Chapter 14
Nanomicrobiology

Introduction

Microbiology plays an important role in practice of medicine. Nanodiagnostics have refined the detection of infectious diseases and many new nanotechnology-based therapies, particularly of viral diseases, are in development.

Nanodiagnosis of Infections

Nanobiotechnology-based molecular diagnostic techniques were described in Chap. 4. Examples of specific applications for detection of infectious agents will be given in this chapter.

Detection of Viruses

Several nanotechnology-based methods have already been described in this chapter, including ferrofluid magnetic nanoparticles, ceramic nanospheres, and nanowire sensors for viruses. Role of cantilevers, SWCNTs, QDs, and surface-enhanced Raman scattering (SERS) will be described in this section.

Cantilever Beams for Detection of Single Virus Particles

Microfabrication and application of arrays of silicon cantilever beams as microresonator sensors with nanoscale thickness has been applied to detect the mass of individual virus particles (Gupta et al. 2004). The dimensions of the fabricated cantilever beams were in the range of 4–5 μm in length, 1–2 μm in width, and 20–30 nm in thickness. The virus particles used in the study were vaccinia virus, which is a member of the Poxviridae family and forms the basis of the smallpox vaccine. The frequency spectra of the cantilever beams, due to thermal and ambient noise, were measured using a laser Doppler vibrometer under ambient conditions.

K.K. Jain, *The Handbook of Nanomedicine*, DOI 10.1007/978-1-61779-983-9_14,

The change in resonant frequency as a function of the virus particle mass binding on the cantilever beam surface forms the basis of the detection scheme. This device can detect a single vaccinia virus particle with an average mass of 9.5 fg. Such devices can be very useful as components of biosensors for the detection of airborne virus particles. This technology has been refined as described under nanocantilever biosensors.

Carbon Nanotubes as Biosensors for Viruses

SWCNTs have been functionalized under ambient conditions with either the Knob protein domain from adenovirus serotype 12 (Ad 12 Knob) or its human cellular receptor, the CAR protein, via diimide-activated amidation (Zhang et al. 2007). The biological activity of Knob protein immobilized on the nanotube surfaces was confirmed by using its labeled conjugate antibody. The activity and specificity of bound CAR on SWCNTs was evaluated first, in the presence of fluorescently labeled Knob, which interacts specifically with CAR, and second, with a negative control protein, YieF, which is not recognized by biologically active CAR proteins. In addition, current-gate voltage measurements on a dozen nanotube devices explored the effect of protein binding on the intrinsic electronic properties of the SWCNTs and also demonstrated the devices' high sensitivity in detecting protein activity. All data show that both Knob and CAR immobilized on SWCNT surfaces fully retain their biological activities, suggesting that SWCNT-CAR complexes can serve as biosensors for detecting environmental adenoviruses.

Electric Fields for Accelerating Detection of Viruses

The rapid detection of viruses in biological samples is of increasing interest, particularly with the recent emergence of new viruses. A device that can quickly and easily detect them is difficult to construct because viral particles are present at such low concentrations in biological samples, such as blood. Typical procedures involve using passive diffusion to get the viral particles to bind to an antibody, a slow process that is not feasible for many applications, such as on the battlefield, where quick results are critical. However, liquid crystals are known to amplify signals from low concentrations of viral particles, quickly indicating whether or not a virus is present on a surface. A study to speed up the collection of viral particles, particularly vesicular stomatitis virus, used directed assembly, in which external electrical and fluid flow fields are designed in order to drive nanoparticles to specific locations and in specific concentrations on a substrate (Docoslis et al. 2007). Electrical fields are advantageous because by designing the electrodes in a certain way, engineers can control directionality and intensity of electrical forces acting on nanoparticles. By using electrodes separated by just a few micrometers together with electrothermally induced fluid flow, one can accelerate the transport of viral particles from aqueous suspensions with physiological ionic strength to specific points on a surface, allowing them to reach local concentrations high enough to enable subsequent rapid detection. These observations provide a potentially useful approach for addressing a bottleneck in the development of devices that allow for rapid sampling and "on-the-spot" detection of infectious biological agents such as viruses.

QD Fluorescent Probes for Detection of Respiratory Viral Infections

Respiratory syncytial virus (RSV) causes about one million deaths annually worldwide. RSV mediates serious lower respiratory tract illness in infants and young children and is a significant pathogen of the elderly and immune-compromised. Although it is only life-threatening in one case out of every 100, it infects virtually all children by the time they are 5 years old. Approximately 120,000 children are hospitalized with RSV in the USA each year. Few children in the USA die from RSV, but it causes 17,000–18,000 deaths annually among the elderly.

Rapid and sensitive RSV diagnosis is important for infection control and efforts to develop antiviral drugs. Current RSV detection methods are limited by sensitivity and/or time required for detection, which can take 2–6 days. This can delay effective treatment. Antibody-conjugated nanoparticles rapidly and sensitively detect RSV and estimate relative levels of surface protein expression. A major development is use of dual-color QDs or fluorescence energy transfer nanobeads that can be simultaneously excited with a single light source.

A QD system can detect the presence of particles of the RSV in a matter of hours. It is also more sensitive, allowing it to detect the virus earlier in the course of an infection. When an RSV virus infects lung cells, it leaves part of its coat containing F and G proteins on the cell's surface. QDs have been linked to antibodies keyed to structures unique to RSV's coat. As a result, when QDs come in contact with either viral particles or infected cells, they stick to their surface. In addition, co-localization of these viral proteins was shown using confocal microscopy. The potential benefits for such an early detection system are that it can:

1. Increase the proper use of antiviral medicines. Although such medicines have been developed for some respiratory viruses, they are not used often as therapy because they are only effective if given early in the course of infection. By the time current tests identify the virus, it is generally too late for them to work.
2. Reduce the inappropriate use of antibiotics. Currently, physicians often prescribe antibiotics for respiratory illnesses. However, antibiotics combat respiratory illness caused by bacteria and are ineffective on viral infections. An early virus detection method would reduce the frequency with which doctors prescribe antibiotics for viral infections inappropriately, thereby reducing unnecessary antibiotic side effects and cutting down on the development of antibiotic resistance in bacteria.
3. Allow hospital personnel to isolate RSV patients. RSV is extremely infectious so early detection would allow hospital personnel to keep the RSV patients separate from other patients who are especially susceptible to infection, such as those undergoing bone-marrow transplants.

