in skin basal carcinoma as an example. They concluded that the NC-Ab conjugates may serve as easy-to-do, highly sensitive, photostable labels for immunofluorescent analysis, immunohistochemical detection, and 3D confocal studies of membrane proteins and cells.

Magnetic Nanoparticles

Magnetic Nanoparticles for Bioscreening

Iron nanoparticles, 15–20 nm in size, having saturation magnetization, have been synthesized, embedded in copolymer beads of styrene and glycidyl methacrylate (GMA), which were coated with poly-GMA by seed polymerization (Maeda et al. 2006). The resultant Fe/St-GMA/GMA beads had diameters of 100–200 nm. By coating with poly-GMA, the zeta potential of the beads changed from -93.7 to -54.8 mV, as measured by an electrophoresis method. This facilitates nonspecific protein adsorption suppression, as revealed by gel electrophoresis method, which is a requisite for nanoparticles to be applied to carriers for bioscreening.

Magnetic nanoparticles can be used for detection of biomolecules and cells based on magnetic resonance effects using a general detection platform termed diagnostic magnetic resonance (DMR) technology, which covers numerous sensing principles, and magnetic nanoparticle biosensors have been designed to detect a wide range of targets including DNA/mRNA, proteins, enzymes, drugs, pathogens, and circulating tumor cells (Haun et al. 2010). The basic principle of DMR is the use of magnetic nanoparticles as proximity sensors that modulate the spin relaxation time of neighboring water molecules, which can be quantified using clinical MRI scanners or benchtop nuclear magnetic resonance (NMR) relaxometers. The capabilities of DMR technology have been advanced considerably with the development of miniaturized, chip-based NMR (μ NMR) detector systems that are capable of performing highly sensitive measurements on microliter sample volumes and in multiplexed format. Thus, DMR biosensor technology holds considerable promise to provide a high-throughput, low-cost, and portable platform for large-scale molecular and cellular screening in clinical and point-of-care settings.

Monitoring of Implanted NSCs Labeled with Nanoparticles

Noninvasive monitoring of stem cells, using high-resolution molecular imaging, will be important for improving clinical neural transplantation strategies. Labeling of human neural stem cells (NSCs) grown as neurospheres with magnetic nanoparticles was shown to not adversely affect survival, migration, and differentiation or alter neuronal electrophysiological characteristics (Guzman et al. 2007). Using MRI, the authors demonstrated that human NSCs transplanted either to the neonatal, the adult, or the injured rodent brain respond to cues characteristic for the ambient

microenvironment resulting in distinct migration patterns. Nanoparticle-labeled human NSCs survive long-term and differentiate in a site-specific manner identical to that seen for transplants of unlabeled cells. The impact of graft location on cell migration and MRI characteristics of graft cell death and subsequent clearance were also described. Knowledge of migration patterns and implementation of noninvasive stem cell tracking might help to improve the design of future clinical NSC transplantation.

Perfluorocarbon Nanoparticles to Track Therapeutic Cells In Vivo

Using perfluorocarbon nanoparticles 200 nm in size to label endothelial progenitor cells taken from human UCB enables their detection by MRI in vivo following administration (Partlow et al. 2007). The MRI scanner can be tuned to the specific frequency of the fluorine compound in the nanoparticles, and only the nanoparticle-containing cells are visible in the scan. This eliminates any background signal, which often interferes with medical imaging. Moreover, the lack of interference means one can measure very low amounts of the labeled cells and closely estimate their number by the brightness of the image. Since several perfluorocarbon compounds are available, different types of cells potentially could be labeled with different compounds, injected and then detected separately by tuning the MRI scanner to each one's individual frequency. This technology offers significant advantages over other cell-labeling technologies in development. Laboratory tests showed that the cells retained their usual surface markers and that they were still functional after the labeling process. The labeled cells were shown to migrate to and incorporate into blood vessels forming around tumors in mice. These could soon enable researchers and physicians to directly track cells used in medical treatments using unique signatures from the ingested nanoparticle beacons. They could prove useful for monitoring tumors and diagnosing as well as treating cardiovascular problems.

Superparamagnetic Nanoparticles for Cell Tracking

Magnetic nanoparticles are a powerful and versatile diagnostic tool in biology and medicine. It is possible to incorporate sufficient amounts of superparamagnetic iron oxide nanoparticles (SPIONs) into cells, enabling their detection in vivo using MRI. Because of their small size, they are easily incorporated into various cell types (stem cells, phagocytes, etc.) allowing the cells to be tracked in vivo, for example, to determine whether stem cells move to the correct target area of the body.

Superparamagnetic iron oxide nanoparticles (SPIONS), used clinically for specific magnetic sorting, can be used as a magnetic cell label for in vivo cell visualization. The fact that SPIONs coated with different commercially available antibodies can bind to specific cell types opens extensive possibilities for cell tracking in vivo. A study has investigated the biological properties, including proliferation, viability, and differentiation capacity of MSCs labeled with clinically approved SPIONs (Jasmin et al. 2011). Rat MSCs were isolated, cultured, and incubated with

dextran-covered SPIONs (ferumoxide) alone or with poly-L-lysine (PLL) or protamine chlorhydrate. Whereas labeling of MSCs incubated with ferumoxide alone was poor, 95 % MSCs were labeled when incubated with ferumoxide in the presence of PLL or protamine. MSCs incubated with ferumoxide and protamine were efficiently visualized by MRI; they maintained proliferation and viability for up to 7 days and remained competent to differentiate. After 21 days, MSCs pretreated with mitomycin C still showed a large number of ferumoxide-labeled cells. The efficient and long-lasting uptake and retention of SPIONs by MSCs using a protocol employing ferumoxide and protamine may be applicable to patients, since both ferumoxide and protamine are approved for human use.

Unfortunately, SPIONs are no longer being manufactured. Second-generation, ultrasmall SPIONs (USPIO), however, offer a viable alternative. Ferumoxytol (Feraheme™) is one USPIO composed of a nonstoichiometric magnetite core surrounded by a polyglucose sorbitol carboxymethylether coat. The colloidal, particle size of ferumoxytol is 17–30 nm. Ferumoxytol has been approved by the FDA as an iron supplement for treatment of iron deficiency in patients with renal failure. This agent has been used “off label” for stem cell labeling (Castaneda et al. 2011). This technique may be applied for noninvasive monitoring of stem cell therapies in preclinical and clinical settings.

SPIONs for Calcium Sensing

A family of calcium indicators for MRI is formed by combining a powerful SPION-based contrast mechanism with the versatile calcium-sensing protein calmodulin and its targets (Atanasijevic et al. 2006). Calcium-dependent protein-protein interactions drive particle clustering and produce up to fivefold changes in T2 relaxivity, an indication of the sensors’ potency. Robust MRI signal changes are achieved even at nanomolar particle concentrations that are unlikely to buffer calcium levels. When combined with technologies for cellular delivery of nanoparticulate agents, these sensors and their derivatives may be useful for functional molecular imaging of biological signaling networks in live, opaque specimens.

Magnetic Nanoparticles for Labeling Molecules

Bound to a suitable antibody, magnetic nanoparticles are used to label specific molecules, structures, or microorganisms. Magnetic immunoassay techniques have been developed in which the magnetic field generated by the magnetically labeled targets is detected directly with a sensitive magnetometer. Binding of antibody to target molecules or disease-causing organism is the basis of several tests. Antibodies labeled with magnetic nanoparticles give magnetic signals on exposure to a magnetic field. Antibodies bound to targets can thus be identified as unbound antibodies disperse in all directions and produce no net magnetic signal.

SPIONs have been functionalized to identify *Mycobacterium avium* spp. paratuberculosis (MAP) through magnetic relaxation (Kaittanis et al. 2007). The results

indicate that the MAP nanoprobe binds specifically to MAP and can quantify the bacterial target quickly in milk and blood with high sensitivity. The advantage of this approach is that detection is not susceptible to interferences caused by other bacteria. The use of these magnetic nanosensors is anticipated in the identification and quantification of bacteria in clinical and environmental samples.

Study of Living Cells by SPIONs

Technologies to assess the molecular targets of biomolecules in living cells are lacking. A technology called magnetism-based interaction capture (MAGIC) can identify molecular targets on the basis of induced movement of SPIONs inside living cells. Intracellular proteins can be painted with fluorescent materials and drugs embedded with SPIONs inserted into the cell. These nanoprobe captured the small molecule's labeled target protein and were translocated in a direction specified by the magnetic field. Use of MAGIC in genome-wide expression screening can identify multiple protein targets of a drug. MAGIC can also be used to monitor signal-dependent modification and multiple interactions of proteins. Internalized SPIONs can be moved inside cells by an external magnetic field. MAGIC can be useful in the development of diagnostics and biosensors. Its ultimate use would be for the analysis of interactions inside living cells of patients.

Imaging Applications of Nanoparticles

Molecular imaging now encompasses all imaging modalities including those used in clinical care: optical imaging, nuclear medical imaging, ultrasound imaging, MRI, and photoacoustic imaging. Molecular imaging always requires accumulation of contrast agent in the target site, often achieved most efficiently by steering nanoparticles containing contrast agent into the target. This entails accessing target molecules hidden behind tissue barriers, necessitating the use of targeting groups. For imaging modalities with low sensitivity, nanoparticles bearing multiple contrast groups provide signal amplification. The same nanoparticles can in principle deliver both contrast medium and drug, allowing monitoring of biodistribution and therapeutic activity simultaneously. Nanoparticles with multiple bioadhesive sites for target recognition and binding share functionalities with many subcellular organelles (ribosomes, proteasomes, ion channels, and transport vesicles), which are of similar sizes. The materials used to synthesize nanoparticles include natural proteins and polymers, artificial polymers, dendrimers, fullerenes and other carbon-based structures, lipid-water micelles, viral capsids, metals, metal oxides, and ceramics. Signal generators incorporated into nanoparticles include iron oxide, gadolinium, fluorine, iodine, bismuth, radionuclides, QDs, and metal nanoclusters (Debbage and Jaschke 2008). Diagnostic imaging applications, now appearing, include sentinel node localization and stem cell tracking.

There is rapid growth in the use of MRI for molecular and cellular imaging. Much of this work relies on the high relaxivity of nanometer-sized, ultrasmall dextran-coated iron oxide particles. Chemical modifications to nanosized virus particles may improve MRI. Attachment of a large number of gadolinium chelates, the chemical compound used in MRI contrast agents, onto the surface of the viral particles resulted in the generation of a very intense signal in a clinical MRI scanner (Anderson et al. 2006). Dendrimers, magnetic nanoparticles, QDs, and ferrofluids are examples of some of the nanoparticles that have been used along with imaging technologies for enhancement of contrast. Some of these are described in the preceding section.

Computer tomography (CT) is among the most convenient imaging/diagnostic tools used currently in terms of availability, efficiency, and cost. In contrast to other imaging modalities, such as PET, single-photon emission computed tomography (SPECT) and MRI, CT is not usually considered as a molecular imaging modality. However, development of nanoparticles as contrast agents is enabling specific applications of CT for molecular imaging. Current clinical CT contrast agents are predominantly based on the high atomic number iodine molecules ($Z=53$), which are effective in absorbing X-rays; but the small size of iodine molecules allows very short imaging times due to rapid clearance by the kidneys. Use of high- Z nanoparticles, for example, polymers, liposomes, and micelles as contrast agents may enable CT imaging at lower radiation doses and with improved sensitivity as well as specificity (Shilo et al. 2012). There is some concern about the toxicity of these nanoparticle contrast agents. Once the toxicity issues are resolved, clinical trials could be initiated in humans. Concomitant encapsulation of metal nanoparticles (diagnosis) and drug molecules (therapy) into carriers, such as liposomes, offers simultaneous *in vivo* imaging and tracking of drug efficacy, which will facilitate development of personalized medicine.

Dendritic Nanoprobes for Imaging of Angiogenesis

Angiogenesis is an important process in ischemia and cancer. A biodegradable positron-emitting dendritic nanoprobe targeted at $\alpha v\beta 3$ integrin, a biomarker known to modulate angiogenesis, was developed for the noninvasive imaging of angiogenesis (Almutairi et al. 2009). The nanoprobe has a modular multivalent core-shell architecture consisting of a biodegradable heterobifunctional dendritic core chemoselectively functionalized with heterobifunctional polyethylene oxide (PEO) chains that form a protective shell, which imparts biological stealth and dictates the pharmacokinetics. Each of the eight branches of the dendritic core was functionalized for labeling with radiohalogens. Placement of radioactive moieties at the core was designed to prevent *in vivo* dehalogenation, a potential problem for radiohalogens in imaging and therapy. Targeting peptides of cyclic arginine-glycine-aspartic (RGD) acid motifs were installed at the terminal ends of the PEO chains to enhance their accessibility to $\alpha v\beta 3$ integrin receptors. This nanoscale design enabled a 50-fold enhancement of the binding affinity to $\alpha v\beta 3$ integrin

receptors with respect to the monovalent RGD peptide alone. Cell-based assays of the ^{125}I -labeled dendritic nanoprobe using $\alpha v\beta 3$ -positive cells showed a sixfold increase in $\alpha v\beta 3$ receptor-mediated endocytosis of the targeted nanoprobe compared with the nontargeted nanoprobe, whereas $\alpha v\beta 3$ -negative cells showed no enhancement of cell uptake over time. In vivo biodistribution studies of ^{76}Br -labeled dendritic nanoprobe showed excellent bioavailability for the targeted and nontargeted nanoprobe. In vivo studies in a murine hind limb ischemia model for angiogenesis revealed high specific accumulation of ^{76}Br -labeled dendritic nanoprobe targeted at $\alpha v\beta 3$ integrins in angiogenic muscles, allowing highly selective imaging of this critically important process.

Gadolinium-Loaded Dendrimer Nanoparticles for Tumor-Specific MRI

A target-specific MRI contrast agent for tumor cells expressing high affinity folate receptor was synthesized using a fifth-generation polyamidoamine dendrimer (Swanson et al. 2008). Surface-modified dendrimer was functionalized for targeting with folic acid, and the remaining terminal primary amines of the dendrimer were conjugated with the bifunctional NCS-DOTA (Dow Chemical) chelator that forms stable complexes with gadolinium. In xenograft tumors in immunodeficient mice induced with human epithelial cancer cells expressing folate receptor, 3D MRI results showed specific and statistically significant signal enhancement in tumors generated with targeted nanoparticle compared with signal generated by nontargeted contrast nanoparticle. The targeted dendrimer contrast nanoparticles infiltrated tumor and were retained in tumor cells up to 48 h following injection. The presence of folic acid on the dendrimer resulted in specific delivery of the nanoparticle to tissues and xenograft tumor cells expressing folate receptor in vivo. The specificity of the dendrimer nanoparticles for targeted cancer imaging with the prolonged clearance time compared favorably with the current clinically approved gadodiamide (Omniscan) contrast agent. Potential applications of this approach include determination of the folate receptor status of tumors and monitoring of drug therapy.

