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Therapeutic and Diagnostic Nanomaterials

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Introduction

Identification or detection of a disease through its signs/symptoms or by analyzing the biomarkers is called diagnosis. Treatment of a disease after diagnosis is called therapy. Agents used for diagnosis and therapy are called diagnostics and therapeutics, respectively. Additionally, some instrumental methods or techniques are also used accordingly. Nanoparticles have wider applications in diagnosis and therapy due to their many unique properties, which are explained elsewhere. Nanomaterials of different morphologies and different forms, such as nanoconjugates and nanocomposites, are widely used for diagnosis and therapeutic purposes. Several reports indicate the high efficacy of nanodiagnostics and nanotherapeutics.

Theranosis combines the diagnosis and therapeutic applications of particles with unique properties into a single platform. This provides the dual benefits of therapy and the monitoring of disease conditions, treatments, and drug efficacy. Theranosis is mostly used in cancer diagnosis and treatment. Certain basic elements of diagnosis and therapy can essentially be coupled into a single platform as modules of theranosis, which are very essential for understanding the biomedical applications of nanoparticles: diagnostic and therapeutic nanomaterials, multifunctional nanoparticles, biomarkers, diagnostic and therapeutic techniques, and toxicity and risk assessment. This chapter systematically describes and illustrates these five modules. The final section of the chapter focuses on a few *in vitro* and *in vivo* applications of select nanomaterials.

Chapter 1
Diagnostic and Therapeutic Nanomaterials

1.1 Why Are Nanoparticles Ideal Diagnostic and Therapeutic Agents?

Several studies on the biomedical applications of nanoparticles clearly reveal that quite a few distinctive properties of nanoparticles hold promise in diagnostic and therapeutic applications (Wang et al. 2012 and Yang et al.):

1. Smaller size, possibly allowing localization in specific sites for imaging and diagnosis. This would also result in high drug/imaging agent loading capacity, thus helping in treatment and diagnostic efficacy.
2. Smaller surface-to-volume ratio, which reduces the rate of glomerular filtration.
3. High circulatory half-life, which increases the time of retention in the blood.
4. Enhanced permeation and retention (EPR) effect and high extravasation effect: EPR refers to the discriminatory accumulation of particles in tumor tissues. The diminished size of nanoparticles enables them to extravasate from the haphazardly dilated and unusually leaky blood vessels of tumor tissues. As a result, the nanoparticles will have a higher degree of retention in the tumor tissue and concomitantly lower degree of lymphatic drainage.
5. Tunable surface area: The high surface area enables functionalization with ligands, as a drug, imaging agent, targeting molecules, and stabilizing agent, among others, thus forming a multifunctional motif.
6. Systemic toxicity is one of the major serious complication associated with anticancer therapy. There is an urgent need to reduce systemic toxicity. This can be achieved if the drug to be delivered is specifically and selectively targeted to the desired tissues; this, however, is a real challenge.
7. Additionally, in terms of an imaging agent, a good contrast can be achieved for a good resolution by elevating the local concentrations of the agent, which in turn increases the target-to-background contrast in imaging.

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8. The innate immune system of the body may recognize theranostic agents as a foreign particle, trying to clear them from the circulation. Nanoparticles, as mentioned earlier, possess enhanced circulatory half-life and may escape the immune clearance. Thus, they are highly suitable for theranostic applications, emphasizing the need for nanotheranostics.

1.2 Nanotheranostic Candidates

Generally, fluorescent particles are used in diagnosis and optical imaging, including quantum dots, gold nanoparticles and magnetic particles. Good photostability, narrow emission spectra, broad excitation wavelength, and feasibility for bioconjugation and surface modificcation makes these particles excel in the biomedical and imaging fields when compared to fluorophores.

(a) **Metals**

Enhanced active surface area, highly tunable optical properties, flexible size, a straightforward green synthesis protocol, and facile surface chemistry are advantages of noble metal nanoparticles (Suganya and Devasena 2015a, b). In addition, noble metal nanoparticles can easily be functionalized with different types of ligands (see Sect. 1.2), thus enabling them for targeting and efficient imaging. Moreover, noble metals, such as gold and silver, can be functionalized in situ by phytochemicals with therapeutic value or by the therapeutic product (Devasena et al 2010; Devi et al 2014). The chances for the thermal ablation of tumors is higher with noble metal nanoparticles such as gold and silver because they are capable of converting the incident radiofrequency radiation or light into heat energy. The rate of tumor selectivity and non-cytotoxicity is more with the metal nanoparticles and selective targeting is mediated by one of the several strategies (Conde et al. 2012), as summarized in Table 1.1.

(i) ***Enhanced permeation and retention effect or passive targeting***

The microenvironment of tumor tissue differs from that of normal tissue. In normal tissue, the microvasculature endothelium is intact and the lymphatic drainage system is normal. Many unusual features of rapidly proliferating tumor cells include rapid formation of new blood vessels (i.e. angiogenesis), disorganization in the otherwise hierarchical vascular network, misalignment and defective architecture of endothelial cells with fenestrations (i.e. the endothelium of the blood vessel is leaky and highly permeable to macromolecules), the presence of wide lumen, and the absence of tumor lymphatic drainage (Fig. 1.1).