Currently, there are three diagnostic tests available for identifying respiratory viruses like RSV. The "gold standard" involves incubating an infected sample in a tissue culture for a few days and then using a fluorescent dye to test for the presence of the virus. The main problem with this technique is that the virus is multiplying in the patient at the same time as it is growing in the culture. This has caused many hospitals to switch to real-time PCR, which is extremely sensitive but still takes several hours because of the need for a technician well trained in molecular biologist to conduct the test in a reference laboratory. The third method, the antigen test, takes ~30 min, but it is not sensitive enough to detect the presence of the virus at the early stages of an infection. By comparison, the QD method takes 1–2 h and is even more sensitive than real-time PCR. It can detect the presence of RSV within an hour after the virus is added to a culture. QDs have an advantage over many traditional fluorophores because their fluorescence properties can be finely tuned and they are resistant to photobleaching (Halfpenny and Wright 2010).

Verigene Respiratory Virus Plus Assay

Verigene (Nanosphere Inc.) platform is based on a direct genomic detection technology that uses DNA probes coated with gold nanoparticles to identify a unique oligonucleotide sequence and combines it with biobarcode protein detection technology. Verigene respiratory virus plus assay, which runs on an automated sample-to-result molecular diagnostic instrument, is more sensitive than currently available rapid tests. It combines optimized ease of use and turnaround time not found in either traditional culture methods or the currently available molecular tests for viruses and is cleared by the FDA for detection of influenza and RSV.

Surface-Enhanced Raman Scattering for Detection of Viruses

Although surface-enhanced Raman scattering (SERS) is well known, previous attempts to use spectroscopy to diagnose viruses failed because the signal produced is inherently weak. A spectroscopic assay based on SERS using silver nanorods, which significantly amplify the signal, has been developed for rapid detection of trace levels of viruses with a high degree of sensitivity and specificity (Shanmukh et al. 2006). The technique measures the change in frequency of a near-infrared laser as it scatters viral DNA or RNA. That change in frequency is as distinct as a fingerprint. This novel SERS assay can detect spectral differences between viruses, viral strains, and viruses with gene deletions in biological media. The method provides rapid diagnostics (<1 min) for detection and characterization of viruses generating reproducible spectra without viral manipulation. It is also quite cheap and is very reproducible.

A dual-mode molecular beacon, based on a combined SERS and fluorescent molecular beacon assay that is assembled on nanobarcode particles, has been developed and used to measure unlabeled human viral RNA (Sha et al. 2007). The molecular beacon probe is a single-stranded oligonucleotide that has been designed with a hairpin structure that holds the dye at 3′-end close to the particle surface when the probe is attached through a 5′-thiol group. In this configuration, the SERS spectrum of the label is obtained and its fluorescence quenched because the dye is in very close proximity to a noble metal surface with nanoscale features. The SERS signal decreases and the fluorescence signal increases when target viral RNA is captured by this molecular beacon probe. In addition, a HCV RT-PCR product is detected using this dual-mode beacon. The development of a multiplexed, label-free assay system with the reassurance offered by detection of two distinctly separate signals offers significant benefits for rapid molecular diagnostics.

Detection of Bacteria

The rapid and sensitive detection of pathogenic bacteria is extremely important in diagnosis of infections at POC. Limitations of most of the conventional diagnostic methods are lack of ultrasensitivity or delay in getting results. Nanobiotechnology has made a significant contribution to improvements in detection of bacterial infections.

Nanoparticle-Based Methods for Bacterial Detection

Bioconjugated nanoparticle-based assays for in situ pathogen quantification can detect a single bacterium within minutes. Such nanoparticles provide high fluorescent signals for bioanalysis and can be easily incorporated in a biorecognition molecule such as an antibody. The antibody-conjugated nanoparticles can readily and specifically identify a variety of bacteria such as *Escherichia coli O157:H7* through antibody-antigen interaction and recognition. This method can be applied to multiple bacterial samples with high throughput and has a potential for application in ultrasensitive detection of disease biomarkers and infectious agents.

Verigene gram-positive blood culture (BC-GP) test (Nanosphere Inc.) is a multiplexed, nanoparticle-based automated nucleic acid test for the identification of genus, species, and genetic resistance determinants for a broad panel of the most common gram-positive blood culture isolates. Whereas conventional microbiological methods may require 2–4 days to produce bacterial identification and resistance results, the Verigene BC-GP test provides results within 2.5 h of blood culture positivity. The Verigene system's unique instrumentation with <5 min of user hands-on time per test enables true random access test processing directly from positive blood culture bottles.

Multifunctional magnetic-plasmonic Fe_3O_4-Au core-shell nanoparticles (Au-MNPs) have been prepared for simultaneous fast concentration of bacterial cells by applying an external point magnetic field and sensitive detection and identification of bacteria using SERS (Zhang et al. 2012a). Surrounded by dense uniformly packed Au-MNPs, bacteria can be sensitively and reproducibly detected directly using SERS. This method can be used in molecular diagnostics of bacterial infections.