Gadonanotubes for MRI

More than 25 million patients in the USA undergo MRI each year, and contrast agents are used in about 30 % of these procedures. Gadolinium agents are the most effective and the most commonly used MRI contrast agents. Gadonanotubes are made of the same highly toxic metal gadolinium (Gd^{3+}) that is used in MRI currently, but the metal atoms are encased inside a carbon nanotube. The ultrashort nanotubes are only about 20–100 times longer than they are wide, and once inside the nanotubes, the gadolinium atoms naturally aggregate into tiny clusters of about 10 atoms each. Clustering causes the unexplained increases in magnetic and MRI effects. Gadonanotubes are at least 40–90 times more effective than Gd^{3+} -based MRI agents now in use. Shrouding the toxic metals inside the benign carbon is expected to significantly reduce or eliminate the metal's toxicity. Currently available methods of attaching disease-specific antibodies and peptides can be applied to gadonanotubes so they can be targeted to malignant and other diseased cells.

Gold Nanorods and Nanoparticles as Imaging Agents

Gold nanorods excited at 830 nm on a far-field laser-scanning microscope produced strong two-photon luminescence (TPL) intensities, and the TPL excitation spectrum can be superimposed on to the longitudinal plasmon band (Wang et al. 2005c). The TPL signal from a single nanorod is 58 times that of the two-photon fluorescence signal from a single rhodamine molecule. Gold nanorods can be used as imaging agents as demonstrated by *in vivo* imaging of single nanorods flowing in mouse ear blood vessels.

Nanoprobes Inc. reported that 1.9 nm gold nanoparticles may overcome many limitations to traditional X-ray contrast agents. Gold has higher X-ray absorption than iodine with less bone and tissue interference, thus achieving better contrast with lower X-ray dose. Because nanoparticles clear the blood more slowly than iodine agents, they permit longer imaging times. In studies in mice, a 5-mm tumor growing in one thigh was clearly evident from its increased vascularity and resultant higher gold content. The gold particles thus enable direct imaging, detection, and measurement of angiogenic and hypervascularized regions. The 1.9-nm gold nanoparticles were found to clear through the kidneys: a closer examination of the kidneys revealed a remarkably detailed anatomical and functional display, with blood vessels less than 100 μm in diameter delineated, thus enabling *in vivo* vascular casting. Toxicity was also low: mice intravenously injected with the gold nanoparticles survived over 1 year without signs of illness.

In Vivo Imaging Using Nanoparticles

Fluorescence provides remarkable results for *in vivo* imaging, but it has several limitations, particularly because of the need for tissue autofluorescence by external illumination and weak tissue penetration of low wavelength excitation light. An alternative optical imaging technique has been developed by using nanoparticles with persisting luminescence suitable for small-animal imaging (le Masne de Chermont et al. 2007). These nanoparticles can be excited before injection, and their *in vivo* distribution can be followed in real time for more than 1 h without the need for an external illumination source. Chemical modification of the nanoparticle surface can be done to target organs such as the lung or the liver or for prolonging luminescence during circulation of the nanoparticles in blood. Tumors have been identified by this technique.

A significant impediment to the widespread use of noninvasive *in vivo* vascular imaging techniques is the current lack of suitable intravital imaging probes. One strategy is the use of viral nanoparticles as a platform for the multivalent display of fluorescent dyes to image tissues deep inside living organisms. The bioavailable cowpea mosaic virus (CPMV) can be fluorescently labeled to high densities with no measurable quenching, resulting in exceptionally bright particles with *in vivo* dispersion properties that allow high-resolution intravital imaging of vascular endothelium for periods of at least 72 h. CPMV nanoparticles can be used to visualize the vasculature and blood flow in living mouse and chick embryos to a depth of up to 500 μm . Intravital visualization of human fibrosarcoma-mediated tumor

angiogenesis using fluorescent CPMV provides a means to identify arterial and venous vessels for monitoring tumor neovascularization.

Manganese Oxide Nanoparticles as Contrast Agent for Brain MRI

A new MRI contrast agent using manganese oxide nanoparticles produces images of the anatomic structures of mouse brain which are as clear as those obtained by histological examination (Na et al. 2007). The new contrast agent will enable better research and diagnosis neurological disorders such as Alzheimer's disease, Parkinson's disease, and stroke. Furthermore, antibodies can be attached to the manganese oxide nanoparticles, which recognize and specifically bind to receptors on the surface of breast cancer cells in mouse brains with breast cancer metastases. The tumors were clearly highlighted by the antibody-coupled contrast agent. The same principle should allow other disease-related changes or physiological systems to be visualized by using the appropriate antibodies.

Nanoparticles Versus Microparticles for Cellular Imaging

Typically, millions of dextran-coated ultrasmall iron oxide particles (USIOPs) must be loaded into cells for efficient detection. Single, micrometer-sized iron oxide particles (MSIOPs) can be detected by MRI *in vivo* in living animals. Experiments studying effects of MRI resolution and particle size indicate that significant signal effects could be detected at resolutions as low as 200 μm . Cultured cells can be labeled with fluorescent MSIOPs in a way that single particles are present in individual cells and can be detected both by MRI and fluorescence microscopy. Single particles injected into single-cell-stage mouse embryos can be detected at later embryonic stages, demonstrating that even after many cell divisions, daughter cells still carry individual particles. These observations show that MRI can detect single particles and indicate that microparticle detection will be useful for cellular imaging for certain purposes and may be preferable to nanoparticles. MSIOPs will be useful in following the division of stem cells and *in vivo* labeling of cells.

Nanoparticles as Contrast Agent for MRI

The determination of brain tumor margins both during the presurgical planning phase and during surgical resection has long been a challenging task in the therapy of brain tumor patients. Multimodal (near-infrared fluorescent and magnetic) nanoparticles were used as a preoperative MRI contrast agent and intraoperative optical probes. Key features of nanoparticle metabolism, namely, intracellular sequestration by microglia and the combined optical and magnetic properties of the probe, allowed delineation of brain tumors both by preoperative MRI and by intraoperative optical imaging. This prototypical multimodal nanoparticle has unique properties that may allow radiologists and neurosurgeons to see the same probe in the same cells and may offer a new approach for obtaining tumor margins.

Alphanubeta3-targeted paramagnetic nanoparticles have been employed to noninvasively detect very small regions of angiogenesis associated with nascent melanoma tumors (Schmieder et al. 2005). Each particle was filled with thousands of molecules of the metal that is used to enhance contrast in conventional MRI scans. The surface of each particle was decorated with a substance that attaches to newly forming blood vessels, which are present at tumor sites. The goal was to create a high density of the glowing particles at the site of tumor growth so they are easily visible. Molecular MRI results were corroborated by histology. This study lowers the limit previously reported for detecting sparse biomarkers with molecular MRI *in vivo* when the growths are still invisible to conventional MRI. Earlier detection can potentially increase the effectiveness of treatment. This is especially true with melanoma, which begins as a highly curable disorder then progresses into an aggressive and deadly disease. A second benefit of the approach is that the same nanoparticles used to find the tumors could potentially deliver stronger doses of anticancer drugs directly to the tumor site with fewer side effects. Targeting the drugs to the tumor site in this way would also allow stronger doses without systemic toxicity than would be possible if the drug were injected or delivered in some other systemic way. The nanoparticles might also allow physicians to more readily assess the effectiveness of the treatment by comparing MRI scans before and after treatment. Other cancer types might be accessible to this approach as well, because all tumors recruit new blood vessels as they grow.

Optical Molecular Imaging Using Targeted Magnetic Nanoprobes

Dynamic magnetomotion of magnetic nanoparticles (MNPs) detected with magnetomotive optical coherence tomography (MM-OCT) represents a new method for contrast enhancement and therapeutic interventions in molecular imaging. *In vivo* imaging of dynamic functionalized iron oxide MNPs using MM-OCT was demonstrated in a preclinical mammary tumor model (John et al. 2010). Using targeted MNPs, *in vivo* MM-OCT images exhibit strong magnetomotive signals in mammary tumor, and no significant signals were measured from tumors of rats injected with nontargeted MNPs or saline. The results of *in vivo* MM-OCT are validated by MRI, *ex vivo* MM-OCT, Prussian blue staining of histological sections, and immunohistochemical analysis of excised tumors and internal organs. The MNPs are antibody functionalized to target the human epidermal growth factor receptor 2 (HER2 neu) protein. Fc-directed conjugation of the antibody to the MNPs aids in reducing uptake by macrophages in the reticuloendothelial system, thereby increasing the circulation time in the blood. These engineered magnetic nanoprobes have multifunctional capabilities enabling them to be used as dynamic contrast agents in MM-OCT and MRI.

QDs for Biological Imaging

Targeted QDs, coated with paramagnetic and pegylated lipids, have been developed for detection by MRI (Mulder et al. 2006). The QDs were functionalized by covalently

linking v3-specific peptides, and the specificity was assessed and confirmed on cultured endothelial cells. The bimodal character, the high relaxivity, and the specificity of this nanoparticulate probe make it an excellent contrast agent for molecular imaging purposes. Among other applications, those in cancer are most important.

Accurate imaging of diseased cells (e.g., primary and metastatic tumors) is of primary importance in disease management. The NIH has developed carbohydrate-encapsulated QDs with detectable luminescent properties useful for imaging of cancer or other disease tissues. Certain carbohydrates, especially those included on tumor glycoproteins, are known to have affinity for certain cell types. One notable glycan used in this technology is the Thomsen-Friedenreich disaccharide (Galbeta1-3GalNAc) that is readily detectable in 90 % of all primary human carcinomas and their metastases. These glycans can be exploited for medical imaging. Encapsulating luminescent QDs with target-specific glycans permits efficient imaging of the tissue to which the glycans bind with high affinity.

Multifunctional nanoparticle probes based on semiconductor QDs have been used for cancer targeting and imaging in living animals. The structural design involves encapsulating luminescent QDs with an ABC triblock copolymer and linking this amphiphilic polymer to tumor-targeting ligands and drug-delivery functionalities. *In vivo* targeting studies of human prostate cancer growing in nude mice indicate that the QD probes accumulate at tumors both by the enhanced permeability and retention of tumor sites and by antibody binding to cancer-specific cell-surface biomarkers (Gao et al. 2004). Using both subcutaneous injection of QD-tagged cancer cells and systemic injection of multifunctional QD, sensitive and multicolor fluorescence imaging of cancer cells have been achieved under *in vivo* conditions. These results raise new possibilities for ultrasensitive and multiplexed imaging of molecular targets *in vivo*.

SPIONs Combined with MRI

Highly lymphotropic SPIONs measuring 2–3 nm on average, which gain access to lymph nodes by means of interstitial-lymphatic fluid transport, have been used in conjunction with high-resolution MRI to reveal small and otherwise undetectable lymph node metastases. In patients with prostate cancer who undergo surgical lymph node resection or biopsy, MRI with lymphotropic SPIONs can identify all patients with nodal metastases, which is not possible with conventional MRI alone, and has implications for the management. In men with metastatic prostate cancer, adjuvant androgen-deprivation therapy with radiation is the mainstay of management.

Sentinel lymph node (SLN) imaging and biopsy is an important part of the workup of some cancers in humans. The presence of lymph node metastases is an important factor in breast cancer patient prognosis. Therefore, the precise identification of SLNs in these patients is critical. Conventional methods have drawbacks including lack of depth, skin staining (blue dye), poor spatial resolution, and exposure to ionizing radiation. Among the newer methods, magnetic resonance

lymphography, in which a gadolinium-labeled nanoparticle is injected and imaged, provides superior anatomic resolution and assessment of lymphatic dynamics, overcoming some of the drawbacks of other methods. Optical imaging employing various nanoparticles, including QDs, also provides the capability of mapping each lymphatic drainage in a different color. However, autofluorescence arising from normal tissues can compromise the sensitivity and specificity of in vivo fluorescence imaging using QDs by lowering the target-to-background signal ratio. Since bioluminescence resonance energy transfer QD (BRET-QD) nanoparticles can self-illuminate in NIR in the presence of the substrate, imaging using BRET-QDs does not produce any autofluorescence. These advantages of BRET-QD enable real-time, quantitative lymphatic imaging without image processing (Kosaka et al. 2011).

Use of lymphatic imaging agents will improve our understanding of the lymphatic system. It is conceivable that anticancer drugs and a tumor vaccine can be incorporated into the imaging agent for the delivery of regional therapy (Ravizzini et al. 2009).

Concluding Remarks and Future Prospects of Nanoparticles for Imaging

Surface functionalization has expanded further the potential of nanoparticles as probes for molecular imaging. Ongoing research of nanoparticles for biomedical imaging focuses on increased selectivity and reduced nonspecific uptake with increased spatial resolution containing stabilizers conjugated with targeting ligands. Structural design of nanomaterials for biomedical imaging continues to expand and diversify. Synthetic methods aim to control the size and surface characteristics of nanoparticles to optimize distribution, half-life, and elimination. Although molecular imaging applications using nanoparticles are advancing into clinical applications, challenges such as storage stability and long-term toxicology should continue to be addressed (Nune et al. 2009).

Applications of Nanopore Technology for Molecular Diagnostics

Nanopore Technology for Detection of Single DNA Molecules

Nanopore sequencing was described in Chap. 3. Nanopores hold great promise as single-molecule analytical devices and biophysical model systems because the ionic current blockades they produce contain information about the identity, concentration, structure, and dynamics of target molecules. Nanopore technology can distinguish between and count a variety of different molecules in a complex mixture. For example, it can distinguish between hybridized or unhybridized unknown RNA and DNA molecules that differ only by a single nucleotide. Nanopore biosensors can enable direct, microsecond-scale nucleic acid characterization without the need for amplification, chemical modification, surface adsorption, or the binding of probes.

A mutant was constructed of porin MspA of *Mycobacterium smegmatis* that is capable of electronically detecting and characterizing single molecules of ssDNA as they are electrophoretically driven through the pore (Butler et al. 2008). A second mutant with additional exchanges of negatively charged residues for positively charged residues in the vestibule region exhibited a factor of ≈ 20 higher interaction rates, required only half as much voltage to observe interaction, and allowed ssDNA to reside in the vestibule ≈ 100 times longer than the first mutant. These results introduce MspA as a nanopore for nucleic acid analysis and highlight its potential as an engineerable platform for single-molecule detection and characterization applications.