In normal environments, only smaller particles will easily diffuse through the endothelium into cells and are cleared by otherwise healthy lymphatic drainage. In the tumor environment, in addition to the smaller particles, macromolecules also gain access into the cells due to a highly permeable endothelium—the so-called

Table 1.1 Selective targeting strategies used by nanoparticles

Strategy	Effect
Enhanced permeation and retention effect or passive targeting	Quantitative accumulation of nanoparticles into tumor cells to inhibit angiogenesis and cell growth
Enhanced circulatory time	Overcoming an attack by macrophages, leading to high therapeutic efficacy of the drug Conservation of the imaging agent for high-resolution diagnosis
Active targeting	Receptor-mediated alterations in the signaling pathways that are related to the cell proliferation Receptor-mediated uptake of imaging ligands for diagnosis
Gene knockdown	Blocking the expression of the mRNA that codes for cancer-promoting proteins
Thermal ablation	Mechanical destruction and killing of cancer cells by high temperatures
Radiosensitizing effect	Destruction of cancer cells by means of free radicals generated via radiation
Enhancing the Raman scattering signal	In vivo imaging and image-guided therapy
In situ NIR probes	High contrast in situ imaging of diseased cells

enhanced permeability effect. However, they are not rapidly cleared away because the lymphatic drainage system is defective. Consequently, the molecules are retained and accumulate within the tumor tissues.

The unusual features of the tumor environment provide more chances for nanoparticles, especially metal ones, to gain quicker and quantitative access into the tumor cells leading to selective accumulation. This phenomenon is called the EPR effect, which enables selective accumulation of the desired material at the tumor site. As the nanoparticle is delivered to the circulatory system for subsequent targeting of the diseased site as a natural physiochemical response of the body to the drug, this phenomenon is called passive targeting. Pegylated metal nanoparticles, especially gold nanoparticles, can result in the passive targeting of tumor cells. An example of this mechanism is discussed in the final section of this chapter.

(ii) ***Enhanced circulatory time***

Upon functionalization with hydrophilic moieties, such as polyethylene glycol (PEG), the metal nanoparticles will stealth themselves from the attack of the macrophages in the reticuloendothelial system (RES) in vivo. In addition, the nanoparticles are deprived from enzymatic degradation, thus prolonging the circulatory half-life. This would result in efficient therapy.

(iii) ***Active targeting***

In an active targeting process, the bioconjugated metal nanoparticles actively target the extracellular or intracellular receptors or the carcinogenesis-related signaling

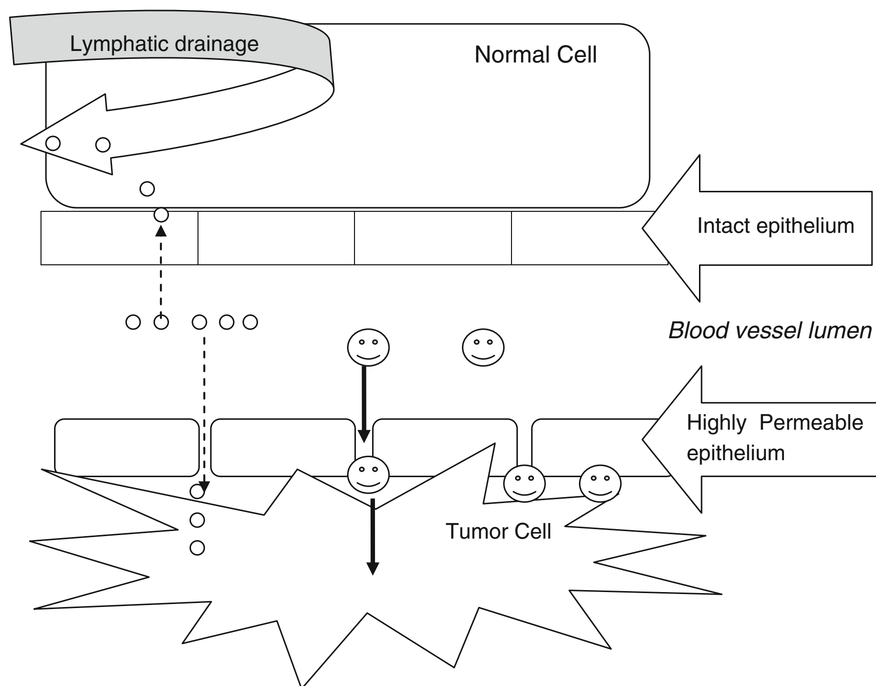

Fig. 1.1 The enhanced permeation and retention effect mediated by an altered tumor microvascular environment. The movement of small molecules is indicated by *dotted arrows*. The movement of larger molecules is indicated using *solid arrows*

pathways to control the tumor growth instead of natural uptake by the RES. Monoclonal antibodies raised against the receptors, peptides, and nucleic acids (DNA and RNA sequences) are useful active targeting biomolecules (Fig. 1.2).

(iv) ***Gene knockdown***

Gene knockdown is the process of blocking the expression of a gene by silencing it. Antisense DNA, small interference RNA, or short interference RNA (Si RNA) are used for this purpose. Si RNA is a short synthetic oligonucleotide tailored to be complementary to a specific mRNA (the target mRNA). It knocks down the target mRNA by interfering with its expression. However, gene knockdown agents possess certain demerits such as: instability, short half-life, more susceptibility to hydrolytic cleavage by nucleases, and vector dissociation. The siRNA and its delivery vector must associate strongly to remain intact during cellular adhesion and subsequent intake. After this, the vector-SiRNA construct should dissociate to execute the intracellular action. These disadvantages can to a certain extent be circumvented by gold nanoparticles, thereby enabling improved cellular uptake of

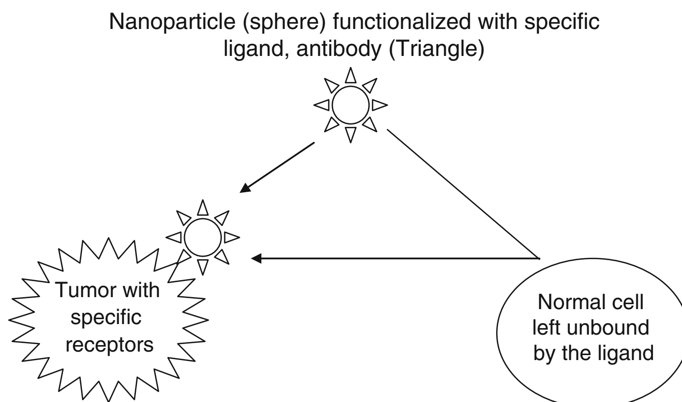

Fig. 1.2 Active targeting of cancer cells by ligand-bound nanoparticles

oligonucleotides and effective gene silencing. Gold nanoparticles can be designed into covalent and noncovalent gene carriers (Ding et al. 2014).