QDs for Detection of Bacterial Infections

Detection of single-molecule hybridization has been achieved by a hybridization detection method using multicolor oligonucleotide-functionalized QDs as nanoprobes

(Ho et al. 2005). In the presence of various target sequences, combinatorial self-assembly of nanoprobes via independent hybridization reactions leads to the generation of discernible sequence-specific spectral codings. This method can be used for genetic analysis of anthrax pathogenicity by simultaneous detection of multiple relevant sequences.

Fluorescent QDs coated with zinc(ii)-dipicolylamine coordination complexes can selectively stain a rough *E. coli* mutant that lacks an O-antigen element and permit optical detection in a living mouse leg infection model (Leevy et al. 2008). QDs have potential use as labeling agents for bacteriophages associated with bacterial infections. A rapid and simple method has been reported that combines in vivo biotinylation of engineered host-specific bacteriophage and conjugation of the phage to streptavidin-coated QDs (Edgar et al. 2006). The method provides specific detection of as few as 10 bacterial cells per ml in experimental samples, with an approximately 100-fold amplification of the signal over background in 1 h. The method can be applied to any bacteria susceptible to specific phages and would be particularly useful for detection of bacterial strains that are slow growing such as Mycobacterium or are highly infectious such as *B. anthracis*. To monitor the infection of *E. coli* cells by light microscopy, procedures have been developed for the tagging of mature bacteriophages with QDs (Edgar et al. 2008). Fluorescent QDs have been used for detection and sorting of pathogenic bacteria by flow cytometry (Zahavy et al. 2012).

Detection of Fungi

Conventional methods for diagnosis of invasive fungal infections in the clinical microbiology laboratory are time-consuming processes or result in misidentification of the fungus due to low sensitivity or low specificity. There is a need for improvement of methods of detection of fungi, and nanobiotechnology-based techniques have been used.

Nano-amplification Technique for the Detection of Fungal Pathogens

In one method, fungal ribosomal DNA was amplified using PCR and the products were hybridized with the species-specific probes immobilized on the surface of a microarray where hybridizing signals were enhanced with gold nanoparticles and silver deposition (Lu et al. 2010). The probes were designed to detect several different clinical pathogenic fungi using a flatbed scanner or visually. The technique showed higher efficiency, specificity, and sensitivity compared with other methods.

Magnetic Nanoparticle-Based Technique

Magnetic resonance detection (T2 Biosystems) uses magnetic nanoparticles coupled with reagents to quickly detect, within minutes, the presence of specific substances in solution using a miniaturized, portable MRI instrument. Detection of the high-level MR signal from the solution enables the detection of low concentrations of target agents or substances. Unlike most existing diagnostic detection techniques which are based on optical detection methods that require pure samples and multiple processing steps, T2's technology is not optical and therefore does not require purification of biological samples. The significant advantage allows the T2 system to perform single-step processing and rapid turnaround times without the need for trained technicians. Furthermore, the technology can accurately identify almost any specimen, including proteins, nucleic acids, or enzymes; microbes; or small molecule drug compounds within almost any sample, including whole blood, plasma, serum, and urine. This method has been used to analyze whole blood specimens from patients with five different types of *Candida* spp. infections and is currently in clinical trials with an aim for regulatory approval for diagnosis of Candida infection.

Nanobiotechnology and Virology

Study of Interaction of Nanoparticles with Viruses

Scanning surface confocal microscopy, simultaneous recording of high-resolution topography, and cell surface fluorescence in a single scan enables imaging of individual fluorescent particles in the nanometer range on fixed or live cells. This technique has been used to record the interaction of single virus-like particles with the cell surface and demonstrated that single particles sink into the membrane in invaginations reminiscent of pinocytic vesicles. This method enables elucidation of the interaction of individual viruses and other nanoparticles, such as gene therapy vectors, with target cells.

Silver nanoparticles undergo a size-dependent interaction with HIV-1 and particles in the range of 1–10 nm attached to the virus (Elechiguerra et al. 2005). The regular spatial arrangement of the attached nanoparticles, the center-to-center distance between nanoparticles, and the fact that the exposed sulfur-bearing residues of the glycoprotein knobs would be attractive sites for nanoparticle interaction suggest that silver nanoparticles interact with the HIV-1 virus via preferential binding to the gp120 glycoprotein knobs. Due to this interaction, silver nanoparticles inhibit the virus from binding to host cells, as demonstrated in vitro.

Study of Pathomechanism of Viral Diseases

Research in nanobiotechnology may be helpful in understanding the pathomechanism of viral diseases and devising strategies for treatment. An example is the neurotropic herpes simplex virus (HSV), which infects mucosal epithelia and enters nerve terminals, from where it travels in axons to dorsal root ganglia neurons and delivers its genome into the nucleus of the cell body. In the nucleus, the genome may give rise to infectious progeny or become latent with little gene expression. The silenced genome can be reactivated upon stress and establish a productive infection in the peripheral nervous system and, later, also in the mucosal periphery. To achieve this, a virus must elude host restrictions at multiple levels, including entry, cytoplasmic transport, replication, innate and adaptive immune recognition, and egress from the infected cell.

Research on virus nanoparticles has provided cues to the regulation of cytoplasmic transport. Viruses that replicate their genomes in the nucleus make use of the microtubule and the actin cytoskeleton as molecular motors for trafficking toward the nuclear membrane during entry and the periphery during egress after replication. Analyzing the underlying principles of viral cytosolic transport will be helpful in the design of viral vectors to be used in research as well as human gene therapy and in the identification of new antiviral target molecules (Dohner and Sodeik 2005).