Nanocytometry

Nanocytometry is a nanotechnology-based approach to flow cytometry. It incorporates previous work on a nanoelectronic technique for detecting the binding of unlabeled antibody-antigen pairs. Nanocytometry uses resistive-pulse sensing and artificial nanopores to detect and measure cell size, which is determined by the change in resistance when an individual cell passes through the pore (Carbonaro et al. 2008). As a proof of principle, it was shown that it was possible to measure the change in size when cells undergo apoptosis. The novel method has an integrated microfluidic chip, which can adapt to sort cancer and other types of cells based on their cell-surface protein expression.

A low-cost, flow-through nanocytometer has been presented that utilizes a colloidal suspension of nonfunctionalized magnetic nanoparticles for label-free manipulation and separation of microparticles (Kose and Koser 2012). The size-based separation is mediated by magnetically excited biocompatible ferrofluid particles with up to 99 % separation efficiency and a throughput of 3×10^4 particles/s per mm^2 of channel cross section. The device is readily scalable and applicable to live cell sorting offering competitive cytometer performance in a simple and inexpensive package.

Nanocytometry is a significant improvement over conventional flow cytometry, because the system permits label-free signal detection, extreme reproducibility and sensitivity, and cell separations using only a few cells. Conventional flow cytometry requires a large sample of cells and usually requires labeling. Nanocytometry could provide an important new technology applicable to cancer. For example, nanocytometry could be used to improve upon physicians' ability to detect minimal residual disease states as well as circulating tumor cells (CTCs) and upon a scientist's ability to study cell populations that occur in very small numbers such as stem cells.

DNA-Protein and Nanoparticle Conjugates

Semisynthetic conjugates composed of nucleic acids, proteins, and inorganic nanoparticles have been synthesized and characterized. For example, self-assembled oligomeric networks consisting of streptavidin and double-stranded DNA are applicable

as reagents in immunoassays. Covalent conjugates of ssDNA and streptavidin are utilized as biomolecular adapters for the immobilization of biotinylated macromolecules at solid substrates via nucleic acid hybridization. This “DNA-directed immobilization” enables reversible and site-selective functionalization of solid substrates with metal and semiconductor nanoparticles or, vice versa, for the DNA-directed functionalization of gold nanoparticles with proteins, such as immunoglobulins and enzymes. This approach is applicable for the detection of chip-immobilized antigens. Moreover, covalent DNA-protein conjugates allow for their selective positioning along single-stranded nucleic acids and thus for the construction of nanometer-scale assemblies composed of proteins and/or nanoclusters. Examples include the fabrication of functional biometallic nanostructures from gold nanoparticles and antibodies, applicable as diagnostic tools in bioanalytics. Gold nanoparticles decorated with fluorescein-modified DNA enables improvement of the detection limit of ascorbic acid quantification by two orders of magnitude due to enhanced cleavage of DNA catalyzed by gold clusters (Malashikhina and Pavlov 2012).

Resonance Light Scattering Technology

Resonance light scattering (RLS) technology, developed at Genicon Sciences Corporation (now acquired by Life Technologies), offers uniquely powerful signal generation and detection capabilities applicable to a wide variety of analytical bioassay formats. RLS exploits submicroscopic metallic particles (e.g., gold and silver) of uniform diameter (in the nanometer range) which scatter incident white light to generate monochromatic colored light that appears as highly intense fluorescence. Each RLS particle produces intense light scattering that can be viewed with the naked eye. Under low-power microscope magnification, individual 80-nm gold particles can be readily observed. The scattering produced by these particles creates a “halo” with an apparent 1- μm diameter. As a result, one can conduct ultrasensitive assays to define location and relative frequency of target molecules. RLS signal generation technology is up to 1,000,000 times more sensitive than current fluorescence signaling technology. Other advantages of RLS technology are that RLS signals do not require computer-enhanced imaging of data as they are so intense. Research applications of RLS technology are:

- Gene expression. Relative gene-expression studies on slide-based cDNA microarrays
- DNA sequencing. RLS-based DNA sequencing on sequence-by-hybridization biochips
- Microfluidics. RLS particles for solution-based assays in nanofluidic flow-through microarrays
- Immunohistology. Rapid in situ localization/quantitation of proteins in tissue sections using RLS-coupled antibodies
- Homogeneous. RLS particles for bimolecular, microvolume studies in solution

Clinical applications of RLS technology are:

- RLS technology is being used to score SNPs for discrimination of therapeutically relevant alleles.
- RLS technology provides ultrahigh-sensitivity probes for in situ hybridizations to quantitate therapeutically important DNA and RNA molecules.
- Antibody-coupled RLS particles can deliver increased sensitivity for detection of rare analytes in diagnostic assays.
- Nanoparticle-labeled bacterial RNA generates reproducible RLS signals that are at least 50 times more intense than state-of-the-art confocal-based fluorescence signals for detection of bacterial pathogens.

Nanobarcodes Technology

Metallic nanobarcodes have been produced with striping patterns prepared by sequential electrochemical deposition of metal ions. The differential reflectivity of adjacent stripes enables identification of the striping patterns by conventional light microscopy. This readout mechanism does not interfere with the use of fluorescence for detection of analytes bound to particles by affinity capture, as demonstrated by DNA and protein bioassays. Among other applications such as SNP mapping and multiplexed assays for proteomics, nanobarcodes can be used for population diagnostics and in point-of-care handheld devices. Multiplexed biodetection based on barcoded nanowires has been described with potential use in cancer detection (Brunker et al. 2007). Key performance advantages relative to existing encoded bead technologies include:

- The ability to use the widely installed base of optical microscopes for readout
- The ability to use multiple colors of fluorophores for quantitation
- The ability to generate hundreds to thousands of unique codes that can be distinguished at high speed

Nanobarcode Particle Technology for SNP Genotyping

Nanobarcode particle technology has been used in universal array for high-throughput SNP genotyping (Sha et al. 2006). The particles are encoded submicron metallic nanorods manufactured by electroplating inert metals such as gold and silver into templates and releasing the resulting striped nanoparticles. The power of this technology is that the particles are intrinsically encoded by virtue of the different reflectivity of adjacent metal stripes, enabling the generation of many thousands of unique encoded substrates. Using SNP found within the cytochrome P450 gene family, and a universal short oligonucleotide ligation strategy, simultaneous genotyping of

15 SNPs was demonstrated, a format requiring discrimination of 30 encoded nanowires (one per allele). To demonstrate applicability in practice, 160 genotypes were determined from multiplex PCR products from 20 genomic DNA samples.

QD Nanobarcode for Multiplexed Gene-Expression Profiling

QD nanobarcode-based microbead random array platform (Life Technologies) has been used for accurate and reproducible gene-expression profiling in a high-throughput and multiplexed format (Eastman et al. 2006). Four different sizes of QDs, with emissions at 525, 545, 565, and 585 nm, are mixed with a polymer and coated onto the magnetic microbeads (8- μm diameter) to generate a nanobarcode bead. Twelve intensity levels for each of the four colors are used. Gene-specific oligonucleotide probes are conjugated to the surface of each spectrally nanobarcode bead to create a multiplexed panel, and biotinylated cRNAs are generated from sample total RNA and hybridized to the gene probes on the microbeads. A fifth streptavidin QD (655 nm or infrared QD) binds to biotin on the cRNA, acting as a quantification reporter. The intensity of the 655-nm Qdot reflects the level of biotinylated cRNA captured on the beads and provides the quantification for the corresponding target gene. The system shows a level of sensitivity, which is better than that with a high-density microarray system, and approaches the level usually observed for quantitative PCR. The QBead nanobarcode system has a dynamic range of 3.5 logs, better than the 2–3 logs observed on various microarray platforms. The hybridization reaction is performed in liquid phase and completed in 1–2 h, at least 1 order of magnitude faster than microarray-based hybridizations. Detectable fold change is lower than 1.4-fold, showing high precision even at close to single copy per cell level. Reproducibility for this proof-of-concept study approaches that of Affymetrix GeneChip microarray. In addition, it provides increased flexibility, convenience, and cost-effectiveness in comparison to conventional gene-expression profiling methods.

Biobarcode Assay for Proteins

An ultrasensitive method for detecting protein analytes relies on nanoparticle probes that are encoded with DNA that is unique to the protein target of interest and antibodies. Magnetic separation of the complexed probes and target followed by dehybridization of the oligonucleotides on the nanoparticle probe surface allows the determination of the presence of the target protein by identifying the oligonucleotide sequence released from the nanoparticle probe. Because the nanoparticle probe carries with it a large number of oligonucleotides per protein-binding event, there is substantial amplification and PSA can be detected at 30 attomolar concentration. Alternatively, a PCR on the oligonucleotide barcodes can boost the sensitivity to 3 attomolar. Comparable clinically accepted conventional assays for detecting the

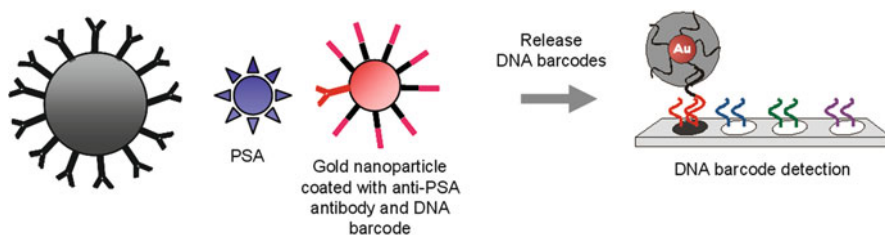


Fig. 4.1 Scheme of biobarcode assay. Schematic illustrating PSA (prostate-specific antigen) detection using the biobarcode assay. Antibody-coated magnetic beads capture and concentrate the protein targets. The captured protein targets are labeled with gold nanoparticle probes that are co-loaded with target-specific secondary antibodies and DNA barcodes. The resulting complexes are separated magnetically and washed to remove excess probe. The DNA barcodes are then released from the complex and detected via hybridization to a surface-immobilized DNA probe and an oligonucleotide-functionalized gold nanoparticle. The gold particles are enlarged through silver deposition, and the light scattered from the particles is detected using the Verigene Reader optical detection system. Increased detection sensitivity is derived from (1) capturing and concentrating protein targets with an antibody-coated magnetic bead, (2) releasing multiple DNA barcodes per captured protein target (hundreds of barcode are attached to a 30-nm-diameter gold particle), and (3) ultrasensitive DNA detection via silver-amplified gold nanoparticles (Courtesy of Nanosphere Inc.)

same target have sensitivity limits of 3 picomolars, six orders of magnitude less sensitive than what is observed with this method. Further development of this technology has resulted in a biobarcode assay with a 500 zeptomolar target DNA sensitivity limit (Nam et al. 2004). Magnetic separation and subsequent release of barcode DNA from the gold nanoparticles leads to a number of barcode DNA strands for every target DNA (see Fig. 4.1).

One reagent is a gold nanoparticle only 30 nm in diameter; the other is a 1- μm magnetic microparticle (MMP). During the assay, the two spheres capture and sandwich the analytes. The MMPs and whatever is bound to them are then captured using a magnet, and unreacted gold NPs are washed away. Thus, only those gold spheres that have captured the analyte remain. Each gold bead also bears an abundance of biobarcodes, custom oligonucleotides that uniquely identify the reaction. The system ultimately detects barcodes released from the beads by heating to 55 $^{\circ}\text{C}$ and not the analytes themselves. Chip-based barcode DNA detection can be done with PCR-like sensitivity but without the use of PCR.

A nanoparticle-based biobarcode assay (BCA) was used to measure the concentration of amyloid β -derived diffusible ligands (ADDLs) in the cerebrospinal fluid (CSF) as a biomarker for Alzheimer's disease (Georganopoulou et al. 2005). Commercial enzyme-linked immunoassays (ELISA) can only detect ADDLs in brain tissue where the biomarker is most highly concentrated. Studies of ADDLs in the CSF have not been possible because of their low concentration. The biobarcode amplification technology, which is a million times more sensitive than ELISA, can detect ADDLs in the CSF where the biomarker is present in very low concentrations. This study is a step toward a diagnostic tool, based on soluble pathogenic markers for Alzheimer's disease. The goal is to ultimately detect and validate the marker in blood.

Using the Verigene ID system (Nanosphere Inc.), one can quantify the barcodes using the kind of technology found in a flatbed scanner, providing results as clear as an at-home pregnancy strip test. Biobarcode system is extremely sensitive for protein detection. At 30 attomolar, it is five orders of magnitude more sensitive than is ELISA (peak sensitivity of around 3 picomolars). The system has enormous potential for multiplexing. It could hypothetically test for 415 different analytes simultaneously by tagging the different gold beads with different barcode sequences. The assay, however, and the fundamental issues with antibodies, such as cross-reactivity, nonspecific binding, and lot-to-lot variability, remain. Antibodies can distort, fall apart, or cling to the wrong analyte. These issues are being addressed. In 2007, the FDA cleared Verigene® Warfarin Metabolism nucleic acid test followed by clearance of Verigene® F5/F2/MTHFR nucleic acid test, which detects disease-associated gene mutations that can contribute to blood coagulation disorders and difficulties metabolizing folate (vitamin B12). Mutations in three specific genes can increase an individual's risk for dangerous blood clots and their leading complication, stroke. Patients that test positively for an increased risk of blood clots can be managed with anticoagulant therapy such as warfarin. Hypercoagulation tests for mutations associated with a predisposition to blood clots are currently among the most frequently conducted human genetic tests. The test is available in single and multitarget (multiplex) formats, allowing users to select the test cartridge that best fits the clinical indications for testing.

A modified form of the BCA called the surface-immobilized biobarcode assay (SI-BCA) is available in a microfluidic chip format (Goluch et al. 2009). The SI-BCA employs microchannel walls functionalized with antibodies that bind with the intended targets. Compared with the conventional BCA, it reduces the system complexity and results in shortened process time, which is attributed to significantly reduced diffusion times in the microscale channels. Raw serum samples, without any pretreatment, were evaluated with this technique. PSA in the samples was detected at concentrations ranging from 40 pM to 40 fM. The detection limit of the assay using buffer samples is 10 fM. The entire assay, from sample injection to final data analysis, was completed in 1 h 20 min. This ability to easily and quickly detect very low levels of PSA, not detectable by conventional assays, may enable diagnosis of prostate cancer recurrence years earlier than is currently possible. Furthermore, the effectiveness of postoperative treatment could be assessed by monitoring a patient's PSA levels. This level of sensitivity in detecting low concentrations of PSA will require revision of the normal laboratory values as currently written in reference manuals.

