Chapter 2
Nanotechnologies

Introduction

This chapter will focus on nanobiotechnologies that are relevant to applications in biomedical research, diagnostics, and medicine. Invention of the microscope revolutionized medicine by enabling the detection of microorganisms and study of histopathology of disease. Microsurgery was a considerable refinement over crude macrosurgery and opened the possibilities of procedure that were either not carried out previously or had high mortality and morbidity. Nanotechnologies, by opening up the world beyond microscale, will have a similar impact on medicine and surgery. Various nanobiotechnologies are described in detail in a special report on this topic (Jain 2012). Those relevant to understanding of diseases, diagnosis, and development of new drugs as well as management of diseases are described briefly in this chapter.

Classification of Nanobiotechnologies

It is not easy to classify the vast range of nanobiotechnologies. Some just represent motion on a nanoscale, but most of them are based on nanoscale structures, which come in a variety of shapes and sizes. A few occur in nature but most are engineered. The word nano is prefixed to just about anything that deals with nanoscale. It is not just biotechnology but many other disciplines such as nanophysics, nanobiology, etc. A simplified classification of basic nanobiotechnologies is shown in Table 2.1. Some technologies such as nanoarrays and nanochips are further developments.

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Table 2.1 Classification of basic nanomaterials and nanobiotechnologies

Nanoparticles
Fluorescent nanoparticles
Fullerenes
Gold nanoparticles
Lipoparticles
Magnetic nanoparticles
Nanocrystals
Nanoparticles assembly into micelles
Nanoshells
Paramagnetic and superparamagnetic nanoparticles
Polymer nanoparticles
Quantum dots
Silica nanoparticles
Nanofibers
Nanowires
Carbon nanofibers
Dendrimers
Polypropylenimine dendrimers
Composite nanostructures
Cochleates
DNA-nanoparticle conjugates
Nanoemulsions
Nanoliposomes
Nanocapsules enclosing other substances
Nanoshells
Nanovesicles
Nanoconduits
Nanotubes
Nanopipettes
Nanoneedles
Nanochannels
Nanopores
Nanofluidics
Nanostructured silicon
Nanoscale motion and manipulation at nanoscale
Cantilevers
Femtosecond laser systems
Nanomanipulation
Surface plasmon resonance
Visualization at nanoscale
Atomic force microscopy
Magnetic resonance force microscopy and nanoscale MRI
Multiple single-molecule fluorescence microscopy
Nanoparticle characterization by Halo™ LM10 technology
Nanoscale scanning electron microscopy
Near-field scanning optical microscopy
Optical imaging with a silver superlens
Partial wave spectroscopy
Photoactivated localization microscopy
Scanning probe microscopy
Super-resolution microscopy for in vivo cell imaging
Ultrananocrystalline diamond
Visualizing atoms with high-resolution transmission electron microscopy

Nanoparticles

Nanoparticles (NPs) form the bulk of nanomaterials. They can be made of different materials, e.g., gold. A NP contains tens to thousands of atoms and exists in a realm that straddles the quantum and the Newtonian. At those sizes, every particle has new properties that change depending on its size. As matter is shrunk to nanoscale, electronic and other properties change radically. NPs may contain unusual forms of structural disorder that can significantly modify materials properties and thus cannot solely be considered as small pieces of bulk material. Two NPs, both made of pure gold, can exhibit markedly different behavior – different melting temperature, different electrical conductivity, and different color – if one is larger than the other. That creates a new way to control the properties of materials. Instead of changing composition, one can change size. Some applications of nanoparticles take advantage of the fact that more surface area is exposed when material is broken down to smaller sizes. For magnetic NPs, the lack of blemishes produces magnetic fields remarkably strong considering the size of the particles. NPs are also so small that in most of them, the atoms line up in perfect crystals without a single blemish.

Zinc sulfide NPs a mere ten atoms across have a disordered crystal structure that puts them under constant strain, increasing the stiffness of the particles and probably affecting other properties, such as strength and elasticity. In similar semiconducting NPs, such as those made of cadmium selenide, slight differences in size lead to absorption and emission of different wavelengths of light, making them useful as fluorescent tracers. The dominant cause of such properties is quantum mechanical confinement of the electrons in a small package. But the disordered crystal structure now found in nanoparticles could affect light absorption and emission also. X-ray diffraction of single nanoparticles is not yet possible, and other methods are used to analyze X-ray diffraction images of nanoparticles so as to separate the effects of size from those of disordered structure.

It is beyond the scope of this handbook to describe all of the NPs. A few selected NPs relevant to nanomedicine are described briefly.

Gold Nanoparticles

Mass spectrometry analysis has determined the formula of gold nanocrystal molecules to be Au333(SR)79 (Qian et al. 2012). This metallic nanocrystal molecule exhibits fcc-crystallinity and surface plasmon resonance (SPR) at approximately 520 nm. Simulations have revealed that atomic shell closing largely contributes to the particular robustness of Au333(SR)79, albeit the number of free electrons is also consistent with electron shell closing based on calculations using a confined free electron model. This work clearly demonstrates that atomically precise nanocrystal molecules are achievable and that the factor of atomic shell closing contributes to their extraordinary stability compared to other sizes.

Ultrashort pulsed laser ablation in liquids represents a powerful tool for the generation of pure gold nanoparticles avoiding chemical precursors and thereby making them useful for biomedical applications. However, there is a concern that

their biochemical properties may change because of their properties of accepting electrons, which often adsorb onto the nanoparticles. A study has shown that cotransfection of plasmid DNA and laser-generated gold nanoparticles does not disturb the bioactivity of GFP-HMGB1 fusion protein – either uptake of the vector through the plasma membrane or protein accumulation in the nucleus (Petersen et al. 2009). Thus, laser-generated gold nanoparticles provide a good alternative to chemically synthesized nanoparticles for use in biomedical applications.

DNA molecules are attached to gold nanoparticles, which tangle with other specially designed pieces of DNA into clumps that appear blue. The presence of lead causes the connecting DNA to fall apart. That cuts loose the individual gold nanoparticles and changes the color from blue to red. Gold nanoparticles are also used as a connecting point to build biosensors for detection of disease. A common technique for a diagnostic test consists of an antibody attached to a fluorescent molecule. When the antibody attaches to a protein associated with the disease, the fluorescent molecule lights up under ultraviolet light. Instead of a fluorescent molecule, a gold nanoparticle can be attached to the antibody, and other molecules such as DNA can be added to the nanoparticle to produce bar codes. Because many copies of the antibodies and DNA can be attached to a single nanoparticle, this approach is much more sensitive and accurate than the fluorescent molecule tests used currently.

Cubosomes

Methods and compositions for producing lipid-based cubic phase nanoparticles were first discovered in the 1990s. Since then, a number of studies have described properties such as particle size, morphology, and stability of cubic phase dispersions, which can be tuned by composition and processing conditions. Stable particle dispersions with consistent size and structure can be produced by a simple processing scheme comprising a homogenization and heat treatment step. Because of their unique microstructure, they are biologically compatible and capable of controlled release of solubilized active ingredients such as drugs and proteins (Garg et al. 2007). As a drug delivery vehicle, high drug payloads, stabilization of peptides or proteins, and simple preparation process are also advantages of a cubosome (Wu et al. 2008). The ability of cubic phase to incorporate and control release of drugs of varying size and polar characteristics, and biodegradability of lipids make it a versatile drug delivery system for various routes of administration, including oral, topical (or mucosal), and intravenous. Furthermore, proteins in cubic phase appear to retain their native conformation and bioactivity and are protected against chemical and physical inactivation.

Fluorescent Nanoparticles

Microwave plasma technique has been used to develop fluorescent nanoparticles. In a second reaction, a layer of organic dye is deposited, and the final step is an outer cover of polymer, which protects the nanoparticles from exposure to environments.

Each layer has characteristic properties. The size of the particles varies, and these are being investigated for applications in molecular diagnostics. Fluorescent nanoparticles can also be used as labels for immunometric assays.

Switchable fluorescent silica nanoparticles have been prepared by covalently incorporating a fluorophore and a photochromic compound inside the particle core (May et al. 2012). The fluorescence can be switched reversibly between an on- and off-state via energy transfer. The particles were synthesized using different amounts of the photoswitchable compound (spiropyran) and the fluorophore (rhodamine B) in a size distribution between 98 and 140 nm and were characterized in terms of size, switching properties, and fluorescence efficiency by TEM, and UV\Vis and fluorescence spectroscopy.

Fullerenes

Fullerene technology derives from the discovery in 1985 of carbon-60, a molecule of 60 carbon atoms that form a hollow sphere one nanometer in diameter. The molecule was named buckyball or fullerene or buckminsterfullerene, because of its similarity to the geodesic dome designed by Buckminster Fuller. Subsequent studies have shown that fullerenes actually represent a family of related structures containing 20, 40, 60, 70, or 84 carbons. C60, however, is the most abundant member of this family. Fullerenes are entirely insoluble in water, but suitable functionalization makes the molecules soluble. Initial studies on water-soluble fullerene derivatives led to the discovery of the interaction of organic fullerenes with DNA, proteins, and living cells. Subsequent studies have revealed interesting biological activity aspects of organic fullerenes owing to their photochemistry, radical quenching, and hydrophobicity to form one- to three-dimensional supramolecular complexes. In these areas of research, synthetic organic chemistry has played an important role in the creation of tailor-made molecules.

Upon contact with water, under a variety of conditions, C60 spontaneously forms a stable aggregate with nanoscale dimensions (25–500 nm), termed "nano-C60," that is both soluble and toxic to bacteria (Fortner et al. 2005). This finding challenges conventional wisdom because buckyballs are notoriously insoluble by themselves, and most scientists had assumed they would remain insoluble in nature. The findings also raise questions about how the aggregates will interact with other particles and living things in natural ecosystems.

A method of application of C60 to cultured cells has been described that does not require water-solubilization techniques (Levi et al. 2006). Normal and malignant cells take up C60 and the inherent photoluminescence of C60 is detected within multiple cell lines. Treatment of cells with up to 200 mg/ml (200 ppm) of C60 does not alter morphology, cytoskeletal organization, and cell cycle dynamics nor does it inhibit cell proliferation. This study shows that pristine C60 is nontoxic to the cells and suggests that fullerene-based nanocarriers may be used for biomedical applications. Fullerenes have important applications in treatment of various diseases such as cancer and as an antioxidant neuroprotective for neurodegenerative disorders in addition to use as contrast agent for brain imaging (Miller et al. 2007).

Lipoparticles

Lipoparticles are nanometer-sized spheres surrounded by a lipid bilayer and embedded with conformationally intact integral membrane proteins. Interactions with integral membrane proteins have been particularly difficult to study because the proteins cannot be removed from the lipid membrane of a cell without disrupting the structure and function of the protein. The ability to solubilize integral membrane proteins has applications for microfluidics, biosensors, high-throughput screening, antibody development, and structural studies of complex receptors.

Nanoparticles Assembly into Micelles

Assembly of gold and silver nanoparticle building blocks into larger structures is based on a novel method that goes back to one of nature's oldest known chemical innovations, i.e., the self-assembly of lipid membranes that surround every living cell (Zubarev et al. 2006). The method makes use of the hydrophobic effect, a biochemical phenomenon that all living creatures use to create membranes, ultrathin barriers of fatty acids that form a strong, yet dynamic, sack around the cell, sealing it from the outside world. Cell membranes are one example of a micelle, a strong bilayer covering that is made of two sheets of lipid-based amphiphiles, molecules that have a hydrophilic end and a hydrophobic end. Like two pieces of cellophane tape being brought together, the hydrophobic sides of the amphiphilic sheets stick to one another, forming the bilayered micelle. All micelles form in three shapes: spheres, cylinders, and sack-like vesicles. By varying the length of the polystyrene arm, the solvents used, and the size of the gold particles, it is possible to form spheres and vesicles and vary the diameter of their cylinders, some of which grew to well over 1,000 nm in length. This method may enable creation of a wide variety of useful materials, including potent cancer drugs and more efficient catalysts for the chemical industry.

Nanoshells

Nanoshells are ball-shaped structures measuring ~100 nm and consist of a core of nonconducting glass that is covered by a metallic shell, which is typically gold or silver. Nanoshells possess highly favorable optical and chemical properties for biomedical imaging and therapeutic applications. These particles are also effective substrates for surface-enhanced Raman scattering (SERS) and are easily conjugated to antibodies and other biomolecules. By varying the relative the dimensions of the core and the shell, the optical resonance of these nanoparticles can be precisely and systematically varied over a broad region ranging from the near-UV to the mid-infrared. This range includes the NIR wavelength region where tissue transmissibility peaks, which forms the basis of absorbing nanoshells in NIR thermal therapy of tumors. In addition to spectral tunability, nanoshells offer other advantages over conventional organic dyes including improved optical properties

and reduced susceptibility to chemical/thermal denaturation. Furthermore, the same conjugation protocols used to bind biomolecules to gold colloid are easily modified for nanoshells. The core/shell ratio and overall size of a gold nanoshell influence its scattering and absorption properties.

Gold Nanoshells (Spectra Biosciences) possess physical properties similar to gold colloid, in particular a strong optical absorption due to the collective electronic response of the metal to light. The optical absorption of gold colloid yields a brilliant red color, which is very useful in consumer-related medical products such as home pregnancy tests. In contrast, the optical response of Gold Nanoshells depends dramatically on the relative sizes of the nanoparticle core and the thickness of the gold shell. Gold Nanoshells can be made either to absorb or scatter light preferentially by varying the size of the particle relative to the wavelength of the light at their optical resonance. Several potential biomedical applications of nanoshells are under development, including immunoassays, modulated drug delivery, photothermal cancer therapy, and imaging contrast agents.

Paramagnetic and Superparamagnetic Nanoparticles

Paramagnetic particles are important tools for cell sorting, protein separation, and single-molecule measurements. The particles used in these applications must meet the following requirements: uniform in size, highly paramagnetic, stable in physiological salt buffer, functionizable, and 100–1,000 nm in size. They have been used for the detection of model pathogens. Paramagnetic nanoparticles, which are linked to antibodies, enable highly specific biological cell separations.