Transdermal Nanoparticles for Immune Enhancement in HIV

DermaVir Patch (Genetic Immunity) is a transdermally delivered nanomedicine to enhance de novo HIV-specific memory T cell responses of HIV-infected individuals and improve the ability of their own immune system to control the disease by killing only HIV-infected cells. Mice receiving DermaVir formulated with HIV-1 Gag plasmid in the presence of IL-7- or IL-15-encoding plasmid have significantly enhanced Gag-specific central memory T cells, as measured by a peptide-based cultured IFN-γ ELISPOT (Calarota et al. 2008). In a DermaVir prime/vaccinia vector boost regimen, the inclusion of IL-15 together with DermaVir significantly improved Gag-specific effector-memory T cell responses. This study demonstrates IL-15 is a promising DermaVir adjuvant to enhance antigen-specific central memory type T cells in a prime-boost setting. It is in phase II clinical trials.

Nanofiltration to Remove Viruses from Plasma Transfusion Products

One of the complications of blood transfusion is transmission of viral infections. Nanofiltration, use of nanotechnology in viral removal filtration systems, is an important safety step in the manufacture of plasma-derived coagulation factor concentrates and other biopharmaceutical products from human blood. Nanofiltration of plasma products has already been carried out since the early 1990s to improve margin of viral safety, as a complement to the viral reduction treatments, such as solvent-detergent and heat treatments, which are applied for the inactivation of HIV as well as hepatitis B and C viruses. The main reason for the introduction of nanofiltration was the need to improve product safety against non-enveloped viruses and to provide a possible safeguard against new infectious agents potentially entering the human plasma pool. Nanofiltration has gained quick acceptance as it is a relatively simple manufacturing step that consists in filtering protein solution through membranes with nanopores (pore size typically 15–40 nm) under conditions that retain viruses by a mechanism largely based on size exclusion. Recent large-scale experience throughout the world has now established that nanofiltration is a robust and reliable viral reduction technique that can be applied to essentially all plasma products. Many of the licensed plasma products are currently nanofiltered. The technology has major advantages as it is flexible and it may combine efficient and largely predictable removal of a wide range of viruses. Compared with other viral reduction means, nanofiltration may be the only method to date permitting efficient removal of enveloped and non-enveloped viruses under conditions where 90–95 % of protein activity is recovered. New data indicate that nanofiltration may also remove prions, opening new perspectives in the development of this technique.

Shortcomings of some membranes are that they often form pin-holes and cracks during the fabrication process, resulting in wasted membranes. Specially designed ceramic membranes have been used as nanomesh for nanofiltration as they are less likely to be damaged during manufacture and have the potential to remove viruses from water, air, and blood. Mesh structure, which is the most efficient form of filtration, has been successfully constructed on a nanoscale with ceramic fibers. This modification has increased the rates of flow that pass through the membranes tenfold compared with current ceramic membranes while maintaining the efficiency of capturing over 96 % of the unwanted particles. This technology could be used to filter airborne viruses such as the severe acute respiratory syndrome (SARS) and the avian flu virus. It may be possible to filter HIV from human blood to treat patients with AIDS.

Role of Nanobacteria in Human Diseases

Nanobacteria are mineral-forming, sterile-filterable, slow-growing gram-negative infectious agents. They are detected in bovine/human blood and urine. Nanobacteria-like particles have been detected in synovial fluids of arthritis patients and were shown to gradually increase in number and in size in culture (Tsurumoto et al. 2006). Nanobacteria have been implicated in a variety of human diseases associated with pathological calcification. Their most remarkable characteristic is the formation of carbonate apatite crystals of neutral pH and at physiologic phosphate and calcium concentrations. The extracellular mineralization forms a hard protective shelter for these hardy microorganisms and enables them to survive conditions of physical stress that would be lethal to most other bacterial species. The Olavi Kajander group (Finland) suggests that the apatite produced by nanobacteria may play a key role in the formation of all kidney stones, by providing a central calcium phosphate deposit around which other crystalline components can collect.

Nanobacteria seem to be causative agents of diseases related to biomineralization processes. Nanobacteria are also associated with calcified geological specimens, human kidney stones, and psammoma bodies in ovarian cancer. Much research has focused attention on the potential role these particles may play in the development of urologic pathology, including polycystic kidney disease, renal calculi, and chronic prostatitis. Nanobacteria may be an important etiological factor for type III prostatitis, which was reproduced in rat prostate infection models by infusing nanobacteria suspension transurethrally (Shen et al. 2010). Recent clinical research on agents targeting nanobacteria has proven effective in treating some patients with refractory category III prostatitis.

Nature of Nanobacteria

According to their 16S rDNA structure, nanobacteria belong to the alpha-2 Proteobacteria, subgroup, which includes the Brucella and Bartonella species. Nanobacterium sanguineum (nanobacteria) is the smallest self-replicating organism ever detected – at 50–500 billionths of a meter, 1/1000th the size of the smallest previously known bacteria. Primordial proteins in nanobacteria, only recently identified in the atmosphere, could play a significant role in clouds, accelerating the formation of cloud droplets and interconnecting nanobacteria (and possibly nanobacteria and other microorganisms), thus enhancing their chances to eventually reach the Earth.

Several research studies indicate that nanobacteria are alive, but it is still unclear whether they represent novel life forms, overlooked nanometer-size bacteria, or some other primitive self-replicating microorganisms. A study has shown that $CaCO_3$ precipitates prepared in vitro are remarkably similar to purported nanobacteria in terms of their uniformly sized, membrane-delineated vesicular shapes, with cellular division-like formations and aggregations in the form of colonies (Martel and Young 2008). The gradual appearance of nanobacteria-like particles in incubated human serum as well as the changes seen with their size and shape can be influenced and explained by introducing varying levels of CO_2 and $NaHCO_3$ as well as other conditions known to influence the precipitation of $CaCO_3$. Western blotting reveals that the monoclonal antibodies, claimed to be specific for nanobacteria, react in fact with serum albumin. Furthermore, nanobacteria-like particles obtained from human blood are able to withstand high doses of irradiation up to 30 kGy, and no bacterial DNA is found by performing broad-range PCR amplifications. These findings provide a more plausible abiotic explanation for the unusual properties of purported nanobacteria.