Single-Molecule Barcoding System for DNA Analysis

Molecular confinement offers new routes for arraying large DNA molecules, enabling single-molecule schemes aimed at the acquisition of sequence information. Such schemes can rapidly advance to become platforms capable of genome analysis

if elements of a nascent system can be integrated at an early stage of development. Integrated strategies are needed for surmounting the stringent experimental requirements of nanoscale devices regarding fabrication, sample loading, biochemical labeling, and detection. Disposable devices featuring both micro- and nanoscale features have been shown to greatly elongate DNA molecules when buffer conditions are controlled for alteration of DNA stiffness (Jo et al. 2007). Analytical calculations that describe this elongation were presented. A complementary enzymatic labeling scheme was developed that tags specific sequences (barcodes) on elongated molecules within described nanoslit devices that are imaged via fluorescence resonance energy transfer. Collectively, these developments enable scalable molecular confinement approaches for genome analysis.

Nanoparticle-Based Colorimetric DNA Detection Method

Nucleic acid diagnostics is dominated by fluorescence-based assays that use complex and expensive enzyme-based target or signal-amplification procedures. Many clinical diagnostic applications will require simpler, inexpensive assays that can be done in a screening mode. Nanosphere Inc.'s Verigene™ platform uses a “spot-and-read” colorimetric detection method for identifying nucleic acid sequences based on optical properties of gold nanoparticles without the need for conventional signal or target amplification. Nucleic acid targets are recognized by DNA-modified gold probes, which undergo a color change that is visually detectable when the solutions are spotted onto an illuminated glass waveguide. Sensitivity of the spot test is improved by monitoring scattered light rather than reflected light from 40- to 50-nm-diameter gold particles. This scatter-based method enables detection of zeptomole quantities of nucleic acid targets without target or signal amplification when coupled to an improved hybridization method that facilitates probe-target binding in a homogeneous format. In comparison to a previously reported absorbance-based method, this method increases detection sensitivity by over four orders of magnitude and has been applied to the rapid detection of *mecA* in methicillin-resistant *Staphylococcus aureus* genomic DNA samples.

Nanoparticle assemblies interconnected with DNA triple helices can be used to colorimetrically screen for triplex DNA binding molecules and simultaneously determine their relative binding affinities based on melting temperatures (Han et al. 2006). Nanoparticles assemble only when DNA triple helices form between DNA from two different particles and a third strand of free DNA. In addition, the triple helix structure is unstable at room temperature and only forms in the presence of triplex DNA binding molecules which stabilize the triple helix. The resulting melting transition of the nanoparticle assembly is much sharper than the analogous triplex structure without nanoparticles. Upon nanoparticle assembly, a concomitant red-to-blue color change occurs. The assembly process and color change do not occur in the presence of duplex DNA binders and therefore provide a significantly better screening process for triplex DNA binding molecules compared to standard methods.

Rapid colorimetric analysis of a specific DNA sequence has been achieved by combining gold nanoparticles (AuNPs) with an asymmetric PCR (Deng et al. 2012). In the presence of the correct DNA template, the bound oligonucleotides on the surface of AuNPs selectively hybridize to form complementary sequences of ssDNA target generated from asymmetric PCR with a concomitant color change from ruby red to blue purple. It is a simple colorimetric method for specific nucleic acid sequence analysis with high specificity and sensitivity and has been used for the detection of *Bacillus anthracis* in clinical samples.

SNP Genotyping with Gold Nanoparticle

Conventional SNP detection techniques are mainly PCR-based. Nanosphere's Verigene technology enables multiplex SNP genotyping in total human genomic DNA without the need for target amplification by PCR. This direct SNP genotyping method requires no enzymes and relies on the high sensitivity of the gold nanoparticle probes.

A simple and rapid MS-based disulfide barcode method relies on magnifying the signal from a dual-modified gold nanoparticle and enables direct SNP genotyping of total human genomic DNA without the need for primer-mediated enzymatic amplification (Yang et al. 2010). Disulfides that are attached to the gold nanoparticle serve as a "barcode" that allows different sequences to be detected. Specificity is based on two sequential oligonucleotide hybridizations, which include two steps: the first is the capture of the target by gene-specific probes immobilized onto magnetic beads; the second is the recognition of gold nanoparticles functionalized with allele-specific oligonucleotides. The sensitivity of this method reaches down to the 0.1 fM range, thus approaching that of PCR. The feasibility of this method was demonstrated by applying it to genomic DNA samples representing all possible genotypes of the SNPs G2677T and C3435T in the human MDR1 gene.

Nanoparticle-Based Up-Converting Phosphor Technology

Up-converting phosphor technology (UPT) is a label detection technology that can be applied to the detection of minute quantities of various substances such as antigens, proteins, and DNA. UPT particles are small ceramic nanospheres composed of rare earth metals and have been shown to be 1,000 times more sensitive than current fluorescent technologies. This particle-based detection provides a stronger signal for each event detected and thereby enhances sensitivity in diagnostic assay systems. UPT has potential in a broad array of DNA testing applications including drug discovery, SNP analysis, and infectious disease testing. Employment of UPT, by bypassing target amplification, brings genetic-based testing a step closer to the point-of-care environment.

A rapid and quantitative UPT-based lateral-flow assay was developed for on-site quantitative detection of different *Brucella* species with high specificity, reproducibility, and stability (Qu et al. 2009). UPT-lateral flow IL-10 assay is a user-friendly, rapid alternative for IL-10 ELISAs, which is suitable for multiplex detection of different cytokines, and can be merged with antibody-detection assays for simultaneous detection of cellular and humoral immunity (Corstjens et al. 2011).

Surface-Enhanced Resonant Raman Spectroscopy

SERRS (surface-enhanced resonant Raman spectroscopy) beads bring various components of the technology into a single robust nanosized polymer-bead support with broad applications in molecular and immunodiagnosics. Focusing on organic fluorescent dyes, because of their strong excitation cross section, compounds are selected experimentally for strong affinity for the silver enhancing surfaces and good spectral resolution. Initially using four dyes, the possibilities for tens to hundreds of unique labels are currently under development. The chosen dyes also have excitation peaks that overlap with the metal plasmon frequency, thereby adding the all-important resonant amplification to the signal intensity.

At the core of the bead is the Raman-active substrate, where silver colloid, with defined physical characteristics, provides the surface-enhancement substrate and is combined with the dye or dyes for specific bead encoding. Control of the various parameters surrounding dye/colloid aggregate permits SERRS response to be modulated as desired.

To protect the SERRS-active complex from degradation, the aggregate is encapsulated in a polymer coating, a process that incorporates a multitude of dye/colloid particles into the same bead. This leads to highly sensitive beads with responses in excess of that achieved using the conformation of single dye molecules on an enhancing surface.

The polymer coating is treated further with a polymer shell to allow a variety of biologically relevant probe molecules (e.g., antibodies, antigens, nucleic acids) to be attached through standard bioconjugation techniques. While most of the development is focused on heterogeneous assays in a 96-well assay sample presentation, other designs include higher plate capacities (384 well) for higher throughput screening and microarray slide reading for DNA and proteomic analysis. Photonic crystal surfaces are used for enhancing the detection of SERR and the development of high-resolution photonic crystal-based laser biosensors, which can be used for gene-expression analysis, and protein biomarker detection (Cunningham 2010).

Near-Infrared (NIR)-Emissive Polymersomes

In vivo fluorescence imaging with near-infrared (NIR) light has enormous potential for a wide variety of molecular diagnostic applications. Because of its quantitative sensitivity, inherent biological safety, and relative ease of use, fluorescence-based

imaging techniques are being increasingly used in small-animal research. Moreover, there is substantial interest in the translation of novel optical techniques into the clinic, where they will prospectively aid in noninvasive and quantitative screening, disease diagnosis, and posttreatment monitoring of patients. Effective deep-tissue fluorescence imaging requires the application of exogenous NIR-emissive contrast agents. Currently, available probes fall into two major categories: organic and inorganic NIR fluorophores (NIRFs). Various studies have used polymersomes (50-nm- to 50- μ m-diameter polymer vesicles) for the incorporation and delivery of large numbers of highly emissive oligo(porphyrin)-based, organic NIRFs (Ghoroghchian et al. 2009). The total fluorescence emanating from the assemblies gives rise to a localized optical signal of sufficient intensity to penetrate through the dense tumor tissue of a live animal. Robust NIR-emissive polymersomes thus define a soft matter platform with exceptional potential to facilitate deep-tissue fluorescence-based imaging for in vivo diagnosis.

Nanobiotechnology for Detection of Proteins

Detection of proteins is an important part of molecular diagnostics. Uses of protein nanobiochips and nanobarcode technology for detection of proteins have been described in preceding sections. Other methods will be included in this section.

Captamers with Proximity Extension Assay for Proteins

Multivalent circular aptamers or “captamers” are formed through the merger of aptameric recognition functions with the DNA as a nanoscale scaffold. Whereas the sequence immobilized to the microtiter plate is termed captamer, the sequence used for detection is called detectamer. Aptamers are useful as protein-binding motifs for diagnostic applications, where their ease of discovery, thermal stability, and low cost make them ideal components for incorporation into targeted protein assays. Captamers are compatible with a highly sensitive protein detection method termed the “proximity extension” assay (Di Giusto et al. 2005). The circular DNA architecture facilitates the integration of multiple functional elements into a single molecule: aptameric target recognition, nucleic acid hybridization specificity, and rolling circle amplification. Successful exploitation of these properties is demonstrated for the molecular analysis of thrombin, with the assay delivering a detection limit nearly three orders of magnitude below the dissociation constants of the two contributing aptamer–thrombin interactions.

Use of liposomes as labels for aptamer-based assays and successful incorporation of cholesteryl–TEG DNA aptamers into liposomal lipid bilayers with subsequent successful function in target recognition further demonstrates the versatility of liposomes as signaling reagents and their potential as a standard platform technology

for various analyses. Such an assay yields a limit of detection of 64 pM or 2.35 ng/mL, corresponding to 6.4 fmol or 235 pg, respectively, in a 100- μ L volume (Edwards et al. 2010).

Real-time signal amplification, detection under isothermal conditions, specificity, and sensitivity would suggest potential application of captamer-based protein assay for further development of personalized medicine.

Nanobiosensors

Nanosensors are devices that employ nanomaterials, exploiting novel size-dependent properties, to detect gases, chemicals, biological agents, electric fields, light, heat, or other targets. The term “nanobiosensors” implies use of nanosensors for detection of chemical or biological materials. Nanomaterials are exquisitely sensitive chemical and biological sensors (Jain 2003a).

The sensors can be electronically gated to respond to the binding of a single molecule. Prototype sensors have demonstrated detection of nucleic acids, proteins, and ions. These sensors can operate in the liquid or gas phase, opening up an enormous variety of downstream applications. The detection schemes use inexpensive low-voltage measurement schemes and detect binding events directly, so there is no need for costly, complicated, and time-consuming labeling chemistries such as fluorescent dyes or the use of bulky and expensive optical detection systems. As a result, these sensors are inexpensive to manufacture and portable. It may even be possible to develop implantable detection and monitoring devices based on these detectors.

Some of the technologies that can be incorporated in biosensing are already covered in earlier sections. An example is nanopore technology, which can form the basis of nanosensors. Some of the biosensor devices are described in the following sections.

Cantilevers as Biosensors for Molecular Diagnostics

Cantilevers (Concentris) are small beams similar to those used in AFM to screen biological samples for the presence of particular genetic sequences. The surface of each cantilever is coated with DNA that can bind to one particular target sequence. On exposure of the sample to beams, the surface stress bends the beams by approximately 10 nm to indicate that the beams have found the target in the sample. This is considered biosensing.

Cantilever technology complements and extends current DNA and protein microarray methods because nanomechanical detection requires no labels, optical excitation, or external probes and is rapid, highly specific, sensitive, and portable. The nanomechanical response is sensitive to the concentration of oligonucleotides in solution, and thus one can determine how much of a given biomolecule is present and

active. In principle, cantilever arrays also could quantify gene-expression levels of mRNA, protein–protein, drug-binding interactions, and other molecular recognition events in which physical steric factors are important. It can detect a single gene within a genome. Furthermore, fabricating thinner cantilevers will enhance the molecular sensitivity further, and integrating arrays into microfluidic channels will reduce the amount of sample required significantly. In contrast to SPR, cantilevers are not limited to metallic films, and other materials will be explored, for example, cantilevers made from polymers. In addition to surface-stress measurements, operating cantilevers in the dynamic mode will provide information on mass changes, and current investigations will determine the sensitivity of this approach. Currently, it is possible to monitor more than 1,000 cantilevers simultaneously with integrated piezoresistive readout, which in principle will allow high-throughput nanomechanical genomic analysis, proteomics, biodiagnostics, and combinatorial drug discovery.

Cantilevers in an array can be functionalized with a selection of biomolecules. Researchers at IBM, Zurich, Switzerland, reported the specific transduction, via surface-stress changes, of DNA hybridization and receptor-ligand binding into a direct nanomechanical response of microfabricated cantilevers. The differential deflection of the cantilevers was found to provide a true molecular recognition signal despite large nonspecific responses of individual cantilevers. Hybridization of complementary oligonucleotides shows that a single-base mismatch between two 12-mer oligonucleotides is clearly detectable. Similar experiments on protein A–immunoglobulin interactions demonstrate the wide-ranging applicability of nanomechanical transduction to detect biomolecular recognition. Microarray of cantilevers has been used to detect multiple unlabeled biomolecules simultaneously at nanomolar concentrations within minutes.

A specific test that uses micrometer-scale beams or “microcantilever” can detect prostate-specific antigen (PSA). PSA antibodies are attached to the surface of the microcantilever, which is applied to a sample containing PSA. When PSA binds to the antibodies, a change in the surface stress on the microcantilever makes it bend enough to be detected by a laser beam. This system is able to detect clinically relevant concentrations of PSA in a background of other proteins. The technique is simpler and potentially more cost-effective than other diagnostic tests because it does not require labeling and can be performed in a single reaction. It is less prone to false positives, which are commonly caused by the nonspecific binding of other proteins to the microcantilever.

Potential applications in proteomics include devices comprising many cantilevers, each coated with a different antibody, which might be used to test a sample rapidly and simultaneously for the presence of several disease-related proteins. One application is for detection of biomarkers of myocardial infarction such as creatine kinase at point-of-care. Other future applications include detection of disease by breath analysis, for example, presence of acetone and dimethylamine (uremia). Detection of a small number of *Salmonella enterica* bacteria is achieved due to a change in the surface stress on the silicon nitride cantilever surface in situ upon binding of bacteria. Scanning electron micrographs indicate that less than 25 adsorbed are required for detection.