Superparamagnetic iron oxide nanoparticles (SPION) with appropriate surface chemistry have been widely used experimentally for numerous in vivo applications such as magnetic resonance imaging (MRI) contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, hyperthermia, drug delivery, in cell separation, etc. These applications require that these nanoparticles have high magnetization values and size smaller than 100 nm with overall narrow particle size distribution, so that the particles have uniform physical and chemical properties. In addition, these applications need special surface coating of the magnetic particles, which has to not only be nontoxic and biocompatible but also allow a targetable delivery with particle localization in a specific area. Nature of surface coatings of the nanoparticles not only determines the overall size of the colloid but also plays a significant role in biokinetics and biodistribution of nanoparticles in the body. Magnetic nanoparticles can bind to drugs, proteins, enzymes, antibodies, or nucleotides and can be directed to an organ, tissue, or tumor using an external magnetic field or can be heated in alternating magnetic fields for use in hyperthermia. Magnetic labeling of cells provides the ability to monitor their temporal spatial migration in vivo by MRI. Various methods have been used to magnetically label cells using SPIONs. Magnetic tagging of stem cells and other mammalian cells has the potential for guiding future cell-based therapies in humans and for the evaluation of cell-based treatment effects in disease models.

Polymer Nanoparticles

Polymer nanoparticles, synthetic as well as biopolymers, are biocompatible, biodegradable, and nontoxic. They can be conjugated with other nanoparticles. Different types of polymer nanoparticles have been designed as drug delivery devices. Biodegradable polymeric nanoparticles are promising drug delivery devices because of their ability to deliver proteins, peptides, and genes as well as targeting therapeutics to specific organs/tissues. Although several synthetic polymers are available, natural polymers are still popular for drug delivery; these include acacia gum, chitosan, gelatin, and albumin. Examples of synthetic biodegradable polymers for controlled release drug delivery are polylactides (PLA), polyglycolides (PLG), and poly(lactide-co-glycolides) or PLGA.

Quantum Dots

Quantum dots (QDs) are nanoscale crystals of semiconductor material that glow or fluoresce when excited by a light source such as a laser. QD nanocrystals of cadmium selenide are 200–10,000 atoms wide and coated with zinc sulfide. The size of the QD determines the frequency of light emitted when irradiated with low energy light. The QDs were initially found to be unstable and difficult to use in solution. Multicolor optical coding for biological assays has been achieved by embedding different-sized QDs into polymeric microbeads at precisely controlled ratios. Their novel optical properties such as size-tunable emission and simultaneous excitation render these highly luminescent QDs ideal fluorophores for wavelength-and-intensity multiplexing. The use of ten intensity levels and six colors could theoretically code one million nucleic acid or protein sequences. Imaging and spectroscopic measurements indicate that the QD-tagged beads are highly uniform and reproducible, yielding bead identification accuracies as high as 99.99 % under favorable conditions. DNA hybridization studies demonstrate that the coding and target signals can be simultaneously read at the single-bead level. This spectral coding technology is expected to open new opportunities in gene expression studies, high-throughput screening, and medical diagnostics.

Latex beads filled with several colors of nanoscale semiconductor QDs can serve as unique labels for any number of different probes. When exposed to light, the beads identify themselves and their linked probes by emitting light in a distinct spectrum of colors – a sort of spectral bar code. The shape and size of QDs can be tailored to fluoresce with a specific color. Current dyes used for lighting up protein and DNA fade quickly, but QDs could allow tracking of biological reactions in living cells for days or longer.

QDs can also be placed in a strong magnetic field, which gives an electron on the dot two allowed energy states separated by an energy gap that depends on the strength of the field. The electron can jump the gap by absorbing a photon of precisely that energy, which can be tuned, by altering the field, to correspond with the energy of a far-infrared photon. Once it is excited by absorption of a photon, the electron can leap onto the terminal of a single-electron transistor, where it “throws the switch” and is detected.

Due to their sheer brightness and high photostability, QDs have the ability to act as molecular beacons. When attached to compounds or proteins of interest, QDs enable researchers to track movements within biological media or whole organisms, significantly impacting the way medical professionals study, diagnose, and treat diseases. Applications of QDs include the following:

- Life sciences research: tracking proteins in living cells
- Fluorescence detection: microscopy, biosensors, multicolor flow cytometry
- Molecular diagnostics
- Ex vivo live cell imaging
- In vivo targeting of cells, tissues, and tumors with monitoring by PET and MRI
- High-throughput screening
- Identification of lymph nodes in live animals by NIR emission during surgery

The new generations of QDs have far-reaching potential for the study of intracellular processes at the single-molecule level, high-resolution cellular imaging, long-term in vivo observation of cell trafficking, tumor targeting, and diagnostics. Best-known commercial preparation is Qdot™ (Life Technologies).

Silica Nanoparticles

In the case of silica, the formation of diatom shell or sponge spicule has attracted much attention in the last decade since it could provide key information to elaborate new hierarchically structured materials and nanodevices. The mineral phase is thought to be formed by the controlled assembly of nanoparticles generated in vivo from diluted precursor solutions, in the presence of biomolecular templates. Biomolecules present in silicifying organisms have been extracted and identified (Lopez et al. 2005). Silicon particles vary in size from 25 to 1,000 nm. Biomimetic approaches have led to the identification of several natural or synthetic molecules that are able to activate silica formation in conditions that closely resemble those found in the living organisms' intracellular compartments. Additionally, several of these systems are able to form silica nanoparticles whose size range and limited polydispersity reproduce colloidal biosilica. Extraction and characterization of biosilicifying molecules from living organisms, however, are still limited. Silicon nanoparticles have been used in drug delivery and gene therapy.

Bacterial Structures Relevant to Nanobiotechnology

Nanostructures Based on Bacterial Cell Surface Layers

Among the most commonly observed bacterial cell surface structures are monomolecular crystalline arrays of proteinaceous subunits termed S-layers, which are the simplest type of biological membrane developed during evolution. As an important component of the bacterial cell envelope, S-layers can fulfill various biological

Table 2.2 Applications of S-layers in nanobiotechnology

As a matrix for controlled immobilization of functional molecules
Binding of enzymes for bioanalytical biosensors
Immobilizing monoclonal antibodies for dipstick style immunoassays
Immobilizing antibodies for preparation of microparticles for ELISA
S-layers as carriers for conjugated vaccines
S-layer-coated liposomes
Immobilization of functional molecules on S-layer-coated liposomes
Entrapping of functional molecules for drug delivery
S-layer-coated liposomes with immobilized antigens and haptens for vaccines
Vehicles for producing fusion proteins
Vaccines
Biosensors
Diagnostics

functions and are usually the most abundantly expressed protein species in a cell (Pavkov-Keller et al. 2011). S-layer plays an important part in interactions of microbial cell with the environment. S-layers are generally 5–10 mm thick, and pores in the protein lattices are of identical size and morphology in the 2 to 8-nm range. S-layers have applications in nanobiotechnology as shown in Table 2.2.

Bacterial Magnetic Particles

Magnetic bacteria synthesize intracellular magnetosomes that impart a cellular swimming behavior referred to as magnetotaxis. The magnetic structures, magnetosomes, aligned in chains are postulated to function as biological compass needles allowing the bacterium to migrate along redox gradients through the Earth's geomagnetic field lines. Despite the discovery of this unique group of microorganisms several years ago, the mechanisms of magnetic crystal biomineralization have yet to be fully elucidated. A lipid bilayer membrane of approximately 2–4 nm in thickness encapsulates individual magnetosomes (50–100 nm in diameter). Magnetosomes are also referred to as bacterial magnetic particles (BMPs) to distinguish them from artificial magnetic particles (AMPs). The aggregation of BMPs can be easily dispersed in aqueous solutions compared with AMPs because of the enclosing organic membrane.

BMPs have potential applications in the interdisciplinary fields of nanobiotechnology, medicine, and environmental management. Through genetic engineering, functional proteins such as enzymes, antibodies, and receptors have been successfully displayed on BMPs, which have been utilized in various biosensors and bioseparation processes (Yoshino et al. 2010). The use of BMPs in immunoassays enables the separation of bound and free analytes by applying a magnetic field. Proteins can be attached covalently to solid supports such as BMPs that prevent desorption of antibodies during an assay. Large-scale production of functionally active antibodies or enzymes expressed on BMP membranes can be accomplished.

Carbon Nanotubes

Carbon nanotubes are rolled-up sheets of carbon atoms that appear naturally in soot, and are central to many nanotechnology projects. These nanotubes can go down in diameter to 1 nm, are stronger than any material in the universe, and can be of any length. These can be used as probes for AFMs that can image individual molecules in both wet and dry environments. This has enormous opportunities for application as conventional structure-based pharmaceutical design is hampered by the lack of high-resolution structural information for most protein-coupled receptors. It is possible to insert DNA into a carbon nanotube. Devices based on the DNA-nanotube combination could eventually be used to make electronics, molecular sensors, devices that sequence DNA electronically, and even gene delivery systems.

Medical Applications of Nanotubes

- Cyclic peptide nanotubes can act as a new type of antibiotic against bacterial pathogens.
- Cyclic peptide nanotubes can be used as artificial ion channels than open and close in response to electrical and chemical stimuli.
- It is easy to chemically functionalize the surfaces of template-synthesized nanotubes, and different functional groups can be attached to the inner versus outer surfaces of the tubes.
- Biomolecules, such as enzymes, antibodies, and DNA chains, can be attached to the nanotube surfaces to make biofunctionalized nanotubes.
- Template-synthesized nanotubes can be used as smart nanophase extraction agents, e.g., to remove drug molecules from solution.
- Template-synthesized nanotube membranes offer new approaches for doing bioseparations, e.g., of drug molecules.
- Nanoscale electromechanical systems (nanotweezers) based on carbon nanotubes have been developed for manipulation and interrogation of nanostructures within a cell.
- Carbon nanotubes can be used as tips for AFM.
- Lumen of a nanotube can carry payloads of drugs.
- Nanotubes can be used in biosensors.
- Blood-compatible carbon nanotubes, with heparin immobilized on the surface, are building blocks for in vivo nanodevices. Activated partial thromboplastin time and thromboelastography studies prove that heparinization can significantly enhance the blood compatibility of nanomaterials (Murugesan et al. 2006).

Studies of electrophoretic transport of ssRNA molecules through 1.5-nm-wide pores of carbon nanotube membranes reveal that RNA entry into the nanotube pores is controlled by conformational dynamics and exit by hydrophobic attachment of RNA bases to the pores. Differences in RNA conformational flexibility and hydrophobicity result in sequence-dependent rates of translocation, which is a prerequisite for nanoscale separation devices.

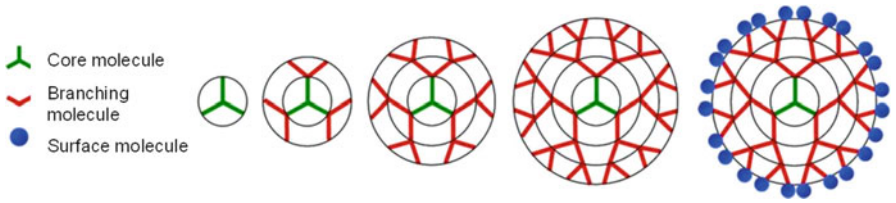

Fig. 2.1 The core, branching, and surface molecules of dendrimers (Source: Starpharma Holding Ltd., by permission)

The uptake of single-walled carbon nanotubes (SWCNTs) into cells appears to occur through phagocytosis. There are no adverse effects on the cells, and the nanotubes retained their unique optical properties suggesting that SWCNTs might be valuable biological imaging agents, in part because SWCNTs fluoresce in the NIR portion of the spectrum, at wavelengths not normally emitted by biological tissues. This may allow light from even a handful of nanotubes to be selectively detected in vivo. Although long-term studies on toxicity and biodistribution must be completed before nanotubes can be used in medical tests, but nanotubes are useful as imaging markers in laboratory in vitro studies, particularly in cases where the bleaching, toxicity, and degradation of more traditional markers are problematic.

Dendrimers

Dendrimers (dendri – tree, mer – branch) are a novel class of three-dimensional nanoscale, core-shell structures that can be precisely synthesized for a wide range of applications. Specialized chemistry techniques allow for precise control over the physical and chemical properties of the dendrimers. They are constructed generation by generation in a series of controlled steps that increase the number of small branching molecules around a central core molecule. Up to ten generations can be incorporated into a single dendrimer molecule. The final generation of molecules added to the growing structure makes up the polyvalent surface of the dendrimer (see Fig. 2.1). The core, branching, and surface molecules are chosen to give desired properties and functions.

As a result of their unique architecture and construction, dendrimers possess inherently valuable physical, chemical, and biological properties. These include:

- Precise architecture, size, and shape control. Dendrimers branch out in a highly predictable fashion to form amplified 3D structures with highly ordered architectures.
- High uniformity and purity. The proprietary stepwise synthetic process used produces dendrimers with highly uniform sizes (monodispersity) possessing precisely defined surface functionality and very low impurity levels.

- High loading capacity. Internal cavities intrinsic to dendrimer structures can be used to carry and store a wide range of metals, organic or inorganic molecules.
- High shear resistance. Through their 3D structure, dendrimers have a high resistance to shear forces and solution conditions.
- Low toxicity. Most dendrimer systems display very low cytotoxicity levels.
- Low immunogenicity when injected or used topically.

Properties

The surface properties of dendrimers may be manipulated by the use of appropriate "capping" reagents on the outermost generation. In this way, dendrimers can be readily decorated to yield a novel range of functional properties. These include:

- Polyvalency – The outer shell of each dendrimer can be manipulated to contain a large number of reactive groups. Each of these reactive sites has the potential to interact with a target entity, often resulting in polyvalent interactions.
- Flexible charge and solubility properties – Through use of appropriate capping groups on the dendrimer exterior, the charge and solubility of dendrimers can be readily manipulated.
- Flexible binding properties – By using appropriate capping groups on the dendrimer exterior, dendrimers can be designed to exhibit strong affinity for specific targets.
- Transfection – Dendrimers are able to move through cell boundaries and transport genetic materials into cell interiors.

Applications

Dendrimers, with their highly customizable properties, are basic building blocks with the promise of enabling specific nanostructures to be built to meet existing needs and solve evolving problems. Dendrimer research and development is currently making an impact on a broad range of fields as shown by exponential growth in the number of dendrimer-based publications. While the potential applications of dendrimers are unlimited, some of their current uses relevant to nanomedicine are shown in Table 2.3.