Nanobacteria and Kidney Stone Formation

Approximately 12 % of men and 5 % of women develop kidney stones by the time they reach the age of 70 years, but exactly how kidney stones form is not known. Kidney stones can be debilitating and recur in 50 % of patients within 5 years. Kidney stone formation is considered to be a multifactorial disease in which the defense mechanisms and risk factors are imbalanced in favor of stone formation. One theory is that if nanoparticles accumulate in the kidney, they can form the focus of subsequent growth into larger stones over months to years. Other factors, such as physical chemistry and protein inhibitors of crystal growth, also play a role.

Mineral-forming nanobacteria are active nidi that attach to, invade, and damage the urinary epithelium of collecting ducts and papilla forming the calcium phosphate center(s) found in most kidney stones. Scientists at NASA have used multiple techniques to determine that nanobacteria infection multiplies faster in spaceflight-simulated conditions than on earth. Nanobacteria are considered to initiate kidney stone formation as they grow faster in a microgravity environment and may explain why astronauts get kidney stones on space missions. This discovery may prove to be critical for future exploratory missions to the moon and Mars. For further proof to this hypothesis, screening of the nanobacterial antigen and antibody level in flight crew before and after flight would be necessary. This concept also opens the door for new diagnostic and therapeutic techniques addressing nanobacterial infection in kidney stones.

Nanoparticles have been isolated and cultured from the majority of renal stones obtained at the time of surgical resection (Kumar et al. 2006). Isolates were susceptible to selected metabolic inhibitors and antibiotics and contained conserved bacterial proteins and DNA. These results suggest that renal stone formation is unlikely to be driven solely by physical chemistry; rather, it is critically influenced by specific proteins and cellular responses, and understanding these events will provide clues toward novel therapeutic targets. Using high-spatial and energy resolution near-edge x-ray absorption fine structure at the 25 nm spatial scale, it is possible to define a biochemical signature for cultured calcified bacteria, including proteins, polysaccharides, nucleic acids, and hydroxyapatite (Benzerara et al. 2006). These preliminary studies suggest that nanoparticles isolated from human samples share spectroscopic characteristics with calcified proteins.

Nanobacteria in Cardiovascular Disease

Nanometer-scale objects, spherical in shape and ranging in size from 30 to 100 nm with a spectral pattern of calcium and phosphorus (high-energy dispersive spectroscopy), have been identified with positive immunostaining in surgical specimens from patients with cardiovascular pathology. Nano-sized particles cultured from calcified but not from non-calcified aneurysms were recognized by a DNA-specific dye and incorporated radiolabeled uridine and, after decalcification, appeared via electron microscopy to contain cell walls. Nanometer-scale particles similar to those described as nanobacteria isolated from geological specimens and human kidney stones can be visualized in and cultured from human-calcified cardiovascular tissue. In further studies, nanoparticles were found near plaque-filled arteries in animal models. These observations suggest that nanoparticles potentially represent a previously unrecognized factor in the development of arteriosclerosis and calcific arterial disease.

Nanotechnology-Based Microbicidal Agents

Nanoscale Bactericidal Powders

Certain formulations of nanoscale powders possess antimicrobial properties. These formulations are made of simple, nontoxic metal oxides such as magnesium oxide (MgO) and calcium oxide (CaO, lime) in nanocrystalline form, carrying active forms of halogens, e.g., MgO.Cl2 and MgO.Br2. When these ultrafine powders contact vegetative cells of *E. coli*, *Bacillus cereus*, or *Bacillus globigii*, over 90 % are killed within a few minutes. Likewise, spore forms of the Bacillus species are decontaminated within several hours. Dry contact with aflatoxins and contact with MS2 bacteriophage (surrogate of human enterovirus) in water also cause decontamination in minutes.

A nanopowder of MgO can scour contaminated rooms of anthrax spores. Unlike antibacterial gases and foams, which are messy, and corrosive and ruin electrical equipment, the powder can be sprayed into rooms and swept or vacuumed up. The chemical specks attract oppositely charged spores. The particles then chemically break down the spores' tough outer shell. Based on this technology, NanoScale Corporation markets a dry powder dubbed FAST-ACT® (First Applied Sorbent Treatment-Against Chemical Threats) that decomposes toxic chemicals. The powder contains reactive nanoparticles that attract and then break down at least 24 commonly transported toxic chemicals, including some acids. Unlike foams, the powder need not be wet to be effective and works on liquids and vapors.

Nanotubes for Detection and Destruction of Bacteria

A simple molecule has been synthesized from a hydrocarbon and an ammonium compound to produce a unique nanotube structure with antimicrobial capability (Lee et al. 2004). The quaternary ammonium compound is known for its ability to disrupt cell membranes and cause cell death, whereas the hydrocarbon diacetylene can change colors when appropriately formulated; the resulting molecule would have the desired properties of both a biosensor and a biocide.

The self-assembled nanotubes are perfectly uniform and organize themselves into an expanse of upright clusters that when magnified a million times resemble the fibers of a shag rug leading to the name "nanocarpet." The self-assembling nanotubes had all the same diameter (89 nm) and wall thickness (27 nm). The nanocarpet measures about 1 μm in height, approximately the same height as the free-form nanotubes. This alignment of nanotubes in the absence of a template is unprecedented and represents an important step toward rational design of bioactive nanostructures. In addition, because they form within hours under room-temperature conditions, the significant costs of synthesizing carbon nanotubes can be reduced. Normally a neutral color, when exposed to ultraviolet light, the nanotubes changed to a permanent deep blue. The process also chemically altered the nanotubes so that they became polymerized, giving them a more firm structure. Polymerized, these nanotubes could change from blue to other colors, depending on its exposure to different materials. For instance, in tests with acids and detergents, they turned red or yellow.