Advantages of Cantilever Technology for Molecular Recognition

Cantilever technology has the following advantages over conventional molecular diagnostics:

- It circumvents the use of PCR.
- For DNA, it has physiological sensitivity and no labeling is required.
- In proteomics, it enables detection of multiple proteins and direct observation of proteins in diseases such as those involving the cardiovascular system.
- It enables the combination of genomics and proteomics assays.
- It is compatible with silicon technology.
- It can be integrated into microfluidic devices.

Antibody-Coated Nanocantilevers for Detection of Microorganisms

Nanocantilevers could be crucial in designing a new class of ultrasensitive sensors for detecting viruses, bacteria, and other pathogens (Gupta et al. 2006). The cantilevers, coated with antibodies to detect certain viruses, attract different densities or quantity of antibodies per area depending on the size of the cantilever. The devices are immersed into a liquid containing the antibodies to allow the proteins to stick to the cantilever surface. Instead of simply attracting more antibodies, the longer cantilevers also contained a greater density of antibodies. The density is greater toward the free end of the cantilevers. The cantilevers vibrate faster after the antibody attachment if the devices have about the same nanometer-range thickness (~20 nm) as the protein layer. Moreover, the longer the protein-coated nanocantilever, the faster the vibration, which could only be explained if the density of antibodies increased with increasing lengths.

The cantilever's vibration frequency can be measured using an instrument called a laser Doppler vibrometer, which detects changes in the cantilever's velocity as it vibrates. This work may have broad impact on microscale and nanoscale biosensor design, especially when predicting the characteristics of nanobioelectromechanical sensors functionalized with biological capture molecules. The nanocantilevers could be used in future detectors because they vibrate at different frequencies when contaminants stick to them, revealing the presence of dangerous substances. Because of the nanocantilever's minute size, it is more sensitive than larger devices, promising the development of advanced sensors that detect minute quantities of a contaminant to provide an early warning that a dangerous pathogen is present. At the nanoscale, just adding the mass of one bacterium, virus or large molecule is enough to change the resonant frequency of vibration of the cantilever by a measurable amount, thereby signaling the presence of the pathogen. If one is trying to detect *E. coli*, other organisms in the fluid can weakly absorb on the detector by electrostatic forces. This is a problem in any biodetection and can be resolved by making the resonator vibrate from side to side. This will shake off loosely adhered materials, while whatever is tightly bound to an antibody will remain.

Cantilevers for Direct Detection of Active Genes

An innovative method for the rapid and sensitive detection of disease- and treatment-relevant genes is based on direct measurement of their transcripts (mRNA), which represent the intermediate step and link to protein synthesis (Zhang et al. 2006a). Short complementary nucleic acid segments (sensors) are attached to silicon cantilevers which are 450 nm thick and therefore react with extraordinary sensitivity. Binding of the targeted gene transcript to its matching counterpart on one of the cantilevers results in optically measurable mechanical bending.

Differential gene expression of the gene 1-8U, a potential marker for cancer progression or viral infections, could be observed in a complex background. The measurements provide results within minutes at the picomolar level without target amplification and are sensitive to base mismatches. An array of different gene transcripts can even be measured in parallel by aligning appropriately coated cantilevers alongside each other like the teeth of a comb. The new method complements current molecular diagnostic techniques such as the gene chip and real-time PCR. It could be used as a real-time sensor for continuously monitoring various clinical parameters or for detecting rapidly replicating pathogens that require prompt diagnosis. These findings qualify the technology as a rapid method to validate biomarkers that reveal disease risk, disease progression, or therapy response. Cantilever arrays have potential as a tool to evaluate treatment response efficacy for personalized medical diagnostics.

Carbon Nanotube Biosensors

Over the years, researchers have sought to tailor carbon nanotubes to detect chemicals ranging from small gas molecules to large biomolecules. The tubes' small size and unique electronic properties make them especially adept at detecting minute changes in the environment. Optical nanosensors can use single-walled carbon nanotubes that modulate their emission in response to the adsorption of specific biomolecules with two distinct mechanisms of signal transduction – fluorescence quenching and charge transfer. The nanotube-based chemical sensors developed so far generate an electric signal in the presence of a particular molecule. The basic design is widely applicable for such analytical tasks as detecting genes and proteins associated with diseases.

To test the feasibility of implanting the sensors in the body, oxidase- and ferricyanide-coated nanotubes were placed inside a sealed glass tube a centimeter long and 200 μm thick. The tube is riddled with pores large enough to let glucose enter but small enough to keep the nanotubes inside. The tube was then implanted in a sample of human skin, and the sensor could be excited with infrared light and detect its fluorescence.

Carbon Nanotube Sensors Coated with ssDNA and Electronic Readout

Nanoscale chemical sensors can be based on ssDNA as the chemical recognition site and single-walled carbon nanotube field-effect transistors (SWCN-FETs) as the

electronic readout component (Staii et al. 2005). SWCN-FETs with a nanoscale coating of ssDNA respond to gas odors that do not cause a detectable conductivity change in bare devices. Responses of ssDNA/SWCN-FETs differ in sign and magnitude for different gases and can be tuned by choosing the base sequence of the ssDNA. ssDNA/SWCN-FET sensors detect a variety of odors, with rapid response and recovery times on the scale of seconds. The arrays of nanosensors could detect molecules on the order of one part per million. The sensor surface is self-regenerating: samples maintain a constant response with no need for sensor refreshing through at least 50 gas exposure cycles. The nanosensors could sniff molecules in the air or taste them in a liquid. This remarkable set of attributes makes sensors based on ssDNA decorated nanotubes promising for “electronic nose” and “electronic tongue” applications ranging from homeland security to disease diagnosis.

Carbon Nanotubes Sensors Wrapped with DNA and Optical Detection

SWCNs wrapped with DNA can be placed inside living cells and detect trace amounts of harmful contaminants using near-infrared light (Heller et al. 2006). The sensor is constructed by wrapping the double-stranded DNA around the surface of a single-walled carbon nanotube, in much the same fashion as a telephone cord wraps around a pencil. The DNA starts out wrapping around the nanotube with a certain shape that is defined by the negative charges along its backbone. Subtle rearrangement of an adsorbed biomolecule can be directly detected by such a carbon nanotube. At the heart of the new detection system is the transition of DNA secondary structure from the native, right-handed “B” form to the alternate, left-handed “Z” form. The thermodynamics that drive the switching back and forth between these two forms of DNA structure would modulate the electronic structure and optical emission of the carbon nanotube. When the DNA is exposed to ions of certain atoms such as calcium or mercury, the negative charges become neutralized and the DNA changes shape in a similar manner to its natural shape-shift from the B form to Z form. This reduces the surface area covered by the DNA, perturbing the electronic structure and shifting the nanotube’s natural, near-infrared fluorescence to a lower energy. The change in emission energy indicates how many ions bind to the DNA. Removing the ions will return the emission energy to its initial value and flip the DNA back to the starting form, making the process reversible and reusable. The viability of this measurement technique was demonstrated by detecting low concentrations of mercury ions in whole blood, opaque solutions, and living mammalian cells and tissues where optical sensing is usually poor or ineffective. Because the signal is in the near infrared, a property unique to only a handful of materials, it is not obscured by the natural fluorescence of polymers and living tissues. The nanotube surface acts as the sensor by detecting the shape change of the DNA as it responds to the presence of target ions. This discovery opens the door to new types of optical sensors and biomarkers that exploit the unique properties of nanoparticles in living systems.

A pair of SWCNs provides at least four modes that can be modulated to uniquely fingerprint agents by the degree to which they alter either the emission band intensity or wavelength. This identification method was validated *in vitro* by demonstrating

the detection of six genotoxic analytes, including chemotherapeutic drugs and reactive oxygen species, which are spectroscopically differentiated into four distinct classes, and also demonstrate single-molecule sensitivity in detecting hydrogen peroxide (Heller et al. 2009). SWCN sensor can be placed in living cells, healthy or malignant, and actually detect several different classes of molecules that damage DNA.

FRET-Based DNA Nanosensor

Rapid and highly sensitive detection of DNA is critical in diagnosing genetic diseases. Conventional approaches often rely on cumbersome, semiquantitative amplification of target DNA to improve detection sensitivity. In addition, most DNA detection systems (e.g., microarrays), regardless of their need for target amplification, require separation of unhybridized DNA strands from hybridized stands immobilized on a solid substrate and are thereby complicated by solution-surface binding kinetics. An ultrasensitive nanosensor is based on fluorescence resonance energy transfer (FRET) capable of detecting low concentrations of DNA in a separation-free format. This system uses quantum dots (QDs) linked to DNA probes to capture DNA targets (Zhang et al. 2005). The target strand binds to a dye-labeled reporter strand thus forming a FRET donor-acceptor ensemble. The QD also functions as a concentrator that amplifies the target signal by confining several targets in a nanoscale domain. Unbound nanosensors produce near-zero background fluorescence, but on binding to even a small amount of target DNA (~50 copies or less), they generate a very distinct FRET signal. A nanosensor-based oligonucleotide ligation assay has been demonstrated to successfully detect a point mutation typical of some ovarian tumors in clinical samples.

Ion-Channel Switch Biosensor Technology

The ion channel switch is a biosensor technology based upon a synthetic self-assembling membrane, which acts like a biological switch and is capable of detecting the presence of specific molecules and signaling their presence by triggering an electrical current. It has the ability to detect a change in ion flow upon binding with the target molecule resulting in a rapid result currently unachievable using existing technologies. An ion-channel biosensor comprised of gramicidin A channels embedded in a synthetic tethered lipid bilayer provides a highly sensitive and rapid detection method, for example, for influenza A in untreated clinical samples (Krishnamurthy et al. 2010).

Electronic Nanobiosensors

A signal-on, electronic DNA biosensor has been described that is label-free and achieves a subpicomolar detection limit (Xiao et al. 2006). The sensor, which is based on a target-induced strand displacement mechanism, is composed of a

“capture probe” attached by its 5' terminus to a gold electrode and a 5' methylene blue-modified “signaling probe” that is complementary at both its 3' and 5' termini to the capture probe. In the absence of target, hybridization between the capture and signaling probes minimizes contact between the methylene blue and electrode surface, limiting the observed redox current. Target hybridization displaces the 5' end of the signaling probe, generating a short, flexible single-stranded DNA element and producing up to a sevenfold increase in redox current. The observed signal gain is sufficient to achieve a demonstrated (not extrapolated) detection limit of 400 fM, which is among the best reported for single-step electronic DNA detection. Moreover, because sensor fabrication is straightforward, the approach appears to provide a ready alternative to the more cumbersome femtomolar electrochemical assays described to date.

Capacitors are critical elements in electrical circuits, and nanocapacitors are capacitors with electrodes spacing in the nano-order. When used with single-stranded DNA probes, target hybridization produces a measurable change in capacitance. When used in arrays, nanocapacitors can enable simultaneous detection of nucleic acids without labeling (Fortina et al. 2005).

Electrochemical Nanobiosensor

An electrochemical biosensor combining microfluidics and nanotechnology has been developed by GeneFluidics with 16 sensors in the array, each consisting of three single-layer gold electrodes – working, reference, and auxiliary. Each of the working electrodes contains one representative from a library of capture probes, which are specific for a clinically relevant bacterial urinary pathogen. The library included probes for *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus* spp., and the *Klebsiella-Enterobacter* group. A bacterial 16S rRNA target derived from single-step bacterial lysis was hybridized both to the biotin-modified capture probe on the sensor surface and to a second, fluorescein-modified detector probe. Detection of the target-probe hybrids is achieved through binding of a horseradish peroxidase (HRP)-conjugated anti-fluorescein antibody to the detector probe. Amperometric measurement of the catalyzed HRP reaction is obtained at a fixed potential of –200 mV between the working and reference electrodes. Species-specific detection of as few as 2,600 pathogenic bacteria in culture, inoculated urine, and clinical urine samples can be achieved within 45 min from the beginning of sample processing. In a feasibility study of this amperometric detection system using blinded clinical urine specimens, the sensor array had 100 % sensitivity for direct detection of gram-negative bacteria without nucleic acid purification or amplification (Liao et al. 2006). Identification was demonstrated for 98 % of gram-negative bacteria for which species-specific probes were available. When combined with a microfluidics-based sample preparation module, the integrated system could serve as a point-of-care device for rapid diagnosis of urinary tract infections.

Metallic Nanobiosensors

Fano resonances have been observed in the optical response of plasmonic nanocavities due to the coherent coupling between their superradiant and subradiant plasmon modes, and multiple Fano resonances occur as structure size is increased (Verellen et al. 2009). By putting together two specific nanostructures made of gold or silver, a prototype device can be constructed, which exhibits a highly sensitive ability to detect particular chemicals in the immediate surroundings once it is optimized. The nanostructures measure about 200 nm. One is shaped like a flat circular disk while the other looks like a doughnut with a hole in the middle. When brought together, they interact with light very differently to the way they behave on their own. When they are paired up, they scatter some specific colors within white light much less, leading to an increased amount of light passing through the structure undisturbed. This is distinctly different to how both structures scatter light separately. Metal nanostructures have been used as sensors but they interact very strongly with light due to so-called localized plasmon resonances. But this is the first time a pair with such a carefully tailored interaction with light has been created. This decrease in the interaction with light is in turn affected by the composition of molecules in close proximity to the structures. These nanosensors could be tailor-made to instantly detect the presence of particular molecules, for example, poisons or explosives in transport screening situations or proteins in patients' blood samples, with high sensitivity.

Quartz Nanobalance Biosensor

Single-strand DNA-containing thin films are deposited onto quartz oscillators to construct a device capable of sensing the presence of the complementary DNA sequences, which hybridize with the immobilized ones. DNA, once complexed with aliphatic amines, appears as a monolayer in a single-stranded form by X-ray small angle scattering. A quartz nanobalance is then utilized to monitor mass increment related to specific hybridization with a complementary DNA probe. The crystal quartz nanobalance, capable of high sensitivity, indeed appears capable of obtaining a prototype of a device capable of sensing the occurrence of particular genes or sequences in the sample under investigation.