Advances in understanding of the role of molecular weight and architecture on the in vivo behavior of dendrimers, together with recent progress in the design of biodegradable chemistries, have enabled the application of dendrimers as antiviral drugs, tissue repair scaffolds, targeted carriers of chemotherapeutics, and optical oxygen sensors. Before such products can reach the market, however, the field must not only address the cost of manufacture and quality control of pharmaceutical-grade materials but also assess the long-term human and environmental health consequences of dendrimer exposure in vivo.

Table 2.3 Potential applications of dendrimers in nanomedicine

Diagnostics
Sensors
Imaging contrast agents
Drug delivery
Improved delivery of existing drugs
Improved solubility of existing drugs
Drug development
Polyvalent dendrimers interacting simultaneously with multiple drug targets
Development of new pharmaceuticals with novel activities
Improving pharmacological activity of existing drugs
Improving bioavailability of existing drugs
Medicine and surgery
Prevention of scar tissue formation after surgery

DNA Octahedron

DNA octahedron is a single strand of DNA that spontaneously folds into a highly rigid, nanoscale octahedron that is several million times smaller than the length of a standard ruler and about the size of several other common biological structures, such as a small virus or a cellular ribosome (Shih et al. 2004). The octahedron consists of 12 edges, 6 vertices, and 8 triangular faces. The structure is about 22 nm in diameter. Making the octahedron from a single strand was a breakthrough. Because of this, the structure can be amplified with the standard tools of molecular biology and can easily be cloned, replicated, amplified, evolved, and adapted for various applications. This process also has the potential to be scaled up so that large amounts of uniform DNA nanomaterials can be produced. These octahedra are potential building blocks for new tools for basic biomedical science. With these we have biological control, and not just synthetic chemical control, over the production of rigid, wire frame DNA objects.

Potential Applications

Because all 12 edges of the octahedral structures have unique sequences, they are versatile molecular building blocks that could potentially be used to self-assemble complex higher-order structures. Possible applications include using these octahedra as artificial compartments into which proteins or other molecules could be inserted, something like a virus in reverse – DNA is on the outside and proteins on the inside. In nature, viruses are self-assembling nanostructures that typically have proteins on the outside and DNA or RNA on the inside. The DNA octahedra could possibly form scaffolds that host proteins for the purposes of X-ray crystallography, which depends on growing well-ordered crystals, composed of arrays of molecules.

Nanowires

The manipulation of photons in structures smaller than the wavelength of light is central to the development of nanoscale integrated photonic systems for computing, communications, and sensing. Assembly of small groups of freestanding, chemically synthesized nanoribbons and nanowires into model structures illustrates how light is exchanged between subwavelength cavities made of three different semiconductors. With simple coupling schemes, lasing nanowires can launch coherent pulses of light through ribbon waveguides that are up to a millimeter in length. Also, interwire coupling losses are low enough to allow light to propagate across several right-angle bends in a grid of crossed ribbons. Nanoribbons function efficiently as waveguides in liquid media and provide a unique means for probing molecules in solution or in proximity to the waveguide surface. These results lay the groundwork for photonic devices based on assemblies of active and passive nanowire elements. There are potential applications of nanowire waveguides in microfluidics and biology. Some nanowire-based nanobiosensors are in development.

Polymer Nanofibers

Polymer nanofibers, with diameters in the nanometer range, possess larger surface areas per unit mass and permit easier addition of surface functionalities compared with polymer microfibers. Research on polymer nanofibers, nanofiber mats, and their applications has seen a remarkable growth over the last few years. Among various methods of manufacture, electrospinning has been used to convert a large variety of polymers into nanofibers and may be the only process that has the potential for mass production. Although measurement of mechanical properties such as tensile modulus, strength, and elongation is difficult because of the small diameters of the fibers, these properties are crucial for the proper use of nanofiber mats. Owing to their high surface area, functionalized polymer nanofibers will find broad applications as drug delivery carriers, biosensors, and molecular filtration membranes.

Nanopores

Nanopores are tiny structures that occur in the cell in nature for specific functions. At the molecular level, specific shapes are created that enable specific chemical tasks to be completed. For example, some toxic proteins such as alpha-hemolysin can embed themselves into cell membranes and induce lethal permeability changes there due to its central pore. The translocation of polymers across nanometer scale apertures in cell membranes is a common phenomenon in biological systems. The first proposed application was DNA sequencing by measuring the size of nanopore,

application of an electric potential across the membrane, and waiting for DNA to migrate through the pore to enable one to measure the difference between bases in the sequence (see Chap. 3). Protein engineering has been applied to ion channels and pores, and protein as well as nonprotein can be constructed. Potential applications of engineered nanopores are:

- Tools in basic cell biology
- Molecular diagnostics: sequencing
- Drug delivery
- Cryoprotection and desiccation of cells
- Components of nanodevices and nanomachines
- Nanomedicine

Nanoporous Silica Aerogel

Nanoporous silica aerogels have been used in nanotechnology devices such as aerogel nanoporous insulation blankets. Silica aerogel substrate enables stable formation of lipid bilayers that are expected to mimic real cell membranes. Typical bilayers are 5 nm in thickness, and the silica beads in aerogel are approximately 10–25 nm in diameter. Silica aerogels have a unique structure and chemistry that allow for the transformation of nanosized liposomes into continuous, surface-spanning lipid bilayers. These lipid bilayers adsorb to the aerogel surface and exhibit the characteristic lateral mobility of real cell membranes. The high (98 %) porosity of aerogel substrates creates an underlying "water well" embedded in the aerogel pore structure that allows these membrane molecules to carry out normal biological activities including transport across the membrane. This porosity could potentially accommodate the movement of membrane proteins or other membrane-extruding molecules.

This aerogel is an improvement over conventional substrates for synthetic biomembranes as it is porous, thus minimizing nonphysiological interaction between membrane proteins and a hard substrate surface. This prevents the proteins from becoming immobilized, denatured, and eventually losing their biological functions. Applications of lipid bilayers are:

- Model biological membranes for research.
- Biosensors and lab-on-chip devices (microfluidic systems, analyte detector, etc.).
- Bio-actuating devices.
- Arrays for use in screening arrays of compounds for membrane-associated drug targets. Lipid bilayer system has been used in immunological screening for drug targets.
- Display libraries of compounds.
- Patterned lipid bilayers can be used for tissue culture and engineering (micropatterns of lipid membranes direct discriminative attachment or growth of living cells).

Advantages of aerogel biomembrane are:

- Best able to mimic the lateral mobility of molecules in real cell membranes.
- Enable membrane transport studies due to liquid permeability of aerogels.
- Both sides of supported membranes are accessible compared to only one side in conventional solid support.
- Can be used to design functional membranes for different applications by incorporating organic, inorganic, polymeric, and/or biologically active components into the aerogel structures.
- Nonphysiological interaction of the membrane-associated components with the underlying support (compared to glass).
- Membranes on the aerogel maintain stability for weeks.

Nanostructured Silicon

Silicon has been used for implants in the human body for several years. Following nanostructuring, silicon can be rendered biocompatible and biodegradable. BioSilicon™ (pSiMedica Ltd.) contains nanosized pores measuring 100 nm. The "silicon skeleton" between the pores comprises tens of silicon atoms in width. Initial applications are in drug delivery. The kinetics of drug release from BioSilicon™ can be controlled by adjusting the physical properties of the matrix, including modifying the pore size. Other potential applications include nanospheres for targeted systemic and pulmonary drug delivery. Nanostructured silicon, as multilayered mirrors, can be used for subcutaneous implants for diagnostics. Nanostructures can be used as prostheses to improve adhesion to bone tissue.

Polymer Nanofibers

Polymer nanofibers, with diameters in the nanometer range, possess larger surface areas per unit mass and permit easier addition of surface functionalities compared with polymer microfibers. Research on polymer nanofibers, nanofiber mats, and their applications has seen a remarkable growth over the last few years. Among various methods of manufacture, electrospinning has been used to convert a large variety of polymers into nanofibers and may be the only process that has the potential for mass production. Although measurement of mechanical properties such as tensile modulus, strength, and elongation is difficult because of the small diameters of the fibers, these properties are crucial for the proper use of nanofiber mats. Owing to their high surface area, functionalized polymer nanofibers will find broad applications as drug delivery carriers, biosensors, and molecular filtration membranes in future.

Nanoparticle Conjugates

DNA-Nanoparticle Conjugates

DNA-DNA hybridization has been exploited in the assembly of nanostructures including biosensors and DNA scaffolds. Many of these applications involve the use of DNA oligonucleotides tethered to gold nanoparticles or nanoparticles may be hybridized with one another. Two types of DNA-nanoparticle conjugates have been developed for these purposes. Both types entail the coupling of oligonucleotides through terminal thiol groups to colloidal gold particles. In one case, the oligonucleotides form the entire monolayer coating the particles, whereas in the other case, the oligonucleotides are incorporated in a phosphine monolayer, and particles containing discrete numbers of oligonucleotides are separated by gel electrophoresis. A minimal length of 50 residues is required, both for separation by electrophoresis and hybridization with complementary DNA sequences. These limitations of shorter oligonucleotides are attributed to interaction between the DNA and the gold. In a new technique, glutathione monolayer-protected gold clusters were reacted with 19- or 20-residue thiolated oligonucleotides, and the resulting DNA-nanoparticle conjugates could be separated on the basis of the number of bound oligonucleotides by gel electrophoresis and assembled with one another by DNA-DNA hybridization (Ackerson et al. 2005). This approach overcomes previous limitations of DNA-nanoparticle synthesis and yields conjugates that are precisely defined with respect to both gold and nucleic acid content.

Networks of Gold Nanoparticles and Bacteriophage

Biological molecular assemblies are excellent models for the development of nanoengineered systems with desirable biomedical properties. A spontaneous biologically active molecular network has been fabricated that consists of bacteriophage (phage) directly assembled with gold (Au) nanoparticles and termed Au-phage (Souza et al. 2006). When the phage is engineered so that each phage particle displays a peptide, such networks preserve the cell surface receptor binding and internalization attributes of the displayed peptide. The spontaneous organization of these targeted networks can be manipulated further by incorporation of imidazole (Au-phage-imid), which induces changes in fractal structure and near-infrared optical properties. The networks can be used as labels for enhanced fluorescence and dark-field microscopy, surface-enhanced Raman scattering detection, and near-infrared photon-to-heat conversion. Together, the physical and biological features within these targeted networks offer convenient multifunctional integration within a single entity with potential for nanotechnology-based biomedical applications such as biological sensors and cell-targeting agents. This genetically programmable nanoparticle with a biologically compatible metal acts as a nanoshuttle that can target specific locations in the body. For example, it could potentially locate damaged areas on arteries that have been caused by heart disease, and then deliver stem cells to the

site that can build new blood vessels. It may be able to locate specific tumors, which could then be treated by either heating the gold particles with laser light and/or using the nanoparticles to selectively deliver a drug to destroy the cancer.

Protein-Nanoparticle Combination

Proteins come in many handy shapes and sizes, which make them major players in biological systems. Chaperonins are ring-shaped proteins found in all living organisms where they play an essential role in stabilizing proteins and facilitating protein folding. A chaperonin can be adapted for technological applications by coaxing it to combine with individual luminescent semiconductor nanoparticles. In bacteria, this chaperonin protein takes in and refolds denatured proteins in order to return them to their original useful shapes. This ability would make the proteins good candidates for drug carriers.

Cadmium sulfite nanoparticles emit light as long as they are isolated from each other; encasing the nanoparticles in the protein keeps the tiny particles apart. The biological fuel molecule ATP releases the nanoparticles from the protein tubes, freeing the particles to clump together, which quenches the light. The protein-nanoparticle combination could be used to detect ATP. This blend of nanotechnology and molecular biology could lead to new bioresponsive electronic nanodevices and biosensors very different from the artificial molecular systems currently available. By adding selective binding sites to the solvent-exposed regions of the chaperonin, the protein-nanoparticle bioconjugate becomes a sensor for specific targets (Xie et al. 2009).

Nanomaterials for Biolabeling

Nanomaterials are suitable for biolabeling. Nanoparticles usually form the core in nanobiomaterials. However, in order to interact with biological target, a biological or molecular coating or layer acting as an interface needs to be attached to the nanoparticle. Coatings that make the nanoparticles biocompatible include antibodies, biopolymers, or monolayers of small molecules. A nanobiomaterial may be in the form of nanovesicle surrounded by a membrane or a layer. The shape is more often spherical but cylindrical, platelike, and other shapes are possible. The size and size distribution might be important in some cases, e.g., if penetration through a pore structure of a cellular membrane is required. The size is critical when quantum-sized effects are used to control material properties. A tight control of the average particle size and a narrow distribution of sizes allow creating very efficient fluorescent probes that emit narrow light in a very wide range of wavelengths. This helps with creating biomarkers with many and well-distinguished colors. The core itself might have several layers and be multifunctional. For example, combining magnetic and luminescent layers one can both detect and manipulate the particles.

The core particle is often protected by several monolayers of inert material, e.g., silica. Organic molecules that are adsorbed or chemisorbed on the surface of the particle are also used for this purpose. The same layer might act as a biocompatible material. However, more often an additional layer of linker molecules is required that has reactive groups at both ends. One group is aimed at attaching the linker to the nanoparticle surface and the other is used to bind various biocompatible substances such as antibodies depending on the function required by the application.

Efforts are being made to improve the performance of immunoassays and immunosensors by incorporating different kinds of nanostructures. Most of the studies focus on artificial, particulate marker systems, both organic and inorganic. Inorganic nanoparticle labels based on noble metals, semiconductor QDs, and nanoshells appear to be the most versatile systems for these bioanalytical applications of nanophotonics. The underlying detection procedures are more commonly based on optical techniques. These include nanoparticle applications generating signals as diverse as static and time-resolved luminescence, one- and two-photon absorption, Raman and Rayleigh scattering as well as surface plasmon resonance, and others. All efforts are aimed at achieving one or more of the following goals:

- Lowering of detection limits (if possible, down to single-molecule level)
- Parallel integration of multiple signals (multiplexing)
- Signal amplification by several orders of magnitude

Potential benefits of using nanoparticles and nanodevices include an expanded range of label multiplexing. Different types of fluorescent nanoparticles and other nanostructures have been promoted as alternatives for the fluorescent organic dyes that are traditionally used in biotechnology. These include QDs, dye-doped polymer, and silica nanoparticles (Dosev et al. 2008). Various nanomaterials for biolabeling are shown in Table 2.4.