Because they display sensitivity to different agents by changing color, these nanotubes can be trained to kill bacteria. In the presence of *E. coli*, some strains of which are food-borne pathogens, the nanotubes turned shades of red and pink. Moreover, with the aid of an electron microscope, the researchers observed the tubes piercing the membranes of the bacteria like a needle being inserted into the cell. Both the polymerized (those that can change color) and the unpolymerized nanotube structures were effective antimicrobials, completely killing all the *E. coli* within an hour's time. The findings have implications for developing products that can simultaneously detect and kill biological weapons. The research, funded by the Department of Defense's Army Research Office, has as its goal the development of a paint that in the event of biological or chemical agents being deployed would change color and simultaneously destroy the deadly substances.

Carbon Nanotubes as Antimicrobial Agents

CNTs have the potential to address the challenges of combating infectious agents by both minimizing toxicity by dose reduction of standard therapeutics and allowing a multiple payload capacity to achieve both targeted activity and combating infectious strains, resistant strains in particular (Rosen and Elman 2009). One of their unique characteristics is the network of carbon atoms in the nanometer scale, allowing the creation of nanochannels via cellular membranes.

Attempts have been made to destroy anthrax spores by antimicrobial agents targeted to bind to carbohydrates on the spore surface but with limited success. SWCNTs have been successfully used as a truly unique scaffold for displaying multivalent monosaccharide ligands that bind effectively to anthrax spores with divalent cation mediation to cause significant spore aggregation (Wang et al. 2006). The work should have far-reaching implications in development of technologies to counteract bioterrorism such as by use of anthrax. For anthrax to be effective, it has to be made into a fine powder that can easily enter the lungs when inhaled. That nanotechnology-based agent clings to the anthrax spores to make their inhalation into the lungs difficult. Similar approach using sugar-coated carbon nanotubes to stop the spread of *E. coli* bacteria was tested successfully.

Nanoemulsions as Microbicidal Agents

The antimicrobial nanoemulsions (NanoBio) are emulsions that contain water and soya bean oil with uniformly sized droplets in the 200–400 nm range. These droplets are stabilized by surfactant and are responsible for the microbicidal activity. In concentrated form, the nanoemulsions appear as a white milky substance with a taste and consistency of cream. They can be formulated in a variety of carriers allowing for gels, creams, liquid products, etc. In most applications, the nanoemulsions become largely water-based and in some cases such as a beverage preservative, comprise 0.01 % or less of the resultant mixture. Laboratory results indicate a shelf life of at least 2 years and virtually no toxicity. NanoBio Corporation's nanoemulsions destroy microbes effectively without toxicity or harmful residual effects. The nanoparticles fuse with the membrane of the microbe, and the surfactant disrupts the membrane, killing the microbe. The classes of microbes eradicated are virus (e.g., HIV, herpes), bacteria (e.g., *E. coli*, *Salmonella*), spores (e.g., anthrax), and fungi (e.g., *Candida albicans*, *Byssochlamys fulva*). NB-402 (NanoBio), a nanoemulsion antimicrobial agent for the treatment of infection due to CF-related opportunistic pathogens, is in development (see Chap. 11). Clinical trials have shown efficacy in healing cold sores due to herpes simplex virus 1 and toenail fungus. The nanoemulsions also can be formulated to kill only one or two classes of microbes. Due in large part to the low toxicity profile, the nanoemulsions are a platform technology for any number of topical, oral, vaginal, cutaneous, preservative, decontamination, veterinary, and agricultural antimicrobial applications.

Since it is nontoxic and non-corrosive, nanoemulsion can be used to decontaminate personnel, equipment, terrain, structures, and water. Further, tests by DTRA (Defense Threat Reduction Agency), an agency of the US Department of Defense, have demonstrated that the nanoemulsion is a chemical decontaminating agent. The US Army tested the nanoemulsion and nine other biodecontamination technologies at against an anthrax surrogate. The nanoemulsion was one of four technologies that proved effective.

Silver Nanoparticle Coating as Prophylaxis Against Infection

The Institute for New Materials (Saarbrucken, Germany), a research institute specializing in applied nanotechnology applications, has developed a silver nanoparticles surface coating that is deadly to fungi and bacteria. The researchers added the germicidal ability by sprinkling copious amounts of silver nanoparticles through the coating material (every square centimeter contains more than one billion of the invisible particles) and aligning them so that they release a tiny number of silver ions. These ions are the death knell for fungus and bacteria that might have succeeded in gathering on the surface despite its already dirt-repellent qualities.

Applications include any surface where germs can gather and possibly endanger people's health. That includes surfaces in hospitals, public buildings, factories, or in the home. The coating could be applied to almost any surface that people touch often such as metal, glass or plastic and would remove the need for constant cleaning with liquid disinfectants, especially in areas where hygienic conditions are crucial. People who normally cannot use hearing aids that lie inside the ear because of the risk of infection of the auditory canal can safely wear nanocoated appliances.

Bio-Gate (Nürnberg, Germany) produces NanoSilver BG, a nanoporous silver powder with particle size ranging from 50 to 100 nm. It has a homogeneous distribution of nanoparticles in the material and anti-infective properties.

Silver nanoparticles have been incorporated in preparations for wound care to prevent infection. Acticoat bandages (Smith & Nephew) contain nanocrystal silver, which is highly toxic to pathogens in wounds.

AcryMed's silver nanoparticle technology, SilvaGard, involves coating with silver nanoparticles with size range of 2–20 nm in a stable solution and antimicrobial treatment levels last for more than a year. With other technologies, nano-based silver coatings must be applied through vapor deposition, which coats only on one side, whereas AcryMed technology is a solution that provides a complete surface treatment rather than a coating.