Viral Nanosensor

Virus particles are essentially biological nanoparticles. Scientists at the Massachusetts General Hospital (Boston, MA) have used herpes-simplex virus (HSV) and adenovirus to trigger the assembly of magnetic nanobeads as a nanosensor for clinically relevant viruses. The nanobeads had a supramagnetic iron oxide core coated with

dextran. Protein G was attached as a binding partner for antiviral antibodies. By conjugating anti-HSV antibodies directly to nanobeads using a bifunctional linker to avoid nonspecific interactions between medium components and protein G and using a magnetic field, the scientists could detect as few as five viral particles in a 10-mL serum sample. This system is more sensitive than ELISA-based methods and is an improvement over PCR-based detection because it is cheaper and faster and has fewer artifacts. Upon target binding, these nanosensors cause changes in the spin–spin relaxation times of neighboring water molecules, which can detect specific mRNA, proteins, and enzymatic activity by (NMR/MRI) techniques.

A QD-DNA nanosensor, based on fluorescence resonance energy transfer (FRET), has been used for the detection of the target DNA and single mismatch in hepatitis B virus (HBV) gene (Wang et al. 2010a). This DNA detection method is simple, rapid, and efficient due to the elimination of the washing and separation steps. In this study, oligonucleotides were attached to the QD surface to form functional QD-DNA conjugates. With the addition of DNA targets and Cy5-modified signal DNAs into the QD-DNA conjugates, sandwiched hybrids were formed leading to fluorescence from the acceptor by means of FRET on illumination of the donor. Oligonucleotide ligation assay was employed to efficiently detect single-base mutants in HBV gene. This simple method enables efficient detection that could be used for high throughput and multiplex detections of viral gene mutations.

PEBBLE Nanosensors

Scientists at the University of Michigan (Ann Arbor, Michigan) have developed PEBBLE (probes encapsulated by biologically localized embedding) nanosensors, which consist of sensor molecules entrapped in a chemically inert matrix by a microemulsion polymerization process that produces spherical sensors in the size range of 20–200 nm. These sensors are capable of real-time inter- and intracellular imaging of ions and molecules and are insensitive to interference from proteins. PEBBLE can also be used for early detection of cancer. PEBBLE nanosensors also show very good reversibility and stability to leaching and photobleaching, as well as very short response times and no perturbation by proteins. In human plasma, they demonstrate a robust oxygen sensing capability, little affected by light scattering and autofluorescence. PEBBLE has been developed further as a tool for diagnosis as well as treatment of cancer.

Detection of Cocaine Molecules by Nanoparticle-Labeled Aptasensors

Metallic or semiconductor nanoparticles (NPs) are used as labels for the electrochemical, photoelectrochemical, or surface plasmon resonance (SPR) detection of

cocaine using a common aptasensor configuration (Golub et al. 2009). The aptasensors are based on the use of two anticocaine aptamer subunits, where one subunit is assembled on an Au support, acting as an electrode or a SPR-active surface, and the second aptamer subunit is labeled with Pt-NPs, CdS-NPs, or Au-NPs. In the different aptasensor configurations, the addition of cocaine results in the formation of supra-molecular complexes between the NPs-labeled aptamer subunits and cocaine on the metallic surface, enabling quantitative analysis of cocaine. The supramolecular Au-NPs-aptamer-subunits–cocaine complex generated on the Au support allows the SPR detection of cocaine through the reflectance changes stimulated by the electronic coupling between the localized plasmon of the Au-NPs and the surface plasmon wave. All aptasensor configurations enable the analysis of cocaine with a detection limit in the range of 10^{-6} to 10^{-5} M. The major advantage of the sensing platform is the lack of background interfering signals.

Nanosensors for Glucose Monitoring

One of the main reasons for developing *in vivo* glucose sensors is the detection of hypoglycemia in people with insulin-dependent (type 1) diabetes. It is possible to engineer fluorescent micro/nanoscale devices for glucose sensing. Deployment of nanoparticles in the dermis may allow transdermal monitoring of glucose changes in interstitial fluid. Using electrostatic self-assembly, an example of nanotechnology for fabrication, two types of sensors are being studied: (1) solid nanoparticles coated with fluorescent enzyme-containing thin films and (2) hollow nanocapsules containing fluorescent indicators and enzymes or glucose-binding proteins. Nanoengineering of the coated colloids and nanocapsules allows precision control over optical, mechanical, and catalytic properties to achieve sensitive response using a combination of polymers, fluorescent indicators, and glucose-specific proteins. Challenges to *in vivo* use include understanding of material toxicity and failure modes and determining methods to overcome fouling, protein inactivation, and material degradation. Noninvasive glucose sensing will maximize acceptance by patients and overcome biocompatibility problems of implants. Near-infrared spectroscopy has been most investigated, but the precision needs to be improved for eventual clinical application.

The nanotube-based optical biosensor could free people with diabetes from the daily pinprick tests now required for monitoring blood sugar concentrations. Carbon nanotubes are coated with glucose oxidase, an enzyme that breaks down glucose molecules. Then ferricyanide, an electron-hungry molecule, is sprinkled onto the nanotubes' surfaces. Ferricyanide draws electrons from the nanotubes, quenching their capacity to glow when excited by infrared light. When glucose is present, it reacts with the oxidase, producing hydrogen peroxide. In turn, the hydrogen peroxide reacts with ferricyanide in a way that reduces that molecule's hunger for electrons. The higher the glucose level, the greater is the nanotube's infrared fluorescence.

Micromechanical detection of biologically relevant glucose concentrations can be achieved by immobilization of glucose oxidase (GOx) onto a microcantilever surface. The enzyme-functionalized microcantilever undergoes bending due to a change in surface stress induced by the reaction between glucose in solution and the GOx immobilized on the cantilever surface.

Nanobiosensors for Protein Detection

High-sensitivity biosensors for the detection of proteins have been developed using several kinds of nanomaterials. The performance of the sensors depends on the type of nanostructures with which the biomaterials interact. 1D structures such as nanowires, nanotubes, and nanorods are proven to have high potential for bio-applications. Different types of nanostructures that have attracted much attention by their performance as biosensors utilize materials such as polymers, carbon, and zinc oxide because of their sensitivity, biocompatibility, and ease of preparation (M et al. 2011). This publication describes the three stages in the development of biosensors: (1) fabrication of biomaterials into nanostructures, (2) alignment of the nanostructures, and (3) immobilization of proteins.

Optical Biosensors

Many biosensors that are currently marketed rely on the optical properties of lasers to monitor and quantify interactions of biomolecules that occur on specially derived surfaces or biochips. An integrated biosensor, based on phototransistor integrated circuits, has been developed for use in medical detection, DNA diagnostics, and gene mapping. The biochip device has sensors, amplifiers, discriminators, and logic circuitry on board. Integration of light-emitting diodes into the device is also possible. Measurements of fluorescent-labeled DNA probe microarrays and hybridization experiments with a sequence-specific DNA probe for HIV-1 on nitrocellulose substrates illustrate the usefulness and potential of this DNA biochip. A number of variations of optical biosensors offer distinct methods of sample application and detection in addition to different types of sensor surface. Surface plasmon resonance technology is the best-known example of this technology.

Laser Nanosensors

In a laser nanosensor, laser light is launched into the fiber, and the resulting evanescent field at the tip of the fiber is used to excite target molecules bound to the antibody molecules. A photometric detection system is used to detect the optical signal (e.g., fluorescence) originating from the analyte molecules or from the analyte-bioreceptor reaction. Laser nanosensors can be used for in vivo analysis of proteins

and biomarkers in individual living cells (Vo-Dinh and Zhang 2011). The nanosensors are made of tapered optical fibers with distal ends having nanometer-sized diameters. Bioreceptors, such as antibody, peptides, and nucleic acids, are immobilized on the fiber tips and designed to be selective to target analyte molecules of interest. A laser beam is transmitted through the fiber and excites target molecules bound to the bioreceptor molecules. The resulting fluorescence from the analyte molecules is detected by a photodetection system. Nanosensors can provide minimally invasive tools to probe subcellular compartments inside individual living cells.

Physicists at University of Rochester have assembled a simple laser system to detect nanoparticles. They split a laser beam in two, sending one half to a sample. When the light hits a small particle, it is scattered back and recombines with the reserve half of the laser beam, producing a detectable interference pattern detectable only when a moving particle is present. This laser method works where others do not because it relies on the amplitude rather than intensity of light. The amplitude is the square root of intensity, so it decays much less than intensity as the particles get smaller. Single particles, as small as 7 nm in diameter, have been detected.

Researchers at the University of Twente (The Netherlands) have developed an ultrasensitive sensor that could potentially be used in a handheld device to detect various viruses and measure their concentration within minutes. It requires only a tiny sample of saliva, blood, or other body fluid. The device uses a silicon substrate containing channels that guide laser light. Light enters into the substrate at one end and is split into four parallel beams. When these beams emerge at the other end, they spread out and overlap with one another, creating a pattern of bright and dark bands, known as an interference pattern, which are recorded. A commercial version of the biosensor is being developed in collaboration with Paradocs Group BV (The Netherlands). Although the sensor has been shown to detect only the herpes-simplex virus, it could be used to quickly screen people at hospitals and emergency clinics for control of outbreaks of diseases such as SARS and avian flu.

Nanoshell Biosensors

Nanoshells can enhance chemical sensing by as much as 10 billion times. That makes them about 10,000 times more effective at Raman scattering than traditional methods. When molecules and materials scatter light, a small fraction of the light interacts in such a way that it allows scientists to determine their detailed chemical makeup. This property, known as Raman scattering, is used by medical researchers, drug designers, chemists, and other scientists to determine what materials are made of. An enormous limitation in the use of Raman scattering has been its extremely weak sensitivity. Nanoshells can provide large, clean, reproducible enhancements of this effect, opening the door for new, all-optical sensing applications. Each individual nanoshell can act as an independent Raman enhancer. That creates an opportunity to design all-optical nanoscale sensors – essentially molecular diagnostic instruments – that could detect as little as a few molecules of a target substance, which could be anything from a drug molecule or a key disease protein to a deadly chemical agent.

The metal cover of the nanoshell captures passing light and focuses it, a property that directly leads to the enormous Raman enhancements observed. Furthermore, nanoshells can be tuned to interact with specific wavelengths of light by varying the thickness of their shells. This tunability allows for the Raman enhancements to be optimized for specific wavelengths of light. The finding that individual nanoshells can vastly enhance the Raman effect opens the door for biosensor designs that use a single nanoshell, something that could prove useful for engineers who are trying to probe the chemical processes within small structures such as individual cells, or for the detection of very small amounts of a material, like a few molecules of a deadly biological or chemical agent. Nanoshells are already being developed for applications including cancer diagnosis, cancer therapy, testing for proteins associated with Alzheimer's disease, drug delivery, and rapid whole-blood immunoassays.

Plasmonics and SERS Nanoprobes

Surface plasmons are collective oscillations of free electrons at metallic surfaces. These oscillations can give rise to the intense colors of solutions of plasmon resonance nanoparticles and very intense scattering. While the use of plasmonic particle absorption-based bioaffinity sensing is now widespread throughout biological research, the use of their scattering properties is relatively less studied. Plasmon scatter can be used for long-range immunosensing and macromolecular conformation studies.

A variety of sensors, metallic nanostructured probes, metallic nanoshells and half-shells, and nanoarrays for SERS sensing have been developed at the Oak Ridge National Laboratory. The SERS technology can detect the chemical agents and biological species (e.g., spores, biomarkers of pathogenic agents) directly. A DNA-based technique based on surface-enhanced Raman gene (SERGen) probes can be also used to detect gene targets via hybridization to DNA sequences complementary to these probes. Advanced instrumental systems designed for spectral measurements and for multi-array imaging as well as for field monitoring (RAMiTS technology) have been constructed. Plasmonics and SERS nanoprobes are useful for biological sensing.

Optical mRNA Biosensors

mRNA quantification is important in molecular diagnostics. Traditional spectrophotometric method cannot distinguish DNA, rRNA, and tRNA species from mRNA. Scheme of an optical mRNA biosensor for examination of pathological samples is shown in Fig. 4.2.

Surface-Enhanced Micro-optical Fluidic Systems

The aim of the surface-enhanced micro-optical fluidic systems (SEMOFS) European project is to develop a new concept for biosensors: a polymer-based card

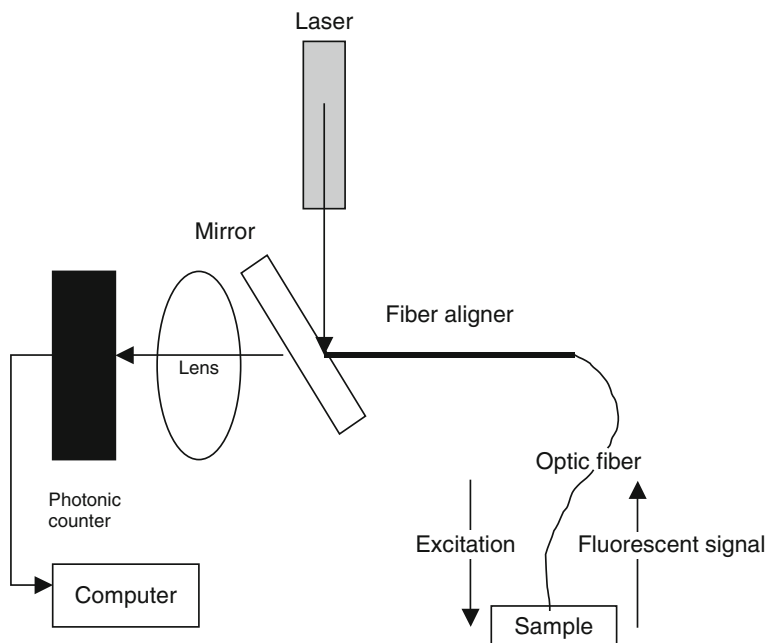


Fig. 4.2 Scheme of an optical mRNA biosensor. Sequence-specific molecular beacons are used as molecular switches. This biosensor detects single molecules in fluids and can be used to search for molecular biomarkers to predict the prognosis of disease

type integrated “plasmon-enhanced SPR” sensor. The card will combine biologically active surfaces with integrated optics (light source, detection) and biocompatible multichannel microfluidics. The project aims to achieve a significant breakthrough, since all functions will be totally integrated on a single polymer-based chip. The final product shall be manufactured with large-scale, mass production techniques. The card will therefore be extremely low cost and disposable while providing increased sensitivity and diagnosis possibilities. The project will focus on:

- Increasing detection sensitivity and access to new information of the biological sample
- Microfluidics on polymer substrate enabling multichanneling (further enhancing sensitivity by parallel analysis) and integrated fluid actuators
- Integrated optical detection concept based on organic light-emitting display (OLED)/waveguide/miniaturized spectrometer enabling card type integrated solution and multichanneling
- Hybrid micromachining to ensure compatibility of the mastering and replication protocols with constraints of industrial scale manufacturing
- Validation of expected applications and evaluation in clinical cancer diagnosis

Nanoparticle-Enhanced Sensitivity of Fluorescence-Based Biosensors

Sensitivity required for high-performance bioassays can be achieved using fluorescence-based techniques for biosensors. There is still a need for enhancement strategies, which can reduce limit of detection and increase sensitivity for the detection of low analyte concentrations in small sample volumes. Possible solutions include the use of SPR effect associated with metal nanostructures, each of which contains a high concentration of dye molecules (McDonagh et al. 2009). The degree of enhancement achieved is dependent on the nanoparticle, dye label, and nanoparticle deposition technique. For optimum assay enhancement, the antibody label must be located outside the quenching range and within the optimum distance from the metal nanoparticle. Nanoparticles with high brightness, low toxicity, biocompatibility, and ease of biomolecule conjugation are selected. For enhancement of bioassays, the nanoparticle is conjugated to the antibody, replacing the single dye label.