(v) ***Thermal ablation***

Noble metals, such as gold nanoparticles, convert light radiation into heat energy and elevate the local temperature of the cancer cells beyond their tolerance limit, thus killing the cells. This is called thermal ablation or hyperthermia. Bimetallic void and branched nanostructures consisting of silver and gold nanorods (Ag–Au NR) are advantageous in the context of a lower dose and lower irradiation power. Surface-bound aptamers require a low-power laser pulse for inducing thermal ablation. Additional details of thermal ablation are discussed in Chap. 4.

(vi) ***Radiosensitizing effect***

Metallic nanoparticles (e.g., platinum, gold, silver) are capable of absorbing X-rays, can function as dose enhancers, and generate free radicals, which in turn can induce cancer cell death. Chapter 4 discusses radiotherapy techniques in detail.

NIR scattering, surface-enhanced Raman scattering (SERS), computed tomography (CT), magnetic resonance imaging (MRI), optical coherence tomography (OCT), and photoacoustic imaging (PAI) are the major diagnostic/therapeutic techniques. Noble metal nanoparticles are used in all of these techniques (see Chap. 4; also Conde et al. 2012). In all of the imaging techniques, noble metals are used to enhance the contrast; in therapeutic techniques, they are used to increase the absorption and scattering of radiation.

(vii) ***Enhancing the Raman scattering signal***

Enhancement of the magnitude of the Raman scattering signal on a metal surface is called SERS. The spectroscopic signal generated by SERS can be translated into an optical signal and used for in vivo imaging and image-guided therapy. Gold nanoparticles are excellent enhancers of Raman signal. The dye molecules (i.e. the

Raman reporters or the Raman tags) are adsorbed on the surface of the gold nanoparticles and then protected by a layer of polymer or silica; they augment the Raman signal of the dye. Gold nanoparticles enhance the Raman scattering of the dye to as high as 10^{14}- to 10^{15}-fold and play a vital role in imaging (Yigit and Medarova 2012).

(viii) **In situ** NIR ***probes***

Conventionally, organic dyes are used as NIR probes for in situ imaging and diagnosis of cancer. However, these probes have the following disadvantages: poor water solubility, instability and susceptibility to photobleaching, low quantum yield, less sensitivity in biological systems, and weak multiplexing capability. Noble metal nanoparticles can very well circumvent these disadvantages and enhance the contrast of the imaging system. For example, silver nanoshells can detect microRNA in lung cancer cells and enhance the emission intensity and photostability. Gold-silver nanoshells enhance the image contrast of live breast cancer cells, overexpressing the herceptin receptor mechanism (Conde et al. 2012). Gold nanoparticles rank first among metallic nanotheranostics, owing to some extraordinary qualities, as outlined in Table 1.2.

The size and the morphology of gold nanoparticles can be tuned by varying the synthesis conditions, thus making them a versatile material. The size determines the optical properties, giving a brownish to pinkish to ruby red color as the size diminishes (Fig. 1.3). As the size of the particle increases, the surface plasmon resonance wavelengths progress towards the infrared segment of the spectrum and most visible wavelengths are reflected, making the nanoparticles almost colorless or translucent. Different morphologies of gold nanoparticles can be used for different applications. For example, nanoparticles, nanorods, nanocages, nanoshells, nanocubes, and nanourchins are highly useful in biomedicine. Gold nanourchins have unique optical properties compared to spherical gold nanoparticles of the same core diameter. Gold nanourchins have uneven surfaces formed of sharp spikes, which results in an entirely different surface plasmon resonance peak when compared to

Table 1.2 Extraordinary qualities of gold nanoparticles that contribute to their biomedical properties

Quality	Application
Enhanced biocompatibility	Sustained drug delivery
Providing room for surface modification	Targeted drug delivery, targeted imaging
Unique optical properties	Imaging of cancer cells
Unique photothermal properties	Cancer therapy
Tunable LSPR (Localized surface plasmon resonance)	Diagnosis
Tunable morphology	Sensor for the diagnosis of molecules in the biomedical field
Ability to form bonding with diagnostic and therapeutic ligands	Nanotheranosis
High X-ray absorption coefficient	CT contrast agent, radiotherapy sensitizer

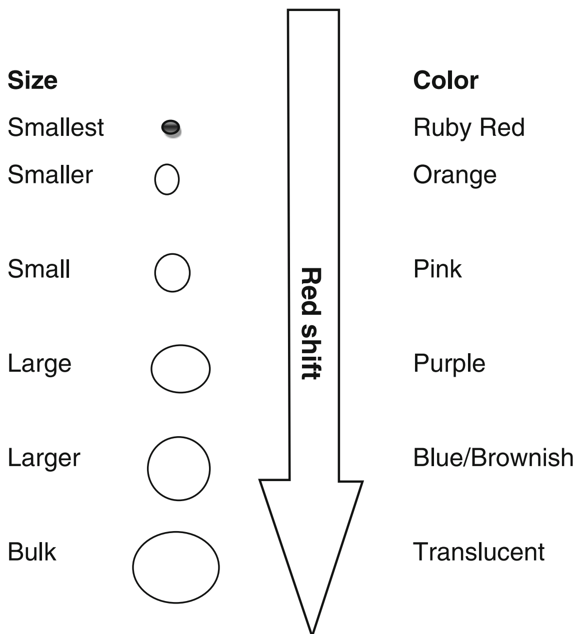

Fig. 1.3 Size-dependent optical properties of gold nanoparticles that are useful in biomedical imaging

nanoparticles of same core diameter, giving a red shift (Fig. 1.4). For instance, 100-nm-diameter gold nanoparticles and nanourchins have peaks at 570 and 680 nm, respectively. As a result, urchins are widely used in applications associated with SERS, such as the label-free sensing of proteins, pollutants, and other molecules. When excited with 700- to 800-nm wavelength light, gold nanoshells and nanorods are capable of absorbing near infrared radiation. They get excited and produce heat to kill tumor cells—so-called photodynamic therapy (discussed in Chap. 4).