Nanotechnology-Based Antiviral Agents

Silver Nanoparticles as Antiviral Agents

Silver nanoparticles possess many unique properties that make them attractive for use in biological applications. Silver nanoparticles are used as surface coatings for prophylaxis of infections. It has been shown that 10 nm silver nanoparticles are bactericidal, and possible use of silver nanoparticles as an antiviral agent is being explored.

Silver nanoparticles undergo a size-dependent interaction with HIV-1 and particles in the range of 1–10 nm attached to the virus (Elechiguerra et al. 2005). The regular spatial arrangement of the attached nanoparticles, the center-to-center distance between nanoparticles, and the fact that the exposed sulfur-bearing residues of the glycoprotein knobs would be attractive sites for nanoparticle interaction suggest that silver nanoparticles interact with the HIV-1 virus via preferential binding to the gp120 glycoprotein knobs. Due to this interaction, silver nanoparticles inhibit the virus from binding to host cells, as demonstrated in vitro.

Silver nanoparticles are capable of inhibiting a prototype arenavirus, Tacaribe virus, at nontoxic concentrations and effectively inhibit arenavirus replication when administered prior to viral infection or early after initial virus exposure (Speshock et al. 2010). This suggests that the mode of action of viral neutralization by silver nanoparticles occurs during the early phases of viral replication.

Fullerenes as Antiviral Agents

A series of bis-fulleropyrrolidines bearing two ammonium groups have been synthesized and their activities against HIV-1 and HIV-2 have been evaluated (Marchesan et al. 2005). Two *trans* isomers were found to have interesting antiviral properties, confirming the importance of the relative positions of the substituent on the C60 cage. None of the compounds showed any inhibitory activity against a variety of DNA and RNA viruses other than HIV.

Cationic, anionic, and amino acid–type fullerene derivatives have shown inhibitory effect against HIV-reverse transcriptase and HCV (Mashino et al. 2005). Out of all derivatives of fullerenes, anionic fullerenes were found to be the most active. All the tried fullerene derivatives were more active than the non-nucleoside analog of HIV-RT inhibitor. The effect of long alkyl chains on fullerenes was not significant; rather, it depressed the inhibition strength. The two important targets for anti-HIV characteristics are the HIV-protease and HIV-reverse transcriptase. The molecular modeling experimental designs exhibit that C60 core could penetrate into hydrophobic binding site of HIV protease. However, the mechanism of this anti-HIV activity is through HIV-protease inhibition, which has not been experimentally demonstrated.

Gold Nanorod-Based Delivery of RNA Antiviral Therapeutics

The emergence of the pandemic 2009 H1N1 influenza virus has become a worldwide health concern. As drug resistance appears, a new generation of therapeutic strategies will be required. Use of RNA immune activator molecule is limited by their instability when delivered into cells, but this can be overcome by using a nanobiotechnology-based delivery system. Gold nanorods protect the RNA from degrading once inside cells while allowing for more selected targeting of cells. Usefulness of delivery of a biocompatible gold nanorod, GNR-5′PPP-ssRNA nanoplex, has been demonstrated for innate immune activation against type A influenza virus (Chakravarthy et al. 2010). In human respiratory bronchial epithelial cells, this nanoplex containing the single-stranded RNA molecule activated the retinoic acid-inducible gene I pathogen recognition pathway, resulting in increased expression of IFN (interferon)-β and other IFN-stimulated genes (e.g., PKR, MDA5, IRF1, IRF7, and MX1), which resulted in a decrease in the replication of H1N1 influenza viruses. The novelty of this approach is that most of RNA viruses share a common host-response immune pathway, and enhancement of the host immune response reduces the ongoing viral resistance generated through mutations. Diseases that could be effectively targeted with this new approach include any viruses that are susceptible to the innate immune response triggered by IFN. Animal studies have started based on these in vitro results, and further evaluation of biocompatible nanoplexes as unique antivirals for treatment of seasonal and pandemic influenza viruses is warranted.

Nanocoating for Antiviral Effect

Laboratory testing of the permanent nanocoating SERQET™ (LaamScience Inc.) showed that coating kills 99.9 % of influenza viruses and 99.99 % of vaccinia viruses that cause rash, fever, and head and body aches. This technology may enable one to protect oneself from virtually all viruses and bacteria by simply exposing a surface to light.

In 2006, Mass Transit Railway (MTR), the corporation that runs Hong Kong subway, announced that nano silver-titanium dioxide coating (NSTDC, a nontoxic disinfectant) will be applied to surfaces that passengers commonly touch in order to enhance hygiene levels in MTR stations and trains. The coating is manufactured using nanotechnology, which maximizes coverage and effectiveness of NSTDC. Developed in Japan, NSTDC is certified to be effective in killing a wide range of bacteria, viruses, and mold, including the H1N1 Influenza Virus A. It is used in hospitals, offices, and homes in Japan. NSTDC's main component, titanium dioxide (TiO2), has been approved for use in foods by the FDA and under the Public Health and Municipal Services Ordinance in Hong Kong.

Nanoviricides

Nanoviricides (NanoViricides Inc.) are polymeric micelles, which act as nanomedicines to destroy viruses. As defined by NanoViricides Inc., "a nanoviricide is a polymeric single chemical chain with covalently attached ligands that specify the virus target. The antiviral spectrum of the drug is determined by the specificity of the set of ligands attached to the chain, in addition to other functionally important aspects inherent in the chemistries." Nanoviricide is designed to seek a specific virus type; attach to the virus particle; engulf or coat the virus particle, thereby neutralizing the virus's infectivity; and destabilize and possibly dismantle the virus particle; and optionally it may also be made capable of attacking the viral genome, thereby destroying the virus completely. Active pharmaceutical ingredients are optional and can be hidden in the core of the nanoviricide missile.