DNA Nanotags

Bright fluorescent dye molecules can be integrated with DNA nanostructure to make nanosized fluorescent labels – DNA nanotags, which improve the sensitivity for fluorescence-based imaging and medical diagnostics. DNA nanotags are useful for detecting rare cancer cells within tissue biopsies. In addition, they offer the opportunity to perform multicolor experiments. This feature is extremely useful for imaging applications because the multiple colors can be seen simultaneously, requiring only one experiment using one laser and one fluorescence-imaging machine. Fluorescent DNA nanotags have been used in a rolling circle amplification immunoassay based as a versatile fluorescence assay platform for highly sensitive protein detection (Xue et al. 2012).

Table 2.4 Nanomaterials for biolabeling

Label/reporter	Characteristics	Function/applications
Dendrimer/silver nanocomposites	Water-soluble, biocompatible, fluorescent, and 3–7-nm diameter stable nanoparticles	Biomarkers for in vitro cell labeling
Electrogenerated chemiluminescence	Tris(2,2′-bipyridyl)ruthenium(II) molecular labels	Nanoscale bioassay
Europium(III)-chelate-doped nanoparticles	Combined with selection of high-affinity monoclonal antibodies coated on label particles and microtitration wells	The sensitivity for virus particle detection is improved compared to immunofluorometric assays
Fluorescent color-changing dyes	3-hydroxychromone derivatives that exhibit two fluorescence bands as a result of excited-state intramolecular proton transfer reaction	Biosensors
Fluorescent lanthanide nanorods	Retain their fluorescent properties after internalization into cells	Multiplexed imaging of molecular targets in living cells
Luminescent core/shell nanohybrid	Luminescent rare earth ions in a nanosized Gd2O3 core (3.5 nm) and FITC molecules entrapped within in a polysiloxane shell (2.5–10 nm)	Two different luminescence emissions: (1) FITC under standard illumination and (2) Tb^{3+} under high-energy source giving highly photostable luminescence
Magnetic nanotags (MNTs)	Alternative to fluorescent labels in biomolecular detection assays	Multiplex protein detection of cancer biomarkers at low concentrations
Nanogold® labels (Nanoprobes Inc.)	Unlike nanogold particles, gold labels are uncharged molecules, which are cross-linked to specific sites on biomolecules	Nanogold® labels have a range and versatility, which is not available with colloidal nanogold particles
Nanophosphors	Nanophosphors contain embedded lanthanide ions, like europium or terbium	Nanophosphor signals hardly fade and can also be used for multiplex testing
Plasmon-resonant nanoparticles	Scatter light with tremendous efficiency	Ultrabright nanosized labels for biological applications, replacing other labeling methods such as fluorescence
QD end-labeling	Multicolor fluorescence microscopy using conjugated QDs	Detection of single DNA molecules
SERS (Surface-enhanced Raman Scattering)-based nanotags	A metal nanoparticle where each type of tag exploits the Raman spectrum of a different small molecule, and SERS bands are 1/50th the width of fluorescent bands	Enables greater multiplexed analyte quantification than other fluorescence-based quantitation tags

Fluorescent Lanthanide Nanorods

Inorganic fluorescent lanthanide (europium and terbium) orthophosphate nanorods can be used as a novel fluorescent label in cell biology. These nanorods, synthesized by the microwave technique, retain their fluorescent properties after internalization into human umbilical vein endothelial cells or renal carcinoma cells. The cellular internalization of these nanorods and their fluorescence properties have been characterized by fluorescence spectroscopy, differential interference contrast microscopy, confocal microscopy, and transmission electron microscopy. At concentrations up to 50 ug/ml, the use of 3H-thymidine incorporation assays, apoptosis assays, and trypan blue exclusion demonstrates the nontoxic nature of these nanorods, a major advantage over traditional organic dyes (Patra et al. 2006). These nanorods can be used for the detection of cancer at an early stage, and functionalized nanorods are potential vehicles for drug delivery.

Magnetic Nanotags

Magnetic nanotags (MNTs) are a promising alternative to fluorescent labels in biomolecular detection assays, because minute quantities of MNTs can be detected with inexpensive sensors. Probe sensors are functionalized with capture antibodies specific to the chosen analyte. During analyte incubation, the probe sensors capture a fraction of the analyte molecules. A biotinylated linker antibody is subsequently incubated and binds to the captured analyte, providing binding sites for the streptavidin-coated MNTs, which are incubated further. The nanotag-binding signal, which saturates at an analyte concentration-dependent level, is used to quantify the analyte concentration. However, translation of this technique into easy-to-use and multiplexed protein assays, which are highly sought after in molecular diagnostics such as cancer diagnosis and personalized medicine, has been challenging. Multiplex protein detection of potential cancer biomarkers has been demonstrated at subpicomolar concentration levels (Osterfeld et al. 2008). With the addition of nanotag amplification, the analytic sensitivity extends into the low femtomolar concentration range. The multianalyte ability, sensitivity, scalability, and ease of use of the MNT-based protein assay technology make it a strong contender for versatile and portable molecular diagnostics in both research and clinical settings.

Molecular Computational Identification

Molecular computational identification, based on molecular logic and computation, has been applied on nanoscale. Examples of populations that need encoding in the microscopic world are cells in diagnostics or beads in combinatorial chemistry. Taking advantage of the small size (about 1 nm) and large “on/off” output ratios of

molecular logic gates and using the great variety of logic types, input chemical combinations, switching thresholds, and even gate arrays in addition to colors, unique identifiers have been produced for small polymer beads (about 100 μm) used for synthesis of combinatorial libraries. Many millions of distinguishable tags become available. This method should be extensible to far smaller objects, with the only requirement being a "wash and watch" protocol. The basis of this approach is converting molecular science into technology concerning analog sensors and digital logic devices. The integration of molecular logic gates into small arrays has been a growth area in recent years (de Silva 2011).

Nanophosphor Labels

Nanostructures based on inorganic phosphors (nanophosphors) are a new emerging class of materials with unique properties that make them very attractive for labeling. The molecular lattice of phosphors contains individual embedded lanthanide ions, like europium or terbium. The crystal lattice or sometimes "activator ions" – such as cerium ions used especially for this purpose – absorbs the stimulating light and transfers the energy to the lanthanide ions, which are the true source of fluorescence. The color emitted depends mainly on the lanthanide ions used. Terbium, e.g., gives off a yellowish green color, while europium produces a red fluorescence. As shown by the "microparticles" in fluorescent lights, the cycle of stimulation and emission can be endlessly repeated, which means that the dye never fades.

Bayer scientists are developing nanophosphors, which many of the advantages of QDs and fewer disadvantages such as high cost and heavy metals content that may not be environmentally friendly. Nanophosphor signals hardly fade and can also be used for multiplex testing. And the major advantage they have over QDs is that the wavelength of their emitted light does not depend on particle size but on the type of lanthanide ions used. For this reason, their particle size, which is also no more than 10 nm, does not need to be monitored so precisely. As a result, the manufacturing process is simpler and less expensive. Moreover, most ions of lanthanides, also called rare earths, are considered less harmful to the environment, and this facilitates their manufacture and disposal.

Background fluorescence from biological components of cells makes it difficult to interpret the signal, e.g., the positive result of a diagnostic test for cancer. Nanophosphors are able to get round this problem because for many types of nanophosphor, the life span of the fluorescence, i.e., the time between stimulation and emission, extends to several milliseconds. Accordingly, when the nanophosphor is exposed to a brief impulse of light, the background fluorescence disappears before the test result is displayed. This considerably enhances the sensitivity of the fluorescent marker in its various applications. Another important advantage of the nanophosphor system, particularly where medical diagnostics are concerned, is its ability to transfer fluorescent energy to a closely related dye. This allows biochemical reactions, like the coupling between antibodies, to be detected without the need

for any additional procedures. So the relevant antibodies in the patient's sample can be detected immediately after the dye has been added to the test solution.

Before the nanophosphors can be used to track down certain segments of DNA, e.g., in cancer tests, they themselves need to be attached to suitable DNA segments. It is always a major challenge to achieve stable coupling of small organic molecules or larger biomolecules with unique, inorganic nanoparticles. The particles have to be painstakingly adapted to the properties of the organic molecules and prevented from lumping together themselves in the process. If this can be done successfully, it will meet the demanding challenges of medical diagnostics in the future.

Photoluminescence imaging in vitro and in vivo has been shown by use of near-infrared to near-infrared (NIR-to-NIR) upconversion in nanophosphors. This NIR-to-NIR upconversion process provides deeper light penetration into biological specimen and results in high-contrast optical imaging due to absence of an autofluorescence background and decreased light scattering. Fluoride nanocrystals (20–30 nm size) codoped with the rare earth ions, Tm^{3+} and Yb^{3+}, have been synthesized and characterized by TEM, XRD, and photoluminescence spectroscopy (Nyk et al. 2009). In vitro cellular uptake was demonstrated with no apparent cytotoxicity. Subsequent animal imaging studies were performed using Balb-c mice injected intravenously with upconverting nanophosphors, demonstrating the high-contrast PL imaging in vivo by photoluminescence spectroscopy. Lanthanide-doped nanocrystals have also been used for imaging of cells and some deep tissues in animals. Polyethylenimine (PEI)-coated $NaYF^4$:Yb,Er nanoparticles produce very strong upconversion fluorescence when excited at 980 nm by an NIR laser, which is resistant to photobleaching and nontoxic to bone marrow stem cells (Chatterjee et al. 2008). The nanoparticles delivered into some cell lines or injected intradermally and intramuscularly into some tissues either near the body surface or deep in the body of rats showed visible fluorescence, when exposed to a 980-nm NIR laser.

Organic Nanoparticles as Biolabels

The use of organic nonpolymeric nanoparticles as biolabels was not considered to be promising or have any advantage over established metallic or polymeric probes. Problems include quenching of fluorescence in organic dye crystals, colloidal stability, and solubility in aqueous environments, but some of these can be circumvented. Labels have been constructed by milling and suspending a fluorogenic hydrophobic precursor, fluorescein diacetate, in sodium dodecyl sulfate (SDS). Thus, a negative surface charge is introduced, rendering the particles (500 nm) colloidally stable and minimizing leakage of fluorescein diacetate molecules into surrounding water. Now it has been shown that the polyelectrolyte multilayer architecture is not vital for the operability of this assay format. Instead of SDS and multilayers, the adsorption of only one layer of an amphiphilic polymeric detergent, e.g., an alkylated poly(ethyleneimine), is sufficient to stabilize the system and to provide an interface for the antibody attachment. This is the basis of a technology

"ImmunoSuperNova®" (invented by 8sens.biognostic AG, Germany). In this the reaction of the analyte molecule with the capture antibody is followed by an incubation step with the antibody-nanoparticle conjugate, which serves as detector. After some washing steps, an organic release solvent is added, dissolving the particle and converting fluorescein diacetate into fluorescein.

Quantum Dots as Labels

The unique optical properties of QDs make them appealing as in vivo and in vitro fluorophores in a variety of biological investigations, in which traditional fluorescent labels based on organic molecules fall short of providing long-term stability and simultaneous detection of multiple signals. The ability to make QDs water soluble and target them to specific biomolecules has led to promising applications in cellular labeling, deep-tissue imaging, assay labeling, and as efficient fluorescence resonance energy transfer donors.

DNA molecules attached to QD surface can be detected by fluorescence microscopy. The position and orientation of individual DNA molecules can be inferred with good efficiency from the QD fluorescence signals alone. This is achieved by selecting QD pairs that have the distance and direction expected for the combed DNA molecules. Direct observation of single DNA molecules in the absence of DNA-staining agents opens new possibilities in the study of DNA-protein interactions. This approach can be applied for the use of QDs for nucleic acid detection and analysis. CdSe QDs can also be used as labels for sensitive immunoassay of cancer biomarker proteins by electrogenerated chemiluminescence. This strategy has been successfully used as a simple, cost-effective, specific, and potential method to detect α-fetoprotein in practical samples (Liu et al. 2011a). In contrast to a QD that is selectively introduced as a label, an integrated QD is one that is present in a system throughout a bioanalysis, and has a simultaneous role in transduction and as a scaffold for biorecognition. The modulation of QD luminescence provides the opportunity for the transduction of these events via fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), charge transfer quenching, and electrochemiluminescence (Algar et al. 2010).

SERS Nanotags

Surface-enhanced Raman scattering (SERS) nanotags (Oxonica Inc.) are silica-coated gold nanoparticles that are active at the glass-metal interface, and are optically detectable tags. Each type of tag exploits the Raman spectrum of a different small molecule, and SERS bands are 1/50th the width of fluorescent bands. These enable a greater degree of multiplexing than current fluorescence-based quantitation tags. The spectral intensity of SERS-based tags is linearly proportional to the number of particles allowing them to be used for multiplexed analyte quantification.

Because they are coated with glass, attachment to biomolecules is straightforward. They can be detected with low-cost instrumentation. The particles can be interrogated in the near-IR, enabling detection in blood and other tissues. Another advantage of these particles is that they are stable and are resistant to photodegradation. Nanoplex biotags are capable of measuring up to 20 biomarkers in a single test without interference from biological matrices such as whole blood. SERS nanotags are also useful for point-of-care diagnostics. There is a great potential for multiplexed imaging in living subjects in cases in which several targeted SERS probes could offer better detection of multiple biomarkers associated with a specific disease (Zavaleta et al. 2009). The primary limitations of Raman imaging in humans are those also faced by other optical techniques, in particular limited tissue penetration. Over the last several years, Raman spectroscopy imaging has advanced significantly, and many critical proof-of-principle experiments have been successfully carried out. It is expected that imaging with Raman spectroscopy will continue to be a dynamic research field over the next decade (Zhang et al. 2010).

Silica Nanoparticles for Labeling Antibodies

Luminescent silicon dioxide nanoparticles with size of 50 nm containing rhodamine (R-SiO2) have been synthesized by sol-gel method. These particles can emit intense and stable room temperature phosphorescence signals. An immune reaction between goat-antihuman IgG antibody labeled with R-SiO2 and human IgG has been demonstrated on polyamide membrane quantitatively, and the phosphorescence intensity was enhanced after the immunoreactions (Jia-Ming et al. 2005). This is the basis of a room temperature phosphorescence immunoassay for the determination of human IgG using an antibody labeled with the nanoparticles containing binary luminescent molecules. This method is sensitive, accurate, and precise. Lissamine rhodamine B sulfonyl chloride and other dyes can be covalently bound to and contained in spherical silica nanoparticles (30–80 nm). Compared to organic molecular markers, these fluorophore hybrid silica particles exhibit superior photostability and detection sensitivity, e.g., for detecting trace levels of hepatitis B surface. Dye-doped fluorescent silica nanoparticles are highly efficient labels for glycans and applied to detect bacteria by imaging as well as to study carbohydrate-lectin interactions on a lectin microarray (Wang et al. 2011a).

Silver Nanoparticle Labels

Silver (Ag) nanoparticles have unique plasmon-resonant optical scattering properties that are useful for nanomedical applications as signal enhancers, optical sensors, and biomarkers. Sensitive electrochemical DNA hybridization detection assay uses Ag nanoparticles as oligonucleotide labeling tags. The assay relies on the hybridization of the target DNA with the Ag nanoparticle-oligonucleotide DNA

probe, followed by the release of the Ag metal atoms anchored on the hybrids by oxidative metal dissolution and the indirect determination of the solubilized Ag ions by anodic stripping voltammetry. Liquid electrode plasma-atomic emission spectrometry requires no plasma gas and no high-power source, which makes it suitable for onsite portable analysis, and can be used for protein-sensing studies employing Ag nanoparticle labeling. Human chorionic gonadotropin (hCG) was used as a model target protein, and the immunoreaction in which hCG is sandwiched between two antibodies, one of which is immobilized on the microwell and the second is labeled with Ag nanoparticles, was performed (Tung et al. 2012). hCG was analyzed in the range from 10 pg/mL to 1 ng/mL. This detection method has a wide variety of promising applications in metal-nanoparticle-labeled biomolecule detection.

Micro- and Nanoelectromechanical Systems

The rapid pace of miniaturization in the semiconductor industry has resulted in faster and more powerful computing and instrumentation, which have begun to revolutionize medical diagnosis and therapy. Some of the instrumentation used for nanotechnology is an extension of MEMS (microelectromechanical systems), which refers to a key enabling technology used to produce microfabricated sensors and systems. The potential mass application of MEMS lies in the fact that miniature components are batch fabricated using the manufacturing techniques developed in the semiconductor microelectronics industry enabling miniaturized, low-cost, high-performance, and integrated electromechanical systems. The "science of miniaturization" is a much more appropriate name than MEMS, and it involves a good understanding of scaling laws and different manufacturing methods and materials that are being applied in nanotechnology.

MEMS devices currently range in size from one to hundreds of micrometers and can be as simple as the singly supported cantilever beams used in AFM or as complicated as a video projector with thousands of electronically controllable microscopic mirrors. NEMS devices exist correspondingly in the nanometer realm – nanoelectromechanical systems (NEMS). The concept of using externally controllable MEMS devices to measure and manipulate biological matter (BioMEMS) on the cellular and subcellular levels has attracted much attention recently. This is because initial work has shown the ability to detect single base pair mismatches of DNA and to quantifiably detect antigens using cantilever systems. In addition is the ability to controllably grab and manipulate individual cells and subsequently release them unharmed.

Surface nanomachining combines the processing methods of MEMS with the tools of electron beam nanofabrication to create 3D nanostructures that move (and thus can do new types of things). Ultrashort pulsed laser radiation, e.g., using femtolasers, is an effective tool for controlled material processing and surface nano/micromodification because of minimal thermal and mechanical damage. Surface nanomachining has potential applications in nanobiotechnology.

BioMEMS

Because BioMEMS involves the interface of MEMS with biological environments, the biological components are crucially important. To date, they have mainly been nucleic acids, antibodies, and receptors that are involved in passive aspects of detection and measurement. These molecules retain their biological activity following chemical attachment to the surfaces of MEMS structures (most commonly, thiol groups to gold), and their interactions are monitored through mechanical (deflection of a cantilever), electrical (change in voltage or current in the sensor), or optical (surface plasmon resonance) measurements. The biological components are in the nanometer range or smaller; therefore, the size of these systems is limited by the minimum feature sizes achievable using the fabrication techniques of the inorganic structures, currently 100 nm–1 μm. Commercially available products resulting from further miniaturization could be problematic because of the expanding cost and complexity of optical lithography equipment and the inherent slowness of electron beam techniques. In addition to size limitations, the effects of friction have plagued multiple moving parts in inorganic MEMS, limiting device speeds and useful lifetimes.

Microarrays and Nanoarrays

Arrays consist of orderly arrangements of samples, which, in the case of biochips, may be cDNAs, oligonucleotides, or even proteins. Macroarraying (or gridding) is a macroscopic scheme of organizing colonies or DNA into arrays on large nylon filters ready for screening by hybridization. In microarrays, however, the sample spot sizes are usually less than 200 μm in diameter and require microscopic analysis. Microarrays have sample or ligand molecules (e.g., antibodies) at fixed locations on the chip, while microfluidics involves the transport of material, samples, and/or reagents on the chip.

Microarrays provide a powerful way to analyze the expression of thousands of genes simultaneously. Genomic arrays are an important tool in medical diagnostic and pharmaceutical research. They have an impact on all phases of the drug discovery process from target identification through differential gene expression, identification, and screening of small molecule candidates to toxicogenomic studies for drug safety. To meet the increasing needs, the density and information content of the microarrays is being improved. One approach is fabrication of chips with smaller, more closely packed features – ultrahigh density arrays, which will yield:

- High information content by reduction of feature size from 200 μm to 50 nm
- Reduction in sample size
- Improved assay sensitivity

Nanoarrays are the next stage in the evolution of miniaturization of microarrays. Whereas microarrays are prepared by robotic spotting or optical lithography, limiting

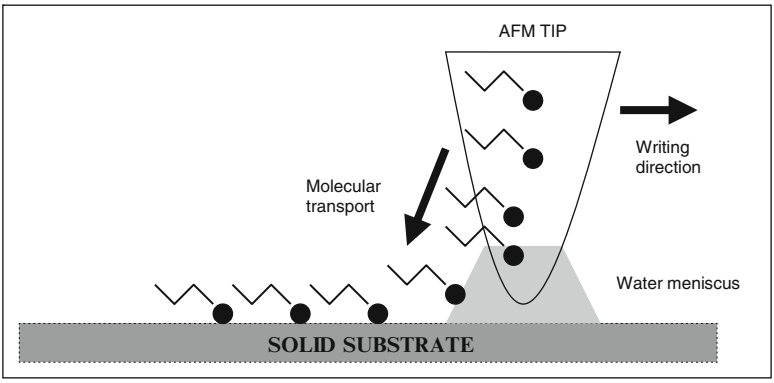

Fig. 2.2 Schematic representation of Dip Pen Nanolithography (*DPN*). A water meniscus forms between the atomic force microscope (*AFM*) tip coated with oligonucleotide (*ODN*) and the Au substrate. The size of the meniscus, which is controlled by relative humidity, affects the ODN transport rate, the effective tip-substrate contact area, and DPN resolution (Modified from Piner et al. 1999. © Jain PharmaBiotech)

the smallest size to several microns, nanoarrays require further developments in lithography strategies. Technologies available include the following:

- Electron beam lithography
- Dip Pen Nanolithography
- Scanning probe lithography
- Finely focused ion beam lithography
- Nanoimprint lithography

Dip Pen Nanolithography for Nanoarrays

Dip Pen Nanolithography™ (DPN™) commercialized by NanoInk Inc. employs the tip of an AFM to write molecular "inks" directly on a surface. Biomolecules such as proteins and viruses can be positioned on surfaces to form nanoarrays that retain their biological activity. DPN is schematically depicted in Fig. 2.2.

Advantages of DPN are as follows:

Ultrahigh resolution. DPN is capable of producing structures with line widths of less than 15 nm. This is compared to photolithography, which supports features of no less than 65-nm line width, and slower e-beam and laser lithography systems, which support features of 15-nm line width.

Flexibility. Direct fabrication is possible with many substances, from biomolecules to metals.

Accuracy. By leveraging existing highly accurate atomic force microscopy technology, DPN utilizes the best possible means for determining exactly where features are being placed on the substrate. This allows for the integration

of multiple component nanostructures and for immediate inspection and characterization of fabricated structures.

Low capital cost. Techniques such as e-beam lithography that approach DPN-scale resolution are considerably more expensive to purchase, operate, and maintain.

Ease of use. DPN systems may be operated by nonspecialized personnel with minimal training. Further, DPN may be performed under normal ambient laboratory conditions with humidity control.

Speed. 100-nm spots can be deposited with a single DPN pen in less than a second. DPN can be used to fabricate arrays of a single molecule with more than 100,000 spots over 100×100 μm in less than an hour.

Applications of Dip Pen Nanolithography

Multiple allergen testing for high throughput and high sensitivity requires the development of miniaturized immunoassays that can be performed with minute amounts of test analyte that are usually available. Construction of such miniaturized biochips containing arrays of test allergens needs application of a technique able to deposit molecules at high resolution and speed while preserving its functionality. DPN is an ideal technique to create such biologically active surfaces, and it has already been successfully applied for the direct, nanoscale deposition of functional proteins, as well as for the fabrication of biochemical templates for selective adsorption. It has potential applications for detection of allergen-specific immunoglobulin E (IgE) antibodies and for mast cell activation profiling (Sekula-Neuner et al. 2012).

Protein Nanoarrays

High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic biomarkers in complex samples. This capability can be enhanced by nanotechnology. DPN technique has been extended to protein arrays with features as small as 45 nm, and immunoproteins as well as enzymes can be deposited. Selective binding of antibodies to protein nanoarrays can be detected without the use of labels by monitoring small (5–15 nm) topographical height increases in AFM images.

Miniaturized microarrays, “mesoarrays,” created by DPN with protein spots 400× smaller by area compared to conventional microarrays, were used to probe the ERK2-KSR binding event of the Ras/Raf/MEK/ERK signaling pathway at a physical scale below that previously reported (Thompson et al. 2011). This study serves as a first step toward an approach that can be used for analysis of proteins at a concentration level comparable to that found in the cellular environment.

Single-Molecule Protein Arrays

The ability to control the placement of individual protein molecules on surfaces could enable advances in many areas ranging from the development of nanoscale biomolecular devices to fundamental studies in cell biology. An approach that combines scanning probe block copolymer lithography with site-selective immobilization strategies has been used to create arrays of proteins down to the single-molecule level with arbitrary pattern control (Chai et al. 2011). Scanning probe block copolymer lithography was used to synthesize individual sub-10-nm single crystal gold nanoparticles to act as scaffolds for the adsorption of functionalized alkylthiol monolayers for facilitating the immobilization of specific proteins. The number of protein molecules that adsorb onto the nanoparticles depends on particle size; when the particle size approaches the dimensions of a protein molecule, each particle can support a single protein. This was demonstrated with both gold nanoparticle and QD labeling coupled with TEM imaging. The immobilized proteins remain bioactive, as demonstrated by enzymatic assays and antigen-antibody binding experiments.

Microfluidics and Nanofluidics

Microfluidics is the handling and dealing with small quantities (e.g., microliters, nanoliters, or even picoliters) of fluids flowing in channels the size of a human hair (~50 μm thick) even narrower. Fluids in this environment show very different properties than in the macroworld. This new field of technology was enabled by advances in microfabrication – the etching of silicon to create very small features. Microfluidics is one of the most important innovations of biochip technology. Typical dimensions of microfluidic chips are 1–50 cm^2 and have channels 5–100 μm. Usual volumes are 0.01–10 μl but can be less. Microfluidics is the link between microarrays and nanoarrays as we reduce the dimensions and volumes.

Microfluidics is the underlying principle of lab-on-a-chip devices, which carry out complex analyses, while reducing sample and chemical consumption, decreasing waste and improving precision and efficiency. The idea is to be able to squirt a very small sample into the chip, push a button and the chip will do all the work, delivering a report at the end. Microfluidics allows the reduction in size with a corresponding increase in the throughput of handling, processing, and analyzing the sample. Other advantages of microfluidics include increased reaction rates, enhanced detection sensitivity, and control of adverse events.

Drawbacks and limitations of microfluidics and designing of microfluidic chips include the following:

- Difficulties in microfluidic connections.
- Because of laminar flows, mixing can only be performed by diffusion.
- Large capillary forces.
- Clogging.
- Possible evaporation and drying up of the sample.

Applications of microfluidics include the following:

- DNA analysis
- Protein analysis
- Gene expression and differential display analysis
- Biochemical analysis

Nanotechnology on a Chip

Nanotechnology on a chip is a new paradigm for total chemical analysis systems. The ability to make chemical and biological information much cheaper and easier to obtain is expected to fundamentally change healthcare, food safety, and law enforcement. Lab-on-a-chip technology involves micro-total analysis systems that are distinguished from simple sensors because they conduct a complete analysis; a raw mixture of chemicals goes in and an answer comes out. Sandia National Laboratories is developing a handheld lab-on-a-chip that will analyze for airborne chemical warfare agents and liquid-based explosives agents. This development project brings together an interdisciplinary team with areas of expertise including microfabrication, chemical sensing, microfluidics, and bioinformatics. Although nanotechnology plays an important role in current efforts, miniaturized versions of conventional architecture and components such as valves, pipes, pumps, and separation columns are patterned after their macroscopic counterparts. Nanotechnology will provide the ability to build materials with switchable molecular functions that could provide completely new approaches to valves, pumps, chemical separations, and detection. For example, fluid streams could be directed by controlling surface energy without the need for a predetermined architecture of physical channels. Switchable molecular membranes and the like could replace mechanical valves. By eliminating the need for complex fluidic networks and microscale components used in current approaches, a fundamentally new approach will allow greater function in much smaller, lower-power total chemical analysis systems.