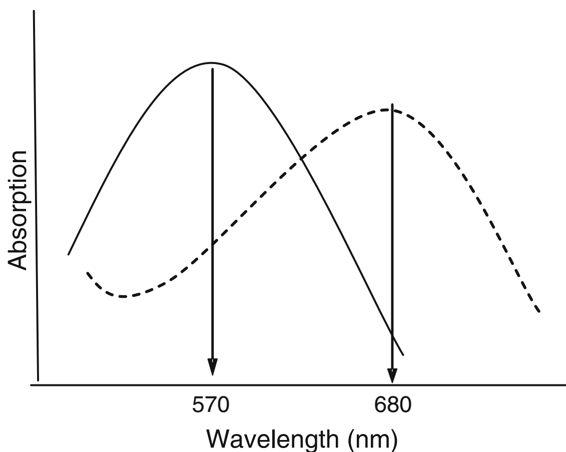

Fig. 1.4 Red shift of gold nanourchins (*dotted peak*) as compared to spherical gold nanoparticles (*solid peak*)

The high surface-to-volume ratio of gold nanoparticles facilitates their surface modification with ligand molecules such as drugs and targeting agents (see Chap. 2). Gold nanoparticles are also used in the sensing of microorganisms, such as *Mycobacterium* to enable the diagnosis of tuberculosis (Wojcik et al. 2013). Multifunctionalized gold nanoparticles containing PEG, biotin, paclitaxel (PTX), and rhodamine B-linked β-cyclodextrin exhibit theranostic applications for cancer treatment without affecting healthy tissues. Some representative applications of gold nanoparticles in diagnosis and treatment are in the last section of this chapter.

(b) ***Quantum dots***

Quantum dots are zero-dimensional semiconductor particles or nanocrystals of 2–10 nm size. The size and optical properties of semiconductor quantum dots are tunable. Therefore, they provide multiple color imaging with a several-fold increase in brightness, thus emerging as a better fluorescent label for cellular imaging. Near-infrared emitting quantum dots are highly suitable for in vivo applications as they penetrate into deeper tissues, owing to their longer wavelength as compared to visible light. Moreover, water and the hemoglobin molecules (the major medium in vivo) do not significantly absorb these rays, thus minimizing the chance for false-positive signals. In spite of their applications, toxicity testing and the assessment of the safety level of the quantum dots, especially while imaging deeper layers of tissues, warrant further study (Choi and Wang 2011).

(c) ***Silica-based nanocrystals***

Silica-based nanoparticles exhibit unique surface properties, such as porosity and functionalization capacity. Moreover, the preparation of silica-based nanoparticles is very easy and inexpensive. Hence, they are widely used as drug carriers, contrast agent protectors, and pharmaceutical additives. High natural abundance, less toxicity, good biocompatibility, and surface-tunable optical properties have made silicon nanocrystals (SNC) as biomedical imaging contrast agents. SNC of 2–5 nm are excellent photosensitizers of singlet oxygen generation; photo-excitation results in the suppression of cancer cell growth and proliferation. Crystalline and porous Si can be mechanically milled into aqueous suspensions of SNC, which can function as an efficient sonosensitizers. A sono-sensitive SNC destroys cancer cells upon excitation with ultrasound radiation (Osminkina et al. 2011; Bitar et al. 2012).

(d) ***Magnetic nanoparticles***

Magnetic nanoparticles (MNPs) and multifunctional magnetic nanoparticles (MFMNPs) show potential for biomedical applications because they can be directly injected into the body and manipulated using an external magnetic field. MNPs and MFNPs are widely used as magnetic resonance contrast agents in MRI, magneto-hyperthermia for cancer treatment, magnetic force-assisted drug delivery, tissue repair, cell and tissue targeting and transfection, and protein isolation. More details of MRI and hyperthermia are discussed in Chap. 4. In nanoscale, magnetic particles shift from paramagnetic to superparamagnetic behavior (the SPM behavior), thus

forming superparamagnetic nanoparticles such as superparamagnetic iron oxide nanoparticles (SPIONs). The magnetic moment of these particles as a whole can be tuned in response to thermal energy, while the individual atomic moments maintain their ordered state relative to each other. The SPIONs can be magnetized only in the presence of an external magnetic energy and do not retain any magnetism in the absence of magnetic fields, which enables them to form stable colloids in a physio-biological medium (Varanda et al. 2011). Owing to their SPM behavior, SPIONs find extensive applications in sensing, diagnosis, and therapy (see Chap. 5).