In contrast to other approaches, a NanoViricide™ micelle can recognize and bind to more than one type of binding site on the virus. The NanoViricide™ system enables design of a drug that binds to more than one type of site – currently as many as three different sites, on the virus – for a highly effective attack. NanoViricides Inc. terms this as "multi-specific targeting." A NanoViricide™ drug goes much further than just blocking all of the binding sites of the virus. The base material of a NanoViricide™ is a specially designed polymeric micelle material. It has the ability to disassemble an HIV particle by itself. Thus, after coating the virus particle, the NanoViricide™ loosens the virus particle and weakens it. Some virus particles will even fall apart (uncoat). This provides a further therapeutic benefit. NanoViricides plans to enhance the viral disassembly capabilities of the NanoViricides™ by attaching specially designed "molecular chisels" to the NanoViricide™. Once the

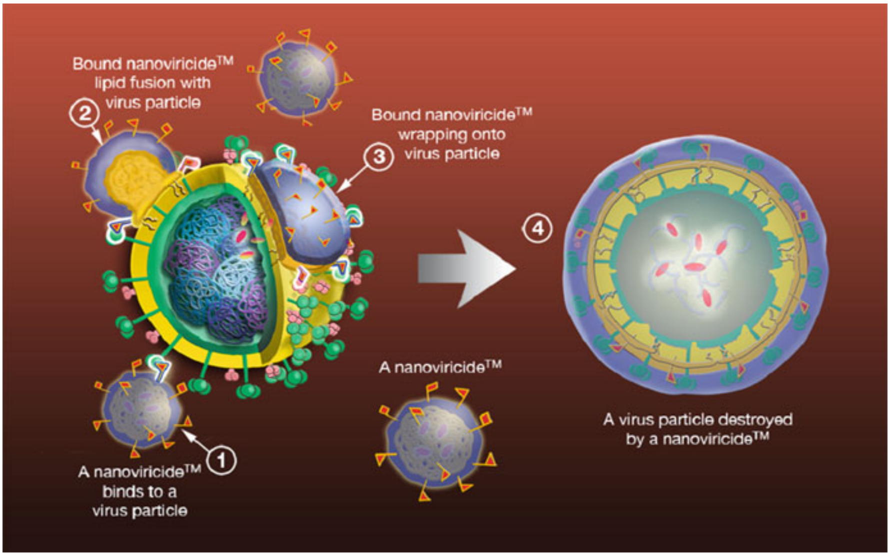

Fig. 14.1 Schematic representation of NanoViricide attacking a virus particle (Reproduced by permission of NanoViricide Inc.)

NanoViricide™ micelles coat the virus particle, the attached "molecular chisels" will go to work. They literally insert themselves into the virus coat at specific vulnerable points and pry apart the coat proteins so that the virus particle falls apart readily. The mechanism of action of NanoViricide is depicted schematically in Fig. 14.1.

NanoViricides have been compared to current approaches to viral diseases, which are seldom curative, and some of the advantages include the following:

- Specific targeting of the virus with no metabolic adverse effects on the host.
- The biological efficacy of NanoViricides drugs may be several orders of magnitude better than that of usual chemical drugs. This in itself may limit the potential for mutant generation.
- There are also other key aspects of the design of NanoViricides that are expected to lead to minimizing mutant generation.
- Nanoviricides are safe because of their unique design and the fact that they are designed to be biodegradable within the body.
- The new technology enables rapid drug development against an emerging virus, which would be important for global biosecurity against natural as well as man-made (bioterrorism) situations. It is possible to develop a research drug against a novel life-threatening viral disease within 3–6 weeks after the infection is found, i.e., as soon as an antibody from any animal source is available.
- It is possible to make a single NanoViricide drug that responds to a large number of viral threats by using targeting ligands against the desired set of viruses

in the construction of the drug. It is possible to "tune" the specificity and range (spectrum) of a NanoViricide drug within a virus type, subtype, or strain, by appropriate choices of the targeting ligand(s).

- The safety of NanoViricide drugs is proven now as they specifically attack the virus and not the host.
- A variety of formulations, release profiles, and routes of administration are possible.
- Low cost of drug development, manufacture, distribution.

Targets for this approach include influenzas, HIV, HCV, rabies, and other viruses. NanoViricide drug candidates are currently in preclinical studies. Clinical trials are planned. Initially injectable products are considered to be most effective but alternative routes of administrations such as nasal sprays and bronchial aerosols can also be developed.

NanoViricides Inc. developed and evaluated NanoViricides against influenza and avian flu H5N1 for efficacy and safety. FluCide™ nanoviricide is designed as a polymeric surfactant micelle which has covalently attached to its ligands that bind to the influenza A virus on conserved sites. The current drug candidate was effective against both H1 and H5 and different strains of H5. This is a direct result of using a conserved binding strategy, very similar to that used by zanamivir. However, FluCide is directed to bind to HA rather than to inhibit neuraminidase. Binding to HA followed by putative engulfment of the virus particle should lead to viral load reduction and therapeutic benefit. Preclinical studies have shown that FluCide does more than an antibody does, in that it completes the task of encapsulating and possibly dismantling the virus, rather than merely tagging it for the immune system as a foreign particle. In mouse model of common murine-adapted influenza A/H1N1, it would require about eight times greater dosage of oseltamivir as compared to FluCide to achieve the same survival advantage results. In cell cultures (MDCK as well as PMKC) against influenza A/H5N1 bird flu strains (Clade 1 as well as Clade 2), these drug candidates have shown as high as 70 % CPE inhibition. An intravenous preparation for systemic administration is in development with a ready-to-use preloaded syringe. Other routes of administration are being explored. A pre-IND briefing document about FluCide has been submitted to the FDA.