A new scheme for the detection of molecular interactions based on optical readout of nanoparticle labels has been developed. Capture DNA probes can be arrayed on a glass chip and incubated with nanoparticle-labeled target DNA probes, containing a complementary sequence. Binding are monitored by optical means, using reflected and transmitted light for the detection of surface-bound nanoparticles. Control experiments' significant influence of nonspecific binding on the observed contrast can be excluded. Distribution of nanoparticles on the chip surface can be demonstrated by scanning force microscopy.

BioForce Nanosciences has taken the technology of the microarray to the next level by creating the "nanoarray," an ultraminiaturized version of the traditional microarray that can actually measure interactions between individual molecules down to resolutions of as little as one nanometer. Here, 400 nanoarray spots can be placed in the same area as a traditional microarray spot. Nanoarrays are the next

evolutionary step in the miniaturization of bioaffinity tests for proteins, nucleic acids, and receptor-ligand pairs. On a BioForce NanoArrayT, as many as 1,500 different samples can be queried in the same area now needed for just one domain on a traditional microarray.

Microfluidic Chips for Nanoliter Volumes

Nanoliter implies extreme reduction in quantity of fluid analyte in a microchip. The use of the word "nano" in nanoliter (nL) is in a different dimension than in nanoparticle, which is in nanometer (nm) scale. Chemical compounds within individual nanoliter droplets of fluid can be microarrayed on to glass slides at 400 spots/cm^2. Using aerosol deposition, subsequent reagents and water can be metered into each reaction center to rapidly assemble diverse multicomponent reactions without cross contamination or the need for surface linkage. Such techniques enable the kinetic profiling of protease mixtures, protease-substrate interactions, and high-throughput screening reactions. From one printing run that consumes <1 nanomole of each compound, large combinatorial libraries can be subjected to numerous separation-free homogeneous assays at volumes much smaller than current high-throughput methods. The rapid assembly of thousands of nanoliter reactions per slide using a small biological sample represents a new functional proteomics format implemented with standard microarraying and spot-analysis tools.

Use of Nanotechnology in Microfluidics

Construction of Nanofluidic Channels

Techniques such as nanoimprinting are used to construct large arrays of nanoscale grooves with efficiency and speed. Such grooves can be sealed with similar ease, to form nanofluidic channels. Laser-assisted direct imprint techniques enable the construction of millions of enclosed nanofluidic channels side by side on a single substrate, which is ideal for such parallel processing. By sputter-depositing silicon dioxide at an angle onto an array of prefabricated nanochannels imprinted into the surface of a biopolymer substrate, not only is an effective and uniform seal formed over the entire array, but the channels are further narrowed down to 10 nm, from an initial width of 55 nm. This process could be easily used for narrowing and sealing micro- and nanofluidic structures formed by other patterning techniques. By minimizing the hollow space in such structures, it could help surpass existing limitations in the spatial resolution of these techniques.

A chip-scale maze for combing out strands of DNA and inserting them into nanoscale channels was made using standard, inexpensive lithographic techniques. Their "gradient nanostructure" might be used to isolate and stretch DNA molecules for analysis, e.g., to look for bound proteins such as transcription factors along the

strand. Such molecules would be obscured in normal solution because DNA, like any other linear polymer, collapses into a random coil as a featureless blob. The strands can, in principle, be straightened by feeding them into channels just a few tens of nanometers wide, using nanofluidic techniques.

Restriction mapping with endonucleases is a central method in molecular biology. Restriction mapping of DNA molecules can be performed using restriction endonucleases in nanochannels with diameters of 100–200 nm. It is based on the measurement of fragment lengths after digestion, while possibly maintaining the respective order. The location of the restriction reaction within the device is controlled by electrophoresis and diffusion of Mg^{2+} and EDTA. It is possible to measure the positions of restriction sites with precision using single DNA molecules with a resolution of 1.5 kbp within 1 min.

A review of nanofluidic systems reveals that these are divided into two large categories: top-down and bottom-up methods. The technology in the region of 1–10 nm is lacking and potentially can be covered by using the pulsed laser deposition method as a controlled way for thin film deposition (thickness of a few nanometers) and further structuring by the top-down method.

The benefits of operating in the nanoliter space include reduction of solvent, waste disposal costs, and human exposure by factors of 1,000×. New routine liquid handling capabilities include a purported 10× increase in MALDI sensitivity for analysis of proteins in proteomics work as demonstrated by various products such as nanoliter syringes based on induction-based fluidics technology, which uses electric fields to launch liquids to targets.

Nanoscale Flow Visualization

Most of the microscale flow visualization methods evolved from methods developed originally for macroscale flow. It is unlikely, however, that developed microscale flow visualization methods will be translated to nanoscale flows in a similar manner. Resolving nanoscale features with visible light presents a fundamental challenge. Although point-detection scanning methods have the potential to increase the flow measurement resolution on the microscale, spatial resolution is ultimately limited by the optical probe volume (length scale on the order of 100 nm), which, in turn, is limited by the wavelength of light employed. Optical spatially resolved flow measurements in nanochannels are difficult to visualize. There is a need for refinement of microscale flow visualization methods and the development of direct flow measurement methods for nanoflows.

Moving (Levitation) of Nanofluidic Drops with Physical Forces

The manipulation of droplets/particles that are isolated (levitated in gas/vacuum) from laboratory samples containing chemicals, cells, and bacteria or viruses is important both for basic research in physics, chemistry, biology, biochemistry, and colloidal science and for applications in nanotechnology and microfluidics. Various optical, electrostatic, electromagnetic, and acoustic methods are used for levitation.

Microfluidic drops can be moved with light – the lotus effect. On a super rough surface, when light shines on one side of a drop, the surface changes, the molecules switch, and the drop moves. This technology has potential applications in drug screening as it can be used for quickly analyzing and screening small amounts of biological materials. Called digital microfluidics, this approach enables one to quickly move small drops around by shining light on them. Hundreds of screens could be done on only one particular surface. The molecules, e.g., protein traces, do not interfere with movements of the drops because the surfaces are hydrophobic and the molecules have little contact with the surface.

The size of diamagnetic levitation devices can be reduced by using micron-scale permanent magnets to create a magnetic micromanipulation chip, which operates with femtodroplets levitated in air. The droplets used are one billion times smaller in volume than has been demonstrated by conventional methods. The levitated particles can be positioned with up to 300 nm accuracy and precisely rotated and assembled. Using this lab-on-a-chip, it might be possible to do the same thing with a large number of fluids, chemicals, and even red blood cells, bacteria, and viruses.

Electrochemical Nanofluid Injection

The ability to manipulate ultrasmall volumes of liquids is required in such diverse fields as cell biology, microfluidics, capillary chromatography, and nanolithography. In cell biology, it is often necessary to inject materials of high molecular weight such as DNA and proteins into living cells because their membranes are impermeable to such molecules. Currently used techniques for microinjection are limited by the relatively large injector size and poor control of the amount of injected material. An electrochemical attosyringe has been devised for electrochemical control of the fluid motion that enables one to sample and dispense attoliter-to-picoliter (10^{-18} to 10^{-12} l) volumes of either aqueous or nonaqueous solutions (Laforge et al. 2007). By changing the voltage applied across the liquid/liquid interface, one can produce a sufficient force to draw solution inside a nanopipette and then inject it into an immobilized biological cell. A high success rate was achieved in injections of fluorescent dyes into human cells in culture. The injection of femtoliter-range volumes can be monitored by video microscopy, and current/resistance-based approaches can be used to control injections from very small pipettes. Other potential applications of the electrochemical syringe include fluid dispensing in nanolithography and pumping in nanofluidic systems.

Nanofluidics on Nanopatterned Surfaces

A very thin layer of liquid behaves on a “nanopatterned” silicon surface, i.e., a surface etched with an ordered array of cavities, each only 20 nm deep. Watching how a liquid adsorbs on a nanopatterned surface is one way to study the basic properties of liquids that are confined in extremely tiny amounts within nanoscale structures. Understanding these properties will help in developing many useful fluid-based nanotechnologies. This work could help improve the “lab-on-a-chip.”

Currently, the knowledge about the microscopic behavior of liquids on solid surfaces, known as "wetting" phenomena, is predominately based on measurements taken using structureless, flat surfaces. In those cases, the behavior of the liquid is based on the strength of attractive molecule-molecule forces known as "van der Waals interactions." But for a surface that contains a regular pattern of cavities, the shape of the surface influences how the liquid will fill those cavities. Analysis of the X-ray data reveals that a liquid layer builds up inside each nanocavity at a faster rate than on a flat surface of the same material. The wetting properties of the surface are considerably enhanced by the nanopatterning.

Nano-interface in a Microfluidic Chip

There are emerging experimental and conceptual platforms for probing living cells with nanotechnology-based tools in a microfluidic chip. Considerable advances have been made in measuring nanoscale mechanical, biochemical, and electrical interactions at the interface between biomaterials and living cells. By merging the fields of microfluidics, electrokinetics, and cell biology, microchips are capable of creating tiny, mobile laboratories. The challenge for the future of designing a nano-interface in a microfluidic chip to probe a living cell lies in seamlessly integrating techniques into a robust and versatile, yet reliable, platform. Potential benefits of nanosystems on a microchip result from real-time detection of numerous events in parallel. In addition to early detection of cell-level dysfunctions, these systems will enable broad screening that encompasses not just a large number of toxic stimuli and disease processes but also population subgroups. This will facilitate the development of personalized medicine. To reach this goal requires advancing the knowledge base of cellular and subcellular functions, perhaps by designing nanosystems that operate in the tissue milieu.

Nanofluidic Channels for Study of DNA

Nanofluidic channels enable molecular biologists to spot the association and dissociation of proteins on fluorescently labeled DNA. The simple system could even help researchers visualize induced tertiary structures such as loops, which push conventional optical or magnetic stretching methods to the limit. This silicon dioxide-glass nanochannel system, also referred to as nanoslit, requires no externally applied forces or fields (Krishnan et al. 2007). To unravel the molecules, scientists place a drop of solution containing DNA at one end of the nanochannel. Capillary action then draws the liquid into channels measuring 2–10 μm wide and 100 nm deep. After 1 min, a drop of buffer solution is added at the other end of the channel to equalize the pressure in the device and stop the flow. In channels of 100 nm depth or less, DNA molecules spontaneously adopt an extended state adjacent to the channel wall. The nanochannel geometry, however, physically confines polymer molecules to two spatial dimensions. Further reduction in configuration results in spontaneous axial stretching of molecules and appears to be electrostatically mediated. The physics for stretching a DNA molecule is built into the structure

of the device. Fabrication of the channels and mass production of the unit are easy. Devices are made by first patterning a silicon substrate using laser lithography and then forming parallel channels 100 nm deep by either reactive ion etching in plasma or wet etching in HF. Cover glass is used to seal the channels from above.

Visualization and Manipulation on Nanoscale

4Pi Microscope

The most prominent restrictions of fluorescence microscopy are the limited resolution and the finite signal. Established conventional, confocal, and multiphoton microscopes resolve at best approximately 200 nm in the focal plane and only 500 nm in depth.

4Pi microscope (Leica Microsystems) uses a special phase- and wavefront-corrected double-objective imaging system linked to a confocal scanner to enable four- to sevenfold increased axial resolution over confocal and two-photon microscope. Even in living specimens, axial sections of ~100 nm are obtained. The system maintains all advantages of fast scanning, Acousto-Optical Beam Splitting (AOBS®), and spectral detection of the Leica TCS SP2 AOBS for routine operation. The first marked leap in resolution in commercial 3D fluorescence microscopy opens up new dimensions for research in cell and developmental biology. Colocalization studies of immunolabeled microtubules and mitochondria demonstrate the feasibility of 4Pi microscopy for routine biological measurements, in particular, to visualize the 3D entanglement of the two networks with unprecedented detail (Medda et al. 2006).

Atomic Force Microscopy

Basic AFM Operation

In its most basic form, atomic force microscopy (AFM) images topography by precisely scanning a probe across the sample to "feel" the contours of the surface. The interaction between the needle and the surface is measured, and an image is reconstructed from the data collected in this manner. With AFM, it is possible to reach an extremely high resolution. Because it can be applied under standard conditions in an aqueous environment, any significant perturbation of the sample can be avoided. In contrast to light microscopy and scanning electron microscopy, AFM provides the most optimal means to investigate the surface structures in three dimensions, with resolutions as high as 0.1–0.2 nm.

A key element of the AFM is its microscopic force sensor, or cantilever. The cantilever is usually formed by one or more beams of silicon or silicon nitride that is 100–500 μm long and about 0.5–5 μm thick. Mounted on the end of the cantilever

is a sharp tip that is used to sense a force between the sample and tip. For normal topographic imaging, the probe tip is brought into continuous or intermittent contact with the sample and raster-scanned over the surface.

Advantages of AFM

In addition to its superior resolution and routine three-dimensional measurement capability, AFM offers several other clear advantages over traditional microscopy techniques. For example, scanning and transmission electron microscopy (SEM, TEM) image biologically inactive, dehydrated samples and generally require extensive sample preparation such as staining or metal coating. AFM eliminates these requirements and, in many cases, allows direct observation of native specimens and ongoing processes under native or near-native conditions.

Further adding to its uniqueness, the AFM can directly measure nanoscale interactive forces, e.g., ligand-receptor binding. Samples can be examined in ambient air or biological fluids without the cost and inconvenience of vacuum equipment. Sample preparation is minimal and allows the use of standard techniques for optical microscopy. The MultiMode AFM provides maximal resolution, while the BioScope AFM integrates the best of optical and atomic force microscopy to help life scientists explore new frontiers.

The ability of the AFM to create 3D micrographs with resolution down to the nanometer and Angstrom scales has made it an essential tool for imaging surfaces in applications ranging from semiconductor processing to cell biology. In addition to this topographical imaging, however, the AFM can also probe nanomechanical and other fundamental properties of sample surfaces, including their local adhesive or elastic (compliance) properties.

Microscopic adhesion affects a huge variety of events, from the behavior of paints and glues, ceramics, and composite materials to DNA replication and the action of drugs in the human body. Elastic properties are similarly important, often affecting the structural and dynamic behavior of systems from composite materials to blood cells. AFM offers a new tool to study these important parameters on the micron to nanometer scale using a technique that measures forces on the AFM probe tip as it approaches and retracts from a surface.

Force Sensing Integrated Readout and Active Tip

Force sensing integrated readout and active tip (FIRAT) is an extremely sensitive AFM technology that is capable of high-speed imaging 100 times faster than current AFM technology. Current AFM scans surfaces with a thin cantilever with a sharp tip at the end. An optical beam is bounced off the cantilever tip to measure the deflection of the cantilever as the sharp tip moves over the surface and interacts with the material being analyzed. FIRAT works a bit like a cross between a pogo stick and a microphone. In one version of the probe, the membrane with a sharp tip moves toward the sample, and just before it touches, it is pulled by attractive forces.

Much like a microphone diaphragm picks up sound vibrations, the FIRAT membrane starts taking sensory readings well before it touches the sample. And when the tip hits the surface, the elasticity and stiffness of the surface determine how hard the material pushes back against the tip. So rather than just capturing a topography scan of the sample, FIRAT can pick up a wide variety of other material properties.

FIRAT can capture additional measurements not possible before with AFM, including parallel molecular assays for drug screening and discovery, as well as material property imaging. This research breakthrough could prove invaluable for many types of nanoresearch, including translating into movies of molecular interactions in real time. FIRAT might eventually replace AFM.

AFM as Nanorobot

An AFM-based nanorobot has been introduced for biological studies (Xi et al. 2011). Using the AFM tip as an end effector, the AFM can be modified into a nanorobot that can manipulate biological objects at the single-molecule level. By functionalizing the AFM tip with specific antibodies, the nanorobot is able to identify specific types of receptors on the cell membrane. It is similar to the fluorescent optical microscopy but with higher resolution. By locally updating the AFM image based on interaction force information and objects' model during nanomanipulation, real-time visual feedback is obtained through the augmented reality interface. The development of the AFM-based nanorobotic system enables us to conduct in situ imaging, sensing, and manipulation simultaneously at the nanometer scale (e.g., protein and DNA levels). The AFM-based nanorobotic system offers several advantages and capabilities for studying structure-function relationships of biological specimens. As a result, many biomedical applications can be achieved by the AFM-based nanorobotic system.

Cantilever Technology

Cantilevers (Concentris) transform a chemical reaction into a mechanical motion on the nanometer scale. Measurements of a cantilever are length 500 μm, width 100 μm, thickness 25–500 μm, and deflection 10 nm. This motion can be measured directly by deflecting a light beam from the cantilever surface. Concentris uses an array of parallel VCSELs (vertical cavity surface emitting lasers) as stable, robust, and proven light source. A state-of-the-art position sensitive detector is employed as detection device.

The static mode is used to obtain information regarding the presence of certain target molecules in the sample substance. The surface stress caused by the adsorption of these molecules results in minute deflections of the cantilever. This deflection directly correlates with the concentration of the target substance. The dynamic mode allows quantitative analysis of mass loads in the subpicogram area.

Table 2.5 Applications of cantilever technology

Basic research
Study of chemical reactions or host-guest interactions on surfaces
Nanocalorimetry
Medical diagnostics
Parallel and label-free detection of disease markers, e.g., serum proteins or autoantibodies
Fast, label-free recognition of specific DNA sequences (SNPs, oncogenes, genotyping)
Detection of microorganisms and antimicrobial susceptibility
Drug discovery and life sciences research
Label-free biochemical assays and investigation of biomolecular interactions
Multiplexed assays
Process and quality control
Process monitoring
Purity analysis
Food analysis
Detection of trace contaminations, e.g., antibiotics, hormones, and pesticides
Detection of microorganisms
Identification and quality control
Environmental monitoring
Detection of heavy metal ions, pesticides, and air pollutants
Water analysis
Fragrance and flavor analysis
Using neural networks to analyze cantilever sensor array signals can identify and characterize complex chemical mixtures (“electronic nose” or “tongue”)
Security devices
Detection of potentially hazardous chemicals and microorganisms
Workplace security

Source: Concentris GmbH

As molecules get adsorbed, minimal shifts in the resonance frequency of an oscillating cantilever can be measured and associated to reference data of the target substance. Both modes can also be operated simultaneously.

The controlled deposition of functional layers is the key to converting nanomechanical cantilevers into chemical or biochemical sensors. Inkjet printing is a rapid and general method to coat cantilever arrays efficiently with various sensor layers (Bietsch et al. 2004). Self-assembled monolayers of alkanethiols are deposited on selected Au-coated cantilevers and rendered them sensitive to ion concentrations or pH in liquids. The detection of gene fragments is achieved with cantilever sensors coated with thiol-linked single-stranded DNA oligomers on Au. A selective etch protocol proves the uniformity of the monolayer coatings at a microscopic level. A chemical gas sensor is fabricated by printing thin layers of different polymers from dilute solutions onto cantilevers. The inkjet method is easy to use, faster, and more versatile than coating via microcapillaries or the use of pipettes. In addition, it is scalable to large arrays and can coat arbitrary structures in noncontact.

The applications of cantilever technology, Cantosens (Concentris), are listed in Table 2.5 and discussed further in Chap. 3.

Further research continues at academic laboratories to develop nanoscale cantilevers, which would be smaller than the wavelength of light and make laser detection more difficult. When these devices are developed, they could be incorporated into AFMs, which are currently designed for standard size cantilevers.

AFM cantilevers have been actuated using a microheater at the bottom and integrated with deflection sensor as well as microactuator for imaging of soft biological samples in fluid (Fantner et al. 2009). Influence of the water was investigated on the cantilever dynamics, the actuation and the sensing mechanisms, as well as the crosstalk between sensing and actuation. Successful imaging of yeast cells in water using the integrated sensor and actuator shows the potential of the combination of this actuation and sensing method. This constitutes a major step toward the automation and miniaturization required to establish AFM in routine biomedical diagnostics and in vivo applications.

CytoViva® Microscope System

Specifically designed to support research in nanotechnology and infectious disease, the CytoViva Microscope System (CytoViva Inc.) employs a proprietary dark-field-based optical illumination technology, which dramatically improves contrast and signal-to-noise ratio. This transmitted-light illumination system enables scientists to observe a wide range of nanomaterials quickly and easily, without any special preparation. In addition, live cells and pathogens can also be viewed at a level of detail not possible with traditional optical imaging techniques such as phase contrast or differential interference contrast. When using the CytoViva Dual Mode Fluorescence system, researchers can also observe the interactions between fluorescently labeled nanoparticles or bacteria and live unlabeled cells. This unique capability can eliminate the need to create computer-enhanced overlay images, which require two different illumination methods and advanced software programs. Finally, when combined with the CytoViva Hyperspectral Imaging system, this high-contrast microscopy method enables researchers to secure spectral data from these images.

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a process by which energy that would normally be emitted as a photon from an excited fluorophore can be directly transferred to a second fluorophore to excite one of its electrons. This, on decay, then generates an even longer wavelength photon. The extent of FRET is critically dependent on the distance between the two fluorophores as well as their spectral overlap. Thus, FRET is a powerful reporter of the separation of the two fluorophores. FRET is a simple but effective tool for measurements of protein-protein interactions.

It is one of the few techniques that are capable of giving dynamic information about the nanometer-range proximity between molecules, as opposed to simply the subcellular colocalization that is provided by fluorescence microscopy.

Magnetic Resonance Force Microscopy and Nanoscale MRI

IBM has been working over a decade to develop nanoscale magnetic resonance imaging technology called magnetic resonance force microscopy (MRFM). The central feature of MRFM is a silicon "microcantilever" that looks like a miniature diving board and is 1,000 times thinner than a human hair. It vibrates at a frequency of about 5,000 times a second, and a tiny but powerful magnetic particle attached to the tip attracts or repels individual electrons. The company claimed a breakthrough in nanoscale MRI by directly detecting for the first time a faint magnetic signal from single electrons buried inside solid samples. MRFM has been combined with 3D image reconstruction to achieve MRI with resolution <10 nm (Degen et al. 2009). The image reconstruction converts measured magnetic force data into a 3D map of nuclear spin density, taking advantage of the unique characteristics of the "resonant slice" that is projected outward from a nanoscale magnetic tip. The basic principles are demonstrated by imaging the 1H spin density within individual tobacco mosaic virus particles sitting on a nanometer-thick layer of adsorbed hydrocarbons. This result, which represents a 100-millionfold improvement in volume resolution over conventional MRI, demonstrates the potential of MRFM as a tool for 3D, elementally selective imaging on the nanometer scale.

With further progress in resolution and sample preparation, force-detected MRI techniques could have significant impact on the imaging of nanoscale biological structures, even down to the scale of individual molecules. Achieving resolution of 1 nm appears to be realistic because the current apparatus operates almost a factor of 10 away from the best demonstrated force sensitivities and field gradients. Even with a resolution >1 nm, MRFM may enable the basic structure of large molecular assemblies to be elucidated. MRFM image contrast can be enhanced beyond the basic spin-density information by using techniques similar to that developed for clinical MRI and NMR spectroscopy. Such contrast may include selective isotopic labeling, selective imaging of different chemical species, relaxation-weighted imaging, and spectroscopic imaging that reflects the local chemical environment.

The development represents a major milestone in the creation of a microscope that can make 3D images of molecules with atomic resolution. Such a device could have a major impact on the study of materials, ranging from proteins and pharmaceuticals to integrated circuits for which a detailed understanding of the atomic structure is essential. The ability to image the detailed atomic structure of proteins directly would also aid the development of new drugs. This new capability should ultimately lead to fundamental advances in nanotechnology and biology.

Multiple Single-Molecule Fluorescence Microscopy

Fitting the image of a single molecule to the point spread function of an optical system greatly improves the precision with which single molecules can be located. In nanometer-localized multiple single-molecule (NALMS) fluorescence microscopy, short duplex DNA strands are used as nanoscale "rulers" for validation. Nanometer accuracy of this microscope has been demonstrated for 2–5 single molecules within a diffraction-limited area. NALMS microscopy will greatly facilitate single-molecule study of biological systems because it covers the gap between fluorescence resonance energy transfer-based (<10 nm) and diffraction-limited microscopy (>100 nm) measurements of the distance between two fluorophores. NALMS microscopy has been applied to DNA mapping with <10-nm resolution.

Near-Field Scanning Optical Microscopy

Near-field scanning optical microscopy (NSOM) was the first technique that has overcome the limits of light microscopy by about one order of magnitude. Typically, the resolution range below 100 nm is accessed for biological applications. Using appropriately designed scanning probes allows for obtaining an extremely small near-field light excitation volume (some tens of nanometers in diameter). Because of the reduction of background illumination, high-contrast imaging becomes feasible for light transmission and fluorescence microscopy. The height of the scanning probe is controlled by atomic force interactions between the specimen surface and the probe tip. The control signal can be used for the production of a topographic (nonoptical) image that can be acquired simultaneously.

Scattering near-field microscopy (s-SNOM) can determine infrared "fingerprint" spectra of individual poly(methyl methacrylate) nanobeads and viruses as small as 18 nm. Amplitude and phase spectra are found surprisingly strong, even at a probed volume of only 10^{-20} l, and robust in regard to particle size and substrate. This makes infrared spectroscopic s-SNOM a versatile tool for chemical and protein-secondary-structure identification.

Nanosized Light Source for Single Cell Endoscopy

A nanosized light source is capable of emitting coherent light across the visible spectrum. Among the potential applications of this nanosized light source are single cell endoscopy and other forms of subwavelength bio-imaging, integrated circuitry for nanophotonic technology, and new advanced methods of cyber cryptography. Working with individual nanowires, scientists have developed the first electrode-free, continuously tunable coherent visible light source that is compatible with physiological environments. It was shown that nanowires with diameters as small

as 20 nm and aspect ratios of more than 100 can be trapped and transported in 3D, enabling the construction of nanowire architectures that may function as active photonic devices (Pauzauskie et al. 2006). They have also demonstrated that it is possible to trap and manipulate single nanowires with optical tweezers, a critical capability not only for bio-imaging but also for wiring together nanophotonic circuitry. This nanowire light source is like a tiny flashlight that can scan across a living cell, enabling visualization of the cell and at the same time, mechanically interacting with it.

Nanoparticle Characterization by Halo™ LM10 Technology

Halo™ LM10 (NanoSight Ltd.) is based on the laser illumination of a specially designed optical element on to which sample is simply placed manually or allowed to flow across the surface. This is the first nanoparticle characterization tool, specifically designed for liquid phase sizing of individual nanoparticles, with the use of a conventional light microscope. Particles as small as 20 nm have been successfully visualized by this method, each particle being seen as an individual point of light moving under Brownian motion within the liquid. The intensity of light scattered by a particle varies as the sixth power of its radius. By doubling the diameter of the particle, 64-fold more light is scattered by the particle. This has significant implications for the early and simple detection of aggregation, flocculation, and dimerization of particulates at the nanometer scale.

Use of a shorter wavelength laser source capable of exciting fluorescent labels enables specific components within the sample to be distinguished from nonspecific background particles. The image can be analyzed by suitable software allowing changes in individual particle position to be followed furnishing real-time information about particle diffusion and particle-to-particle interactions. In the fluorescence mode, correlation techniques can be used to derive information by use of the technique known as fluorescence correlation spectroscopy. Halo™ LM10 is supported by Halo™ GS10 software.

The laser source need only be a few mW in power and can be delivered to the optical device via fiber optic connection, or the laser diode can be coupled directly to the edge of the optical element. The optical element can be manufactured in optical quality plastic or in glass or silica. The optical element need only be a few mm square and 2–5 mm in depth.

Larger volumes of sample containing dilute concentrations of particles of interest can be analyzed by being configured within a flow cell. Fabrication of the optical element is by industry standard metal coating techniques such as those found in the electronics and optical devices industries. Applications relevant to nanobiotechnology are shown in Table 2.6.

Table 2.6 Applications of optical nanoscopy

Molecular diagnostics
Detection of viral particles
DNA analysis
Mycoplasma detection in animal cell culture
Contaminant monitoring
Drug delivery
Drug carriers
Monitoring drug efficacy in body fluids
Biofilm production and implants
Nanoparticles
Environmental
Biodefense
Airborne contaminants such as asbestos particles
Medical
Clinical diagnosis of viral diseases, e.g., cerebrospinal fluid
Cancer cell detection, e.g., metastases

Source: Nanosight Ltd.

Nanoscale Scanning Electron Microscopy

Pharmaceutical enterprises require a range of imaging products that provide high-quality information, allowing them to reach their own targets on technology, productivity, and ultimately profitability. With the increasing expectations upon drug delivery systems for efficient and controlled delivery of the active material, there is a matching need for accurate information on these mechanisms. One of the most effective instruments in this area is a scanning electron microscope (SEM) from Carl Zeiss that has a unique ability to provide high-resolution images of a specimen under investigation. One example of its application in EVO® EP instrument (Carl Zeiss) is in manufacture of aspirin. The interaction of water with soluble aspirin demonstrates the mechanisms by which tablets lose mechanical strength and stability and hence release the active material. This process can be observed in real time in the SEM by introducing water vapor into the chamber at sufficiently high pressures that liquid water is condensed onto the specimen. During the wetting phase, the particle absorbs water and fragments. During the drying phase, the reverse processes can be followed in detail. The latest product from Carl Zeiss, ULTRA 55 field emission SEM, features a totally new "complete detection system," which enables simultaneous surface, compositional, and crystallographic imaging down to the nanometer level with high signal contrast and unsurpassed clarity.

Use of SEM to Reconstruct 3D Tissue Nanostructure

3D structural information is important in biological research. Excellent methods are available to obtain structures of molecules at atomic, organelles at electron microscopic, and of tissue at light-microscopic resolution. However, there is a need to reconstruct 3D tissue structure with a nanoscale resolution to identify small organelles such as synaptic vesicles. Such 3D data are essential to understand cellular networks that need to be completely reconstructed throughout a substantial spatial volume, particularly in the nervous system. Datasets meeting these requirements can be obtained by automated block-face imaging combined with serial sectioning inside the chamber of a SEM. Backscattering contrast is used to visualize the heavy-metal staining of tissue prepared using techniques that are routine for TEM. The resolution is sufficient to trace even the thinnest axons and to identify synapses. Stacks of several hundred sections, 50–70 nm thick, have been obtained at a lateral position jitter of typically under 10 nm. This opens up the possibility of automatically obtaining the electron-microscope-level 3D datasets needed to completely reconstruct the neuronal circuits.

Optical Imaging with a Silver Superlens

A superlens has been created using a thin film of silver as the lens and ultraviolet light that can overcome a limitation in physics that has historically constrained the resolution of optical images. The superlens has been used to record the images of an array of nanowires at a resolution of about 60 nm, whereas current optical microscopes can only make out details down to 400 nm. This work has a far-reaching impact on the development of detailed biomedical imaging. With current optical microscopes, scientists can only make out relatively large structures within a cell, such as its nucleus and mitochondria. With a superlens, optical microscopes could reveal the movements of individual proteins traveling along the microtubules that make up a cell's skeleton. SEM and AFM are now used to capture detail down to a few nanometers. However, such microscopes create images by scanning objects point by point, which means they are typically limited to nonliving samples, and image capture times can take up to several minutes. Optical microscopes can capture an entire frame with a single snapshot in a fraction of a second, opening up nanoscale imaging to living materials, which can help biologists better understand cell structure and function in real time and ultimately help in the development of new drugs to treat human diseases.

Photoactivated Localization Microscopy

Photoactivated localization microscopy (PALM) enables scientists peering inside cells to discern individual proteins at nanometer ~2 to 25 nm resolution (Betzig et al. 2006). The basic concepts behind this technology are simple: The researchers

label the molecules they want to study with a photoactivatable probe, and then expose those molecules to a small amount of violet light. The light activates fluorescence in a small percentage of molecules, and the microscope captures an image of those that are turned on until they bleach. The process is repeated approximately 10,000 times, with each repetition capturing the position of a different subset of molecules. When a final image is created that includes the center of each individual molecule, it has a resolution previously only achievable with an electron microscope. Unlike electron microscopy, however, the new technique allows for more flexibility in labeling molecules of interest. The method is demonstrated in thin sections by imaging specific target proteins in lysosomes and mitochondria and in fixed, whole cells by imaging vinculin at focal adhesions, actin within a lamellipodium, and the distribution of the retroviral protein Gag at the plasma membrane. A great feature of PALM is that it can be readily used with electron microscopy, which produces a detailed image of very small structures, but not proteins, in cells. By correlating a PALM image showing protein distribution with an electron microscope image showing cell structure of the same sample, it becomes possible to understand how molecules are individually distributed in a cellular structure at the molecular scale. Correlative PALM unites the advantages of light and electron microscopy, producing a revolutionary new approach for looking at the cell in molecular detail. As the PALM technology advances, it may prove to be a key factor in unlocking at the molecular level secrets of intracellular dynamics that are unattainable by other methods. However, the time needed to collect the thousands of single-molecule images that go into each PALM picture is cumbersome. With the camera snapping one to two pictures each second, it can take 2–12 h to image a single sample. Activating more molecules per frame would reduce the number images that must be collected, and making the molecules brighter would reduce the time needed to take each image. Either would help to speed the PALM process. The technique is still undergoing refinements with an aim to developing a practical tool for use by biologists.

Scanning Probe Microscopy

The scanning probe microscope (SPM) system is an important tool for nonintrusive interrogation of biomolecular systems in vitro. Its particular merit is that it retains complete functionality in a biocompatible fluid environment and can track the dynamics of cellular and molecular processes in real time and real space at nanometer resolution, as an imaging tool, and with pN force sensing/imposing resolution, as an interaction tool. The capability may have relevance as a test bed for monitoring cellular response to environmental stimuli and pharmaceutical intervention. Best-known contributions of SPM are toward explanatory and predictive descriptions of biomolecular interactions at surfaces and interfaces, and there are some attempts to reconfigure the SPM platform for demonstration of novel biodevice applications. SPM enables high resolution without any of the drawbacks of electron microscopy, which can damage sensitive molecules by electrons. SPM enables investigation of

biomolecules in fluid environments under physiological conditions and is useful for study of biology on nanoscale.

Scanning ion-conductance microscopy (SICM) is part of the larger family of SPM. It was specially designed for the submicrometer resolution scanning of soft nonconductive materials that are bathed in electrolyte solution. It consists of an electrically charged glass micro- or nanopipette probe filled with electrolyte lowered toward the surface of the sample (which is nonconducting for ions) in an oppositely charged bath of electrolyte. As the tip of the micropipette approaches the sample, the ion conductance and therefore current decreases since the gap through which ions can flow is reduced in size. SICM is a suitable tool for imaging surfaces of living cells in a contact-free manner and enables one to trace the outlines of entire cell soma and to detect changes in cellular shape and volume. SICM can also be employed to quantitatively observe cellular structures such as cell processes of living cells as well as cell soma of motile cells within hours (Happel and Dietzel 2009).

Partial Wave Spectroscopy

An optical microscopy technique, partial wave spectroscopy (PWS), is capable of quantifying statistical properties of cell structure at the nanoscale (Subramanian et al. 2009). PWS has been used to show for the first time the increase in the disorder strength of the nanoscale architecture not only in tumor cells but also in the microscopically normal-appearing cells outside of the tumor. Although genetic and epigenetic alterations have been previously observed in the field of carcinogenesis, these cells were considered morphologically normal. PWS can show organ-wide alteration in cell nanoarchitecture. This seems to be a general event in carcinogenesis, which is supported by data in three types of cancer: colon, pancreatic, and lung. These results have important implications in that PWS can be used as a new method to identify patients harboring malignant or premalignant tumors by interrogating easily accessible tissue sites distant from the location of the lesion. Once optimized, PWS could be used to detect cell abnormalities early and help physicians assess who might be at risk for developing cancer. Like a pap smear of the cervix, a simple brushing of cells is all that is needed to get the specimen required for testing. PWS can look inside the cell and see those critical building blocks, which include proteins, nucleosomes, and intracellular membranes, and detect changes to this nanoarchitecture. Conventional microscopy cannot do this, and other techniques that can (to some degree) are expensive and complex. PWS is simple, inexpensive, and minimally invasive.

Super-Resolution Microscopy for In Vivo Cell Imaging

Super-resolution microscopy comprises a variety of new approaches such as structured illumination (3D-SIM), localization microscopy (PALM, STORM), and

stimulated emission depletion (STED) that have been developed to surpass the limits of conventional optical microscopes. These methods allow precise visualization and measurement of features that are below the diffraction limit. 3D-SIM (Applied Precision Inc./GE Healthcare) projects a structured light pattern onto the sample. The illumination pattern interacts with the fluorescent probes in the sample to generate interference patterns know as moiré fringes. By modulating the illumination pattern and collecting and reconstructing the subsequent images, super-resolution images with double the lateral and axial resolution are obtained.

Normal microscopes enable visualization of cell contents that are >200 nm in size but would be unable to detect a very small molecules such as insulin, which is about 10 nm in size. Super-resolution microscopy enables nanoscale observation of cells in vivo. In vivo study of cells provides invaluable information for study of pathomechanism of disease at cell level and for cell-based drug discovery. Electron microscopes have similar resolution to a super-resolution microscope, but they do not allow observation of cells in vivo. Exploration of cellular functions at nanoscale enables a better understanding of the processes that occur in a dysfunctional cell. Super-resolution microscopes have been used to study how the HIV virus penetrates cells and provide information for developing new drugs.

Ultrananocrystalline Diamond

A common problem in AFM is the deterioration of the tip apex as surfaces are scanned. To overcome this problem, an ultrananocrystalline diamond (UNCD) was used to fabricate a hard, low-wear probe for contact-mode writing techniques such as Dip Pen Nanolithography. Diamond, the hardest known material, is probably the optimal tip material for many applications. In addition to hardness, diamond is stiff, biocompatible, and wear resistant. Diamond tips with radii down to 30 nm were obtained through growth of UNCD films followed by selective etching of the silicon template substrate. The probes were monolithically integrated with diamond cantilevers and subsequently integrated into a chip body obtained by metal electroforming. The probes were characterized in terms of their mechanical properties, wear, and atomic force microscopy imaging capabilities. The developed probes performed exceptionally well in DPN molecular writing/imaging mode. Furthermore, the integration of UNCD films with appropriate substrates and the use of directed microfabrication techniques are particularly suitable for fabrication of one- and two-dimensional arrays of probes that can be used for massive parallel fabrication of nanostructures by the Dip Pen Nanolithography method. The technology can be employed for a variety of AFM scanning modes, from regular surface scanning in air or fluids to conductive AFM. It can also be employed as a nanofabrication tool. Examples include nanopatterning of biomolecules (for sequencing, synthesis, and drug discovery) and scanning probe electrochemistry (scanning electrode imaging, localized electrochemical etching or deposition of materials, and nanovoltametry). Potential markets include those industries where it is pivotal to preserve the performance of

the tips or that require two-dimensional arrays for high throughput in which the cost of manufacturing is such that minimum possible tip wear is paramount. These include the chemical and biological sensor industry where high throughput and spatial resolution are important.

Visualizing Atoms with High-Resolution Transmission Electron Microscopy

The characterization of nanostructures down to the atomic scale is essential to understand some physical properties. Such a characterization is possible today using direct imaging methods such as aberration-corrected high-resolution transmission electron microscopy (HRTEM), when iteratively backed by advanced modeling produced by theoretical structure calculations and image calculations (Bar Sadan et al. 2008). Aberration-corrected HRTEM is therefore extremely useful for investigating low-dimensional structures, such as inorganic fullerene-like particles and inorganic nanotubes. The atomic arrangement in these nanostructures can lead to new insights into the growth mechanism or physical properties, where imminent commercial applications are unfolding. HRTEM study combined with modeling reveals new information regarding the chirality of the different shells and provides a better understanding of their growth mechanism. The next frontier will be seeing atoms in 3D.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) is an optical-electrical phenomenon involving the interaction of light with the electrons of a metal. Light is coupled into the surface plasmon by means of either a prism or a grating on the metal surface. Depending on the thickness of a molecular layer at the metal surface, the SPR phenomenon results in a graded reduction in intensity of the reflected light. The optical-electronic basis of SPR is the transfer of the energy carried by photons of light to a group of electrons (a plasmon) at the surface of a metal.

In Biacore systems (Fig. 2.3), SPR arises when light is reflected under certain conditions from a conducting film at the interface between two media of different refractive index. The media are the sample and the glass of the sensor chip, and the conducting film is a thin layer of gold on the chip surface. SPR causes a reduction in the intensity of reflected light at a specific angle of reflection (the SPR angle). When molecules in the sample bind to the sensor surface, the concentration, and therefore the refractive index, at the surface changes, and an SPR response is detected. Plotting the response against time during the course of an interaction provides a quantitative measure of the progress of the interaction. This plot is called a sensorgram.

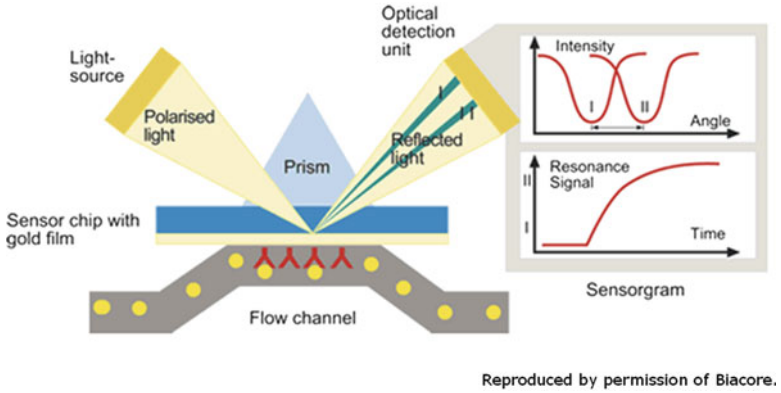

Fig. 2.3 Surface plasmon resonance (SPR) technology (Reproduced by permission of Biacore)

Biomedical applications take advantage of the exquisite sensitivity of SPR to the refractive index of the medium next to the metal surface, which makes it possible to measure accurately the adsorption of molecules on the metal surface and their eventual interactions with specific ligands. Applications of this technique include the following:

- Measurement in real time of the kinetics of ligand-receptor interactions
- Screening of lead compounds in the pharmaceutical industry
- Measurement of DNA hybridization
- Enzyme-substrate interactions
- Polyclonal antibody characterization
- Protein conformation studies
- Label-free immunoassays