Nanowire Biosensors

Since their surface properties are easily modified, nanowires can be decorated with virtually any potential chemical or biological molecular recognition unit, making the wires themselves independent of the analyte. The nanomaterials transduce the chemical binding event on their surface into a change in conductance of the nanowire in an extremely sensitive, real-time, and quantitative fashion. Boron-doped silicon nanowires (SiNWs) have been used to create highly sensitive, real-time electrically based sensors for biological and chemical species. Biotin-modified SiNWs were used to detect streptavidin down to at least a picomolar concentration range. The small size and capability of these semiconductor nanowires for sensitive, label-free, real-time detection of a wide range of chemical and biological species could be exploited in array-based screening and in vivo diagnostics.

A novel approach to synthesizing nanowires (NWs) allows their direct integration with microelectronic systems for the first time, as well as their ability to act as highly sensitive biomolecule detectors that could revolutionize biological diagnostic applications. An interdisciplinary team of engineers in Yale University's Institute for Nanoscience and Quantum Engineering has overcome hurdles in NW synthesis by using a tried-and-true process of wet-etch lithography on commercially available silicon-on-insulator wafers. These NWs are structurally stable and demonstrate an unprecedented sensitivity as sensors for detection of antibodies and other biologically important molecules. According to researchers, not only can the NWs detect extremely minute concentrations (as few as 1,000 individual molecules in a cubic millimeter), they can do it without the hazard or inconvenience of any added fluorescent or radioactive detection probes. The study demonstrated ability of the NWs to monitor antibody binding and to sense real-time live cellular immune response using T-lymphocyte activation as a model. Within approximately 10 s, the NW could register T-cell activation as the release acid to the device. The basis for the sensors is the detection of hydrogen ions or acidity, within the physiological

range of reactions in the body. Traditional assays for detection of immune system cells such as T cells or for antibodies usually take hours to complete.

When biological molecules bind to their receptors on the nanowire, they usually alter the current moving through the sensor and signal the presence of substance of interest. This direct detection dispenses with the time-consuming labeling chemistry and speeds up the detection process considerably. Nanowire biosensors are used for the detection of proteins, viruses, or DNA in a highly sensitive manner. They can be devised to test for a complex of proteins associated with a particular cancer and used for diagnosis as well as monitoring the progress of treatment.

Nanowire Biosensors for Detection of Single Viruses

Rapid, selective, and sensitive detection of viruses is crucial for implementing an effective response to viral infection, such as through medication or quarantine. Established methods for viral analysis include plaque assays, immunological assays, transmission electron microscopy, and PCR-based testing of viral nucleic acids. These methods have not achieved rapid detection at a single virus level and often require a relatively high level of sample manipulation that is inconvenient for infectious materials.

Direct, real-time electrical detection of single virus particles with high selectivity has been reported by using nanowire field-effect transistors (Patolsky et al. 2004). Measurements made with nanowire arrays modified with antibodies for influenza A showed discrete conductance changes characteristic of binding and unbinding in the presence of influenza A but not paramyxovirus or adenovirus. Simultaneous electrical and optical measurements using fluorescently labeled influenza A were used to demonstrate conclusively that the conductance changes correspond to binding/unbinding of single viruses at the surface of nanowire devices. pH-dependent studies further show that the detection mechanism is caused by a field effect and that the nanowire devices can be used to determine rapidly isoelectric points and variations in receptor-virus binding kinetics for different conditions. Larger arrays of reproducible nanowire devices might simultaneously screen for the presence of 100 or more different viruses. Finally, studies of nanowire devices modified with antibodies specific for either influenza or adenovirus show that multiple viruses can be selectively detected in parallel. The possibility of large-scale integration of these nanowire devices suggests potential for simultaneous detection of a large number of distinct viral threats at the single virus level.

Nanowires for Detection of Genetic Disorders

The surfaces of the silicon nanowire devices have been modified with peptide nucleic acid (PNA) receptors designed to recognize wild type versus the F508 mutation site in the cystic fibrosis transmembrane receptor gene (Hahm and Lieber 2004). Conductance measurements made while sequentially introducing wild type or mutant DNA samples exhibit a time-dependent conductance increase consistent with the PNA-DNA hybridization and enabled identification of fully complementary

versus mismatched DNA samples. Concentration-dependent measurements show that detection can be carried out to at least the tens of femtomolar range. It provides more rapid results than current methods of DNA detection. This nanowire-based approach represents a step forward for direct, label-free DNA detection with extreme sensitivity and good selectivity and could provide a pathway to integrated, high-throughput, multiplexed DNA detection for genetic screening.

Nanowires Biosensor for Detecting Biowarfare Agents

A multi-stripped biosensing nanowires system can be used for detecting biowarfare agents in the field (Tok et al. 2006). It is constructed from submicrometer layers of different metals including gold, silver, and nickel that act as “barcodes” for detecting a variety of pathogens ranging from anthrax, smallpox, and ricin to botulinum. Antibodies of specific pathogens are attached to the nanowires producing a small, reliable, sensitive detection system. The system could also be used during an outbreak of an infectious disease.

Concluding Remarks and Future Prospects of Nanowire Biosensors

A review has shown that nanowire biosensors modified with specific surface receptors represent a powerful nanotechnology-enabled diagnostic/detection platform for medicine and the life sciences (Patolsky et al. 2006). Key features of these devices include direct, label-free, and real-time electrical signal transduction, ultrahigh sensitivity, exquisite selectivity, and potential for integration of addressable arrays on a massive scale, which sets them apart from other sensor technologies that are currently available. Nanowire biosensors have unique capabilities for multiplexed real-time detection of proteins, single viruses, DNA, enzymatic processes, and small organic molecule-binding to proteins. Apart from their value as research tools, they have a significant impact on disease diagnosis, genetic screening, and drug discovery. They will facilitate the development of personalized medicine. Because these nanowire sensors transduce chemical/biological-binding events into electronic/digital signals, they have the potential for a highly sophisticated interface between nano-electronic and biological information processing systems in the future.

Future Issues in the Development of Nanobiosensors

New biosensors and biosensor arrays are being developed using new materials, nanomaterials, and microfabricated materials including new methods of patterning. Biosensor components will use nanofabrication technologies. Use of nanotubes, buckminsterfullerenes (buckyballs), and silica and its derivatives can produce nanosized devices. Some of the challenges will be:

- Development of real-time noninvasive technologies that can be applied to detection and quantitation of biological fluids without the need for multiple calibrations using clinical samples.

- Development of biosensors utilizing new technologies that offer improved sensitivity for detection with high specificity at single-molecular level.
- Development of biosensor arrays that can successfully detect, quantify, and quickly identify individual components of mixed gases and liquid in an industrial environment.

It would be desirable to develop multiple integrated biosensor systems that utilize doped oxides, polymers, enzymes, or other components to give the system the required specificity. A system with all the biosensor components, software, plumbing, reagents, and sample processing is an example of an integrated biosensor. There is also a need for reliable fluid handling systems for “dirty” fluids and for relatively small quantities of fluids (nanoliter to attoliter quantities). These should be low cost, disposable, reliable, and easy to use as part of an integrated sensor system. Sensing in picoliter to attoliter volumes might create new problems in development of micro-reactors for sensing and novel phenomenon in very small channels.

Applications of Nanodiagnostics

Applications of nanotechnologies in clinical diagnostics have been expanding. Although some of these were mentioned along with individual technologies in the preceding section, other important applications will be identified here. Applications for diagnosis in special areas such as cancer are described in chapters dealing with these therapeutic areas.

Nanotechnology for Detection of Biomarkers

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise, and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on DNA, RNA, metabolite, or protein level can be referred to as a molecular biomarker. From a practical point of view, the biomarker would specifically and sensitively reflect a disease state and could be used for diagnosis as well as for disease monitoring during and following therapy (Jain 2010). Currently available molecular diagnostic technologies have been used to detect biomarkers of various diseases such as cancer, metabolic disorders, infections, and diseases of the central nervous system. Nanotechnology has further refined the detection of biomarkers. Some biomarkers also form the basis of innovative molecular diagnostic tests.

The physicochemical characteristics and high surface areas of nanoparticles make them ideal candidates for developing biomarker harvesting platforms. Given the variety of nanoparticle technologies that are available, it is feasible to tailor

nanoparticle surfaces to selectively bind a subset of biomarkers and sequestering them for later study using high-sensitivity proteomic tests (Geho et al. 2006). Biomarker harvesting is an underutilized application of nanoparticle technology and is likely undergo substantial growth. Functional polymer-coated nanoparticles can be used for quick detection of biomarkers and DNA separation.

DNA Y-junctions have been used as fluorescent scaffolds for EcoRII methyltransferase-thioredoxin fusion proteins and covalent links were formed between the DNA scaffold and the methyltransferase at preselected sites on the scaffold containing 5FdC (Singer and Smith 2006). The resulting thioredoxin-targeted nanodevice was found to bind selectively to certain cell lines but not to others. The fusion protein was constructed so as to permit proteolytic cleavage of the thioredoxin peptide from the nanodevice. Proteolysis with thrombin or enterokinase effectively removed the thioredoxin peptide from the nanodevice and extinguished cell line-specific binding measured by fluorescence. Potential applications for devices of this type include the ability of the fused protein to selectively target the nanodevice to certain tumor cell lines suggesting that this approach can be used to probe cell-surface receptors as biomarkers of cancer and may serve as an adjunct to immunohistochemical methods in tumor classification.

A magnetic nanosensor technology that is up to 1,000 times more sensitive than any technology now in clinical use is accurate regardless of which bodily fluid is being analyzed and can detect biomarker proteins over a range of concentrations three times broader than any existing method (Gaster et al. 2009). The nanosensor chip also can search for up to 64 different proteins simultaneously and has been shown to be effective in early detection of tumors in mice, suggesting that it may open the door to significantly earlier detection of even the most elusive cancers in humans. The magnetic nanosensor can successfully detect cancerous tumors in mice when levels of cancer-associated proteins are still well below concentrations detectable using the current standard method, ELISA. The sensor also can be used to detect biomarkers of diseases other than cancer.

Nanotechnology for Genotyping of Single-Nucleotide Polymorphisms

Nanoparticles for Detecting SNPs

There are two types of DNA-nanoparticles aggregation assays: one of the methods relies on cross-linking of the gold nanoparticle by hybridization, and the other is a non-cross-linking system (Sato et al. 2007). The cross-linking system has been used not only to detect target DNA sequences but also to detect metal ions or small molecules, which are recognized by DNAzymes. The non-cross-linking approach shows high performance in the detection of SNPs. These methods do not need special equipment and open up a new possibility of POC diagnoses.

The primer extension (PEXT) reaction is the most widely used approach to genotyping of SNPs. Current methods for analysis of PEXT reaction products are

based on electrophoresis, fluorescence resonance energy transfer, fluorescence polarization, pyrosequencing, mass spectrometry, microarrays, and spectrally encoded microspheres. A dry-reagent dipstick method has been devised that enables rapid visual detection of PEXT products without instrumentation (Litos et al. 2007). The genomic region that spans each SNP of interest is amplified by PCR. Two primer extension reactions are performed with allele-specific primers (for one or the other variant nucleotide), which contain an oligo(dA) segment at the 5'-end. Biotin-dUTP is incorporated in the extended strand. The product is applied to the strip followed by immersion in the appropriate buffer. As the DNA moves along the strip by capillary action, it hybridizes with oligo(dT)-functionalized gold nanoparticles, such that only extended products are captured by immobilized streptavidin at the test zone, generating a red line. A second red line is formed at the control zone of the strip by hybridization of the nanoparticles with immobilized oligo(dA). The dipstick test is complete within 10 min. The described PEXT-dipstick assay is rapid and highly accurate; it shows 100 % concordance with direct DNA sequencing data. It does not require specialized instrumentation or highly trained technical personnel. It is appropriate for a diagnostic laboratory where a few selected SNP markers are examined per patient with a low cost per assay.

Nanopores for Detecting SNPs

Use of nanopore technology for sequencing was described earlier in this chapter. The focus in this section is the application for detection of SNPs. A voltage threshold has been discovered for permeation through a synthetic nanopore of dsDNA bound to a restriction enzyme that depends on the sequence (Zhao et al. 2007). Molecular dynamic simulations reveal that the threshold is associated with a nanonewton force required to rupture the DNA-protein complex. A single mutation in the recognition site for the restriction enzyme, that is, an SNP, can easily be detected as a change in the threshold voltage. Consequently, by measuring the threshold voltage in a synthetic nanopore, it may be possible to discriminate between two variants of the same gene (alleles) that differ in one base.

Nanobiotechnologies for Single-Molecule Detection

Various nanobiotechnologies for single-molecule detection are shown in Table 4.2. These have been described in preceding sections.