(e) ***Polymers***

Polymers are endowed with excellent encapsulation properties, especially when designed as multifunctional carriers. Examples of polymeric nanocarriers with successful biomedical applications are summarized in Table 1.3. Formulations of

Table 1.3 Polymeric nanocarriers suitable for drug delivery and imaging

Polymer	Example	Ideal properties	Proven applications
Chitosan	5β-cholanic acid-modified chitosan	Rapid cellular uptake	Live imaging using Cy5.5 and targeted delivery of paclitaxel for cancer treatment
Gelatin	PEG-coated gelatin nanoparticles	Biodegradability and biocompatibility, presence of different accessible functional groups	Efficient delivery of photodynamic agent, hypocrellin B
Dendrimers	Poly amidoamine dendrimer	Tunable surface function and polyvalent surface	Efficient gene delivering capacity
Amphiphilic block copolymers	Maleimide-terminated PEG-*co*-poly(d-, l-lactic acid), (Mal-PEG-PLA) and methoxy-terminated PEG-*co*-poly(d-, l-lactic acid) (MeO-PEG-PLA))	Micelle formation and accommodation of hydrophobic and/or hydrophilic cargos	Ultrasensitive MR detection
Polymeric core shell structures	Poly(propargyl acrylate): PEG	Enhancement of imaging signal	Controlled growth of liver tumor cells
Grafted poly-aminoacid	Octadecyl-grafted poly-aspartatic acid	Self-assembly and biodegradation	Delivery of doxorubicin and imaging via iron oxide nanocrystals
PLGA	PEGylated PLGA	High encapsulation efficiency, high cellular uptake	Targeted delivery of doxorubicin
Metal-loaded polymeric capsules	Gold nanoparticle-loaded PEGylated dendrimers	Photothermogenic properties	Computed tomography imaging and thermal killing of cancer cells

BDMCA (Bisdemethoxy curcumin analog) can be prepared by encapsulation into polymeric nanovesicles or polymeric nanocomposites. For example, chitosan or chitosan-starch nanocomposite can be used. The BDMCA–chitosan-starch nanocomposite is more effective than free BDMCA in killing the MCF-7 breast cancer cell line, as evidenced by MTT assay (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) and cytotoxic activity. Additionally, the chitosan-starch nanocomposite is an ideal carrier that shows good encapsulation efficiency and sustained release efficacy (Subramanian et al. 2014).

(f) ***Carbon-based nanomaterials***

Carbon-based nanomaterials include zero-dimensional carbon dots, nanodiamond and fullerene, one-dimensional carbon nanotubes (CNTs), and two-dimensional graphene sheets. These materials hold superior mechanical and optoelectronic properties. Green synthesized carbon dots have bright luminescence, aqueous stability, and low cytotoxicity, thus exhibiting high potential for biomedical applications such as imaging and detection. (Kim et al. 2014). Carbon quantum dots synthesized from bagasse using hydrothermal carbonization show high monodispersity, photoluminescence, superior photostability, and aqueous dispersibility. Moreover, they show good biocompatibility and rapid internalization through the biological membrane. Hence, they are useful in biolabelling and bioimaging of cancer cells (Du et al. 2014).

Nanodiamonds (NDs) are classified into three types based on size: diamond nanocrystals (10–150 nm), ultrananocrystalline diamond particles (2–10 nm), and diamondoids (1–2 nm). NDs show great possibility for drug delivery due to their high biocompatibility, superior payload, and capacity to traverse the lipid membrane. NDs are an ideal tool to deliver genes as well as hydrophobic drugs and chemotherapeutic molecules with sustained release. Additionally, their high refractive index and Raman optical activity have bestowed NDs with scattering properties, thus making them suitable for cellular imaging (Kaur and Badea 2013).

Ligand-functionalized single-walled carbon nanotubes (SWCNTs) are good carriers for the sustained targeted delivery of anticancer drugs, proteins, and genes. SWCNTs possess intrinsic fluorescence in the near-infrared range, which makes them an excellent imaging tool. The intrinsic Raman and photoacoustic signals produced by SWCNTs are useful in detection techniques in the biomedical field.

Fullerene (C60) possesses a cage-like structure with a delocalized double bond. Owing to its structure, C60 functions as an effective free radical scavenger superior to conventional antioxidants. Hence, it has potential biomedical applications. Nevertheless, the high hydrophobicity of C60 affects its direct biomedical applications. To overcome this problem, functionalized C60 is used as an effective nanotheranostic agent. C60 is used in photodynamic therapy, neuroprotection, apoptosis, drug and gene delivery, and magnetic resonance imaging (Partha and Conyers 2009).

Table 1.4 Diagnostic applications of graphene and its derivatives

Target sensed	Material used
Glucose	Palladium-functionalized graphene
	Nickel oxide-functionalized graphene
	Copper-functionalized graphene
	Palladium-functionalized graphene
	Platinum-nickel alloy nanoparticle functionalized graphene
Dopamine	EDTA-graphene-nafion
	Nitrogen-doped graphene
Cholesterol	Gold-functionalized graphene
DNA	Graphene oxide functionalized with gold nanorods
Escherichia coli	Graphene sheets with anti-*E. coli* antibodies
Acetyl choline esterase	Graphene sheets with titanium oxide nanoparticles

Graphene, a two-dimensional carbon allotrope, has attracted attention because of its extraordinary physiochemical and electrochemical properties. Its unique and remarkable properties paved the way for the development of electrochemical biosensors. The electrochemical behavior of graphene makes it an ideal sensor for current molecular diagnostic techniques, as reviewed by Pandey et al. (2014). The diagnostic applications of graphene-based sensors are reviewed by Devasena and Francis 2014 and shown in Table 1.4.

(g) ***Upconversion nanoparticles***

Upconversion is an optical process by which lower energy photons of near-infrared radiation are converted to higher energy photons via a nonlinear optical process. Luminescent that are capable of upconversion (i.e. capable of producing high-energy visible light under near-infrared irradiation) are called upconversion nanoparticles (UCNPs). Examples are rare earth-doped UCNPs, such as lanthanide-doped nanocrystals and some fluorophores. UCNPs possess wider applications in imaging and biodetection in vitro and in vivo owing to exclusive luminescence, the ability to penetrate deeply into biological tissues, high signal-to-noise ratio, greater Stokes shifts, sharp absorption and emission lines, long half-life, and high photo-stability (i.e. lesser susceptibility for photobleaching; Chen and Zhao 2012).

1.3 Safety Concerns with Medically Important Nanoparticles

As the biomedical applications of nanoparticles continue to expand, the safety concerns associated with their use are equally important. The response of the biological system to a nanoparticle is different than that of the bulk counterpart (Devasena and Rajasekar 2014). For example, binary quantum dots are one of the

best tagging materials, but they pose severe health risks. Occupational and intentional exposure to carbon nanotubes and nanofibers may result in inhalation toxicity beyond a certain level (Prakash et al 2015). In this regard, it is essential to assess the toxicity, mechanism of action, biodistribution pathway, and fate of the nanoparticles in the living system. This is indeed a complicated task because the toxicity data of one particle cannot be correlated with that of the other; also, the same material with different properties and surface functions would have different biological effects. Hence, the assays related to the safety concerns should be done separately for each type of particle in vitro and in vivo, which results in a new emerging subdiscipline of nanotechnology called nanotoxicology. Nanotoxicology is a part of nanomedicine, without which marketing of the drugs would be in question. This is also a social and public health issue to be addressed. Interesting examples of the toxicity of nanoparticles are discussed in Chap. 6.

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Chapter 2
Multifunctional Nanoparticles

The functionalization of nanoparticles with one or more specific chemical moieties, so-called ligands, results in multifunctional nanoparticles. They are widely used in biomedical applications, especially drug delivery, cancer therapy, diagnostics, tissue engineering, and molecular biology (Svenson and Prudhomme 2012). Ligands are named based on their functions, such as stabilization, targeting, imaging, anti-immunogenics, and biocompatibility improvement (Table 2.1). Therefore, it is important to discuss the ligands used for the functionalization of nanoparticles.

2.1 Stabilizing Ligands

Generally, surfactants are used for stabilizing nanoparticles. These ligand molecules adhere to the nanoparticles in situ during the synthesis process itself, usually by chemisorption, electrostatic attraction, or hydrophobic interaction. Various chemical functional groups possess a certain affinity for inorganic surfaces, the most common example being thiol-gold affinity. These molecules exert repulsive forces due to electrostatic repulsion, steric exclusion, or the hydration layer on the surface; they serve to prevent the aggregation of the particles and stabilize them. This is an important aspect because the stability of the drug or the drug carrier influences the efficacy of the therapy. For example, the surface charge of a gold nanoparticle becomes neutral, causing aggregation. Therefore, the color changes from ruby red to blue. However, surface modification with ligands such as polymers, small molecules, and biological recognition molecules prevents aggregation. Gold nanoparticles can be stabilized using thiol surface functions. Surface-stabilizing ligands are of three types (Table 2.2): polar, nonpolar, and amphiphilic (Sperling and Parak 2010).

The stability factor, the reducing factor, and part of the therapeutic factor are one and the same if the nanoparticles are generated using a green synthesis protocol.

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Table 2.1 Ligands used for biomedical applications

S. No	Ligand type	Function	Examples
1	Stabilizing ligand	Stabilizes the drug or the drug delivery system	Flavonoid-stabilized metal nanoparticles
2	Targeting ligand	Site-specific binding of the drug delivery/imaging system	Lung targeting peptide (LCP), hyaluronic acid
3	Imaging ligand	Monitoring and diagnosis of tumor and drug distribution	Indocyanin green, fluorophores
4	Biocompatibility ligand	Decreasing the host–implant or host–drug carrier interaction	Self-assembled monolayers, carboxyl groups, hydroxyl groups, amino groups, plasma filming
5	Anti-immunogenic ligand	Preventing an immune response against biomedical agents, enhancing the circulatory time, restricting the clearance by the reticuloendothelial system (RES)	Polyethylene glycol (PEG)

Table 2.2 Surface-stabilizing ligands

Stabilizing ligand type	Example
Polar	Carboxylic Sulphonic acid groups
Nonpolar	Trioctylphosphine oxide (TOPO) Triphenylphosphine (TPP) Dodecanethiol (DDT) Tetraoctylammonium bromide (TOAB) Oleic acid (OA)
Amphiphilic	Poly(ethylene imine) (PEI) Poly(acrylic acid)

This is the advantage of a green synthesis approach. For example, silver nanorods synthesized using germinated fenugreek seed extract utilized phytochemicals for reducing the substrate silver nitrate, for stabilizing the nanoparticles, and also in the therapy by acting as an anticancer agent (Suganya and Devasena 2015). Similarly, during the synthesis of gold nanocubes, the polyphenol curcumin analog served a dual role of reduction and stabilization (Devi et al. 2014).

Polyethylene glycol (PEG), also called poly(ethylene oxide) or polyoxyethylene, is an amphiphilic polymer that is widely used as a ligand for drug delivery. The process of the coupling of PEG to a host molecule is called PEGylation; the modified molecules can be referred as PEGylated carriers. The following advantages and unique properties of PEG make it an excellent ligand for biomedical applications:

- High stability and inertness
- High biocompatibility
- Nontoxic nature
- High steric effects
- High hydrophilicity, leading to an increase in the solubility of the nanocarrier
- Decreased immunogenicity and lesser probability to bind to the antibody molecules
- Ability to confer higher circulatory half-life to the host molecules
- Ability to shield the core of nanocarriers from degradation by steric hindrance
- Capacity to increase the hydrodynamic size in order to reduce the renal clearance, increasing the solubility of nanocarriers as a result of its hydrophilicity
- When the cargo is a protein molecule, PEG is useful in preventing the enzymatic attack by proteases.

To conjugate PEG with the drug or the drug carrier, the reacting end of the PEG should be pre-activated. This can be done by using cyanuric chloride, or by the method of succinimidyl succinate, imidazoyl formate, succinimidyl carbonates, or succinimidyl esters. The activated PEG can form conjugates with drugs such as proteins, peptides, enzymes, and cytokines or with carriers such as liposomes, polymers, micelles, hydrogels, nanoparticles, or antibody-conjugated nanoparticles. For example, PEG can be attached to the α- or ε- amino groups of lysine, the thiol moiety of the cysteine, or the N-terminal amino groups of protein drugs.

PEGylated interferons were synthesized for the treatment of hepatitis C and hepatitis B viruses. PEG-conjugated human growth hormone antagonist has potential for treating acromegaly. PEG molecules were reported to increase the thrombopoietic activity of interferon-6 and the antitumor activity of tumor necrosis factor by several fold. PEGylation is known to increase the penetration of polymeric nanocarriers across the blood-brain barrier for drug delivery into the brain. PEGylated liposomes show greater circulatory half-life than the unPEGylated ones. Polymeric hydrogels formed with PEG exhibit superior wound-healing activity and biodegradability. PEG has the potential to enhance the gene delivery efficacy of vectors. The circulatory half-life of insulin can be enhanced by conjugation with PEGylated nanoparticles for biological and pharmaceutical applications (Otsuka et al. 2003).

2.1.1 Biomolecules

Biomolecules such as lipids, vitamins, peptides, and sugars and biopolymers such as proteins, enzymes, DNA and RNA molecules can be used as ligands and can be attached to the nanoparticles (so-called bioconjugation). The resulting product is referred to as a nano-bioconjugate or a nano-biohybrid. Such hybrids will combine the unique properties of both the components—that is, the biological specificity (the

molecular recognition property) of the biomolecule and the fluorescent/magnetic property of the nanoparticles (Sperling and Parak 2010).

Bioconjugation can be carried out by following methods:

- Chemisorption, where the ligand binds to the core surface without establishing covalent bonds
- Electrostatic adsorption, which can be established due to attraction between the oppositely charged moieties in the core and the ligand
- Covalent bonding
- Molecular affinity as in the case of biotin-avidin system and protein tags or a polyhistidine system.

2.1.2 Avidin-Biotin Conjugate

The avidin-biotin system is used to attach biomolecules to nanoparticles. Biotin, a small molecule (vitamin H) with one free carboxylic function is first attached to the nanoparticles via conjugation chemistry or through ligands. Biotin-modified nanoparticles can bind to avidin via covalent interaction. Avidin is a tetramer in which each subunit has stronger binding affinity for biotin. At pH below the Pi, avidin has a positive charge, thus enabling electrostatic interaction with negatively charged nanoparticles. Streptavidin can be conjugated through its carboxyl or primary amine function to the quantum dots or alternatively adsorbed directly via a polyhistidine tag (Sperling and Parak 2010). This would help in targeted imaging. The avidin/biotin-liposome system is used for sustained peritoneal drug delivery and also delivery into associating lymph nodes in an ovarian cancer xenograft model (Zavaleta et al. 2007).

2.2 Targeting Ligands

Cancer treatment by cytotoxic drugs is usually associated with a risk of non-specific off-target damaging and collateral toxicity. Hence, there is a need for selective targeting of cancer cells based on the recognition of cancer-specific antigens (the tumor-associated antigens), which are overexpressed in the cancer cells alone. This can be achieved by conjugating the drug with another molecule that has affinity for the surface antigens. Consequently, the drugs will hamper the proliferation and induce apoptosis of cancer cells. These molecules are called targeting ligands. The ligand is usually attached to a spacer, which in turn is linked to the drug-loaded nanoparticles by means of a dissociable bridge, thus forming an assembly (Fig. 2.1). The ligand will direct the assembly towards the cancer cell, thereafter

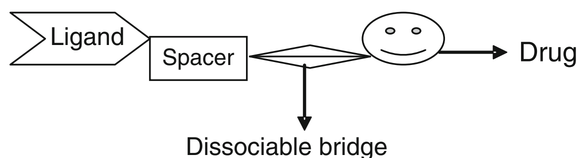


Fig. 2.1 Targeting ligand-drug assembly

entering into the cell by endocytosis. The bridge is capable of dissociating and releasing the drug once the assembly is engulfed after endocytosis. This is called the "magic bullet approach" of drug targeting, as first proposed by Paul Ehrlich (Muro 2012). The most important criteria for a targeting ligand are (i) high binding affinity to the target, (ii) low immunogenicity to the host, and (iii) high penetrating capacity into the target cell.

Table 2.3 shows the classification of targeting ligands. Monoclonal antibodies require a more detailed explanation because they are the best targeting ligands in view of their specificity and selectivity towards tumor cells. Other targeting ligands include antibody derivatives, peptide, aptamers, and proteins such as DARPins, transferrin, lactoferrin, and lectins, as well as small targeting molecules such as folates and mannose derivatives.

Table 2.3 Summary of targeting ligands

Targeting ligand type	Examples	Target
Antibodies	Cetuximab	Epidermal growth factor receptors overexpressed on the surface of certain cancer cells
	Herceptin or rituxumab	CD20 proteins expressed on the surface of B cells
Antibody fragments/antibody derivatives	Fab fragment	Used to target drug-loaded liposomal vesicles Also used for targeting specific receptors in the cells of breast, colon, lung and ovary
Peptides	DARPins	Targeting the amyloid β protein to treat Alzheimer disease
	Arginine–glycine–aspartate (RGD)	Targeting the integrins of endothelial cells, epithelial cells, and glioblastoma cells and delivery of paclitaxel and doxorubicin
Aptamers	Oligonucleotides of DNA	Diagnosis and treatment of viral infection
	Oligonucleotides of RNA	Diagnosis and treatment of viral infection
Small targeting molecules	Transferrin	Transferrin receptors upregulated on the surface of cancer cells
	Lectin	Luminal surface of small intestine
	Folate	Folate receptors expressed in many malignancies

2.2.1 Antibodies

Among antibodies, IgG is mostly used. Antibodies can be used as such, in the form of fragments (e.g., Fab fragment obtained by enzymatic cleavage of antibodies or by genetic engineering), or as immunoconjugates.

2.2.2 Antibody Fragments/Antibody Derivatives

Fab fragments of antibodies (Fig. 2.2) can be covalently cross-linked to a drug carrier for targeting. The Fab fragment of antibody may be coupled to a nanocarrier encapsulated with the drug. This may result in site-specific drug release. For example, a Fab fragment with a projected thiol group forms a stable disulphide bridging with the pyridothiol derivative of phosphotidyl ethanolamine, which constitutes the liposome carrier (Attarwala 2010).

Immunoconjugates are antibodies linked to an effector molecule, which is the cytotoxic agent. The immunoconjugates are either an immune-drug conjugate, immunotoxin, or radioimmuno-conjugate, depending on whether the effector molecule is a drug, protein, or radionuclide, respectively (Attarwala 2010). An immune-drug conjugate is internalized into the cells by endocytosis and thereafter enzymatically hydrolysed by the lysosomal hydrolases to release the drug. The effector proteins of immunotoxins are usually cytotoxic enzymes of plant or bacterial origin or proapoptotic proteins. After recognition and internalization, the toxin induces cell death. A radio-immunoconjugate induces cell death after being engulfed into the cells, due to the toxicity of its constituent radioactive materials.

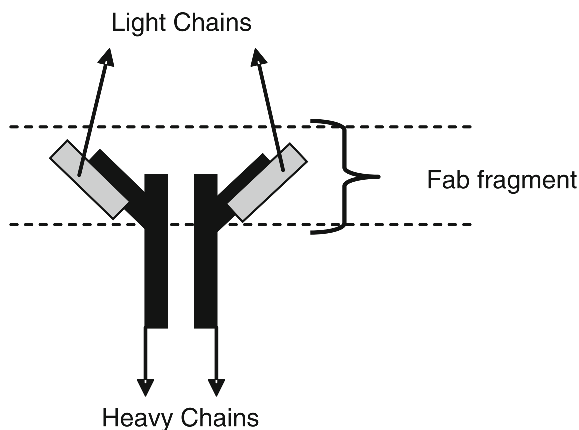


Fig. 2.2 The Fab fragment of an antibody

2.2.3 Peptides

RGD peptide is a synthetic peptide (Arginyl-glycyl-aspartic acid) with binding affinity for cell surface integrins. RGD can therefore be used for the targeted delivery of drugs, drug-loaded nanoparticles, and imaging agents into cells. RGD functions as an apoptotic anticancer agent by activating procaspase-3. It is usually PEGYlated and linked to the drug via an amide or hydrazone spacer (Fig. 2.3). RGD has the advantages of rapid cellular uptake and ease of production by solid-phase peptide synthesis; they are widely used for targeting doxorubicin and paclitaxel. Proteins containing an RGD motif include fibronectin, fibrinogen alpha chain, *E. coli* lambda receptor, sindbis coat protein, alpha lytic protease, and testis-specific basic protein (Schaffner and Dard 2003).

Designed ankyrin repeat proteins (DARPins) are genetically tailored antibody-like proteins (i.e. antibody mimetics) consisting of three to five repeated motifs of ankyrin proteins (Fig. 2.4). The repeats are tightly packed with a hydrophobic core, resulting in high stability, solubility, and dispersibility. DARPins possess specific affinity towards their target protein, thus enabling protein-protein interactions; they have diagnostic and therapeutic applications.

DARPins are capable of effectively crossing the blood-brain barrier, thus emerging as a therapeutic tool for treating neurodegenerative disorders. For example, they are used for treating Alzheimer disease by targeting the amyloid-β peptide (ABP). The ABP plays a pivotal role in the initiation and progression of Alzheimer disease, which is characterized by cognitive defects. ABP-specific DARPins treat Alzheimer disease by preventing the aggregation of ABP, reducing soluble ABP levels, and ameliorating ABP-mediated neurotoxicity in vivo.

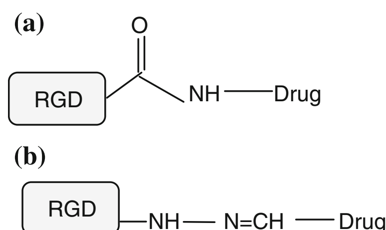


Fig. 2.3 RGD peptide-based targeting assembly formed by **a** an amide linker and **b** a hydrazone linker

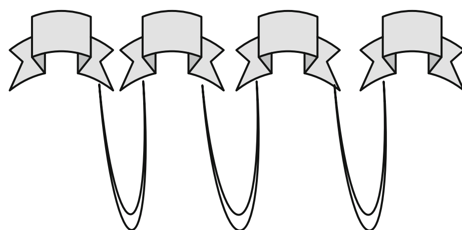


Fig. 2.4 DARPin with three repeated motifs

Vascular endothelial growth factor (VEGF) is another good example of a therapeutic DARPin. It is used in the treatment of wet macular degeneration (Hanenberg et al. 2014).

2.2.4 Aptamers

In Latin, *Aptus* means 'to fit' and *meros* means 'part'. Aptamers are short strands of DNA or RNA that are capable of fitting into a specific part of a target molecule. Aptamers are produced by a method called *systematic evolution of ligands by exponential enrichment* (SELEX). They are competent with monoclonal antibodies. Aptamers are used in the treatment of cancer because they can bind to cancer-specific proteins and nucleic acid targets. The binding affinity between aptamers and their target proteins is useful in fabricating biosensors for detecting cancer markers. Aptamers can conjugate with effector molecules such as drugs, photosensitizers, imaging agents, or Si RNA and