Protease-Activated QD Probes

QDs have been programmed to glow in presence of enzyme activity and give off NIR light only when activated by specific proteases. Altered expression of particular

Table 4.2 Nanobiotechnologies for single-molecule detection**Visualization of biomolecules by near nanoscale microscopy**

Atomic force microscope

Scanning probe microscope

3D single-molecular imaging by nanotechnology

Near-field scanning optical microscope

Spectrally resolved fluorescence lifetime imaging microscopy

Nanolaser spectroscopy for detection of cancer in single cells**Nanoproteomics**

Study of protein expression at single-molecule level

Detection of a single molecule of protein

Erenna™ Bioassay System: digital single-molecule detection platform**Nanofluidic/nanoarray devices: detection of a single molecule of DNA****Carbon nanotube transistors for genetic screening****Nanopore technology****Portable nanocantilever system for diagnosis****Nanobiosensors**

QD-FRET nanosensors for single-molecule detection

proteases is a common hallmark of cancer, atherosclerosis, and many other diseases. NIR light also passes harmlessly through skin, muscle, and cartilage, so the new probes could detect tumors and other diseases at sites deep in the body without the need for a biopsy or invasive surgery. The probe's design makes use of a technique called "quenching" that involves tethering a gold nanoparticle to the QD to inhibit luminescence. The tether, a peptide sequence measuring only a few nanometers, holds the gold close enough to prevent the QD from giving off its light. The peptide tether used is one that is cleaved by the enzyme collagenase. The luminescence of the QDs is cut by more than 70 % when they are attached to the gold particles. They remain dark until the nanostructures were exposed to collagenase after which the luminescence steadily returns. The ultimate aim of the research is to pair a series of QDs, each with a unique NIR optical signature, to an index of linker proteases. This probe would be important for understanding and monitoring the efficacy of therapeutic interventions, including the growing class of drugs that act as protease inhibitors. An important feature of the protease imaging probes described in this study is the combination of the contrast enhancement achievable through a probe that can be activated and is combined with the brightness, photostability, and tunability of QDs.

Labeling of MSCs with QDs

QDs are useful for concurrently monitoring several intercellular and intracellular interactions in live normal cells and cancer cells over periods ranging from less than a second to over several days (several divisions of cells). QDs offer an alternative to organic dyes and fluorescent proteins to label and track cells *in vitro* and *in vivo*.

These nanoparticles are resistant to chemical and metabolic degradation, demonstrating long-term photostability. The cytotoxic effects of in vitro QD labeling on MSC proliferation and differentiation and use as a cell label in an in vitro cardiomyocyte coculture model have been investigated (Muller-Borer et al. 2007). Fluorescent QDs were shown to label MSC effectively, were easy to use, and showed a high yield as well as survival rate with minimal cytotoxic effects. Dose-dependent effects, however, suggest limiting MSC QD exposure.

The peptide CGGGRGD has been immobilized on CdSe–ZnS QDs coated with carboxyl groups by cross-linking with amine groups. These conjugates are directed by the peptide to bind with selected integrins on the membrane of hMSCs. Upon overnight incubation with optimal concentration, QDs effectively labeled all the cells. Long-term labeling of bone marrow-derived hMSCs with RGD-conjugated QDs was demonstrated during self-replication and differentiation into osteogenic cell lineages. Labeling of hMSCs with QDs has been carried out during self-replication and multilineage differentiations into osteogenic, chondrogenic, and adipogenic cells (Shah et al. 2007). QD-labeled hMSCs remained viable as unlabeled hMSCs from the same subpopulation suggesting the use of bioconjugated QDs as an effective probe for long-term labeling of stem cells.

Nanotechnology for Point-of-Care Diagnostics

Point-of-care (POC) or near patient testing means that diagnosis is performed in the doctor's office or at the bedside in case of hospitalized patients or in the field for several other indications including screening of populations for genetic disorders and cancer. POC involves analytical patient testing activities provided within the healthcare system but performed outside the physical facilities of the clinical laboratories. POC does not require permanent dedicated space but includes kits and instruments, which are either hand carried or transported to the vicinity of the patient for immediate testing at that site. The patients may even conduct the tests.

An example of POC test is CD4 T-cell count as guide to treatment of HIV/AIDS. The number of circulating CD4 T cells drops significantly when patients are infected with HIV/AIDS. CD4 counts assist in the decisions on when to initiate and when to stop the treatment, which makes this test so important at POC. While such testing is routine in Western countries and used repeatedly over the course of treatment to see if interventions are effective, it is unavailable to many people in the developing world, especially in rural areas. A cheap test for CD4+ T lymphocytes in the blood is in development using biosensor nanovesicles to enhance the signal.

After the laboratory and the emergency room, the most important application of molecular diagnostics is estimated to be at the POC. Nanotechnology would be another means of integrating diagnostics with therapeutics. Nanotechnology-based diagnostics provides the means to monitor drugs administered by nanoparticle carriers. A number of devices based on nanotechnology are among those with potential applications in POC testing.

Nanotechnology-Based Biochips for POC Diagnosis

The use of metal nanoparticles as labels represents a promising approach. They exhibit a high stability in signal and new detection schemes that would allow for robustness and low-cost readout in biochips. First examples of this kind have been established and are in the market, and more are in the development pipeline (Festag et al. 2008). Nanosphere Inc.'s Verigene™ platform will be suitable for development of POC testing.

Carbon Nanotube Transistors for Genetic Screening

Carbon nanotube network field-effect transistors (NTNFETs) have been reported that function as selective detectors of DNA immobilization and hybridization (Star et al. 2006). NTNFETs with immobilized synthetic oligonucleotides have been shown to specifically recognize target DNA sequences, including H63D SNP discrimination in the HFE gene, responsible for hereditary hemochromatosis, a disease in which too much iron accumulates in body tissues. The electronic responses of NTNFETs upon single-stranded DNA immobilization and subsequent DNA hybridization events were confirmed by using fluorescence-labeled oligonucleotides and then were further explored for label-free DNA detection at picomolar to micromolar concentrations. A strong effect of DNA counterions on the electronic response was observed, suggesting a charge-based mechanism of DNA detection using NTNFET devices. Implementation of label-free electronic detection assays using NTNFETs constitutes an important step toward low-cost, low-complexity, highly sensitive, and accurate molecular diagnostics. Label-free electronic detection of DNA has several advantages over state-of-the-art optical techniques, including cost, time, and simplicity. The sensitivity of the new device is good enough to detect a single-base mutation in an amount of DNA present in 1 mL of blood. This technology can bring to market handheld, POC devices for genetic screening, as opposed to laboratory methods using labor-intensive labeling and sophisticated optical equipment. This device will be commercially developed by Nanomix Inc. as Sensation™ technology.

POC Monitoring of Vital Signs with Nanobiosensors

Researchers at the University of Arkansas (Fayetteville, AR) have worked with pentacene, a hydrocarbon molecule, and carbon nanotubes (CNTs) to develop the two types of nanobiosensors for vital signs: a temperature sensor and a strain sensor for respiration. The two similar but slightly different biosensors are integrated into "smart" fabrics – garments with wireless technology and will be able to monitor a patient's respiration rate and body temperature in real time. The addition of CNTs with pentacene increases biosensor sensitivity. As an organic semiconductor, pentacene is efficient and easy to control. Both biosensors were fabricated directly on flexible polymeric substrates. The strain sensor, which would monitor respiration rate, consisted of a Wheatstone bridge, an instrument that measures unknown

electrical resistance, and a thin pentacene film that acted as a sensing layer. The system would work when a physiological strain, such as breathing, creates a mechanical deformation of the sensor, which then affects the electrical current's resistance. For the temperature sensor, the researchers used a thin-film transistor that helped them to observe electrical current in linear response to temperature change. Most importantly, in low-voltage areas, the current displayed the highest sensitivity to temperature changes. This device is useful for patients whose vital signs must be continuously monitored on bedside either at home or in hospital. The sensors and wireless networks can fit on garments such as undershirts. With this technology, the smart fabric can monitor vital signs and collect and send data to an information center in real time. The information can enable immediate detection of physiological abnormalities, which will allow physicians to begin treatment or prevent illness before full-blown disease manifestation.

Shri Lakshmi Nano Technologies Ltd. is collaborating with the University of Arkansas to optimize utilization of upcoming nanotechnologies to invent, design, and manufacture advanced conductive fabric incorporating a biosensor that will allow the monitoring of body temperature, blood pressure, ECG, heart rate, and other vital health signs.

Nanodiagnostics for the Battle Field and Biodefense

One of the areas of interest at the MIT's Institute for Soldier Nanotechnologies (Cambridge, MA) concerns ultrasensitive nanoengineered chemical detectors. Researchers have taken a major step toward making an existing miniature lab-on-a-chip fully portable, so the tiny device can perform hundreds of chemical experiments in any setting including the battlefield. This will make testing soldiers to see if they have been exposed to biological or chemical weapons much faster and easier. Neither of the previous approaches, such as mechanically forcing fluid through microchannels or capillary electroosmosis, offers portability. Within the lab-on-a-chip, biological fluids such as blood are pumped through channels about 10 μm wide. Each channel has its own pumps, which direct the fluids to certain areas of the chip so they can be tested for the presence of specific molecules. In the new system, known as a 3D AC electroosmotic pump, tiny electrodes with raised steps generate opposing slip velocities at different heights, which combine to push the fluid in one direction, like a conveyor belt. Simulations predict a dramatic improvement in flow rate, by almost a factor of 20, so that fast (mm/s) flows, comparable to pressure-driven systems, can be attained with battery voltages. If exposure to chemical or biological weapons is suspected, the device can automatically and rapidly test a miniscule blood sample, rather than sending a large sample to a laboratory and waiting for the results. The chips are so small and cheap to make that they could be designed to be disposable or they could be made implantable. Another project focuses on research to develop different approaches to sensing and characterization of materials, including toxins, with identifiable chemical signatures. Each project

exploits manipulation of nanoscale features of materials to achieve one or more of specificity, spatial resolution, convenience of use, reduced power demand, or multifunctionality.

An Integrated Nanobiosensor

DINAMICS (DIagnostic NANotech and MICrotech Sensors) is a Sixth Framework European project for develop an integrated cost-effective nanobiosensor assay for detection of bioterrorism and harmful environmental agents. The project (<http://www.dinamics-project.eu/>) started in 2007 and concluded at the end of 2011. The prime deliverable is an exploitable lab-on-a-chip device for detection of pathogens in water using on-the-spot recognition and detection based on the nanotechnological assembly of unlabeled DNA. DINAMICS has integrated DNA hybridization sensors with microfluidics and signal conditioning/processing both on silicon and polymer substrates avoiding the use of external apparatus for fluid handling, electrical signal generation, and processing, based on DNA hybridization. Measurements based on electrical signals and detection is through UV light absorption. The aim is development of a system where each sensing site in the microarray contains a UV microfabricated sensor. After DNA hybridization, the whole array is illuminated with UV light, and the absorption of each site is measured by the sensor. The project has culminated in an integrated multi-technology product that will be high tech, low-cost, and time-efficient sensing device applicable for use in the water industry. This will ensure a reliable source of cost reduction through a drastic shortening of the sensing pipeline and without the need of transferring the samples to an analytical laboratory.

Nanodiagnostics for Integrating Diagnostics with Therapeutics

Molecular diagnostics is an important component of personalized medicine. Improvement of diagnostics by nanotechnology has a positive impact on personalized medicine. Nanotechnology has potential advantages in applications in point-of-care (POC) diagnosis: on patient's bedside, self-diagnostics for use in the home, and integration of diagnostics with therapeutics. All of these will facilitate the development of personalized medicines.

Concluding Remarks About Nanodiagnostics

It is now obvious that direct analysis of DNA and protein could dramatically improve speed, accuracy, and sensitivity over conventional molecular diagnostic methods. Since DNA, RNA, protein, and their functional subcellular scaffolds and compartments are in the nanometer scale, the potential of single-molecule analysis

approach would not be fully realized without the help of nanobiotechnology. Advances in nanotechnology are providing nanofabricated devices that are small, sensitive and inexpensive enough to facilitate direct observation, manipulation and analysis of single biological molecule from single cell. This opens new opportunities and provides powerful tools in the fields such as genomics, proteomics, molecular diagnostics, and high-throughput screening. A review of articles published over the past 10 years investigating the use of QDs, gold nanoparticles, cantilevers, and other nanotechnologies concluded that nanodiagnostics promise increased sensitivity, multiplexing capabilities, and reduced cost for many diagnostic applications as well as intracellular imaging. Further work is needed to fully optimize these diagnostic nanotechnologies for clinical laboratory setting and to address the issues of potential health and environmental risks related to QDs.

Various nanodiagnostics that have been reviewed will improve the sensitivity and extend the present limits of molecular diagnostics. Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It seems quite likely that there will be numerous applications of inorganic nanostructures in biology and medicine as biomarkers. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive, and more flexible when certain nanoscale particles are put to work as tags or labels. Nanoparticles are the most versatile material for developing diagnostics.

Nanomaterials can be assembled into massively parallel arrays at much higher densities than is achievable with current sensor array platforms and in a format compatible with current microfluidic systems. Currently, quantum dot technology is the most widely employed nanotechnology for diagnostic developments. Among the recently emerging technologies, cantilevers are the most promising. This technology complements and extends current DNA and protein microarray methods, because nanomechanical detection requires no labels, optical excitation, or external probes and is rapid, highly specific, sensitive, and portable. This will have applications in genomic analysis, proteomics and molecular diagnostics. Nanosensors are promising for detection of bioterrorism agents that are not detectable with current molecular diagnostic technologies, and some have already been developed.

Future Prospects of Nanodiagnostics

Within the next decade, measurement devices based on nanotechnology, which can make thousands of measurements very rapidly and very inexpensively, will become available. The most common clinical diagnostic application will be blood protein analysis. Blood in systemic circulation reflects the state of health or disease of most organs. Therefore, detection of blood molecular fingerprints will provide a sensitive assessment of health and disease. Another important area of application will be

cancer diagnostics. Molecular diagnosis of cancer including genetic profiling would be widely used by the year 2017. Nanobiotechnology would play an important part, not only in cancer diagnosis but also in linking diagnosis with treatment.

In the near future, nanodiagnosics would reduce the waiting time for the test results. For example, the patients with sexually transmitted diseases could give the urine sample when they first arrive at the outpatient clinic or physician's practice; the results could then be ready by the time they go in to see the doctor. They could then be given the prescription immediately, reducing the length of time worrying for the patient and making the whole process cheaper.

Future trends in diagnostics will continue in miniaturization of biochip technology to nanorange. The trend will be to build the diagnostic devices from bottom up starting with the smallest building blocks. Whether interest and application of nanomechanical detection will hold in the long range remains to be seen. Another trend is to move away from fluorescent labeling as miniaturization reduces the signal intensity, but there have been some improvements making fluorescent viable with nanoparticles.

Molecular electronics and nanoscale chemical sensors will enable the construction microscopic sensors capable of detecting patterns of chemicals in a fluid. Information from a large number of such devices flowing passively in the bloodstream allows estimates of the properties of tiny chemical sources in a macroscopic tissue volume. Such devices should be cabled to discriminate a single-cell-sized chemical source from the background chemical concentration *in vivo*, providing high-resolution sensing in both time and space. With currently used methods for blood analysis, such a chemical source would be difficult to distinguish from background when diluted throughout the blood volume and withdrawn as a blood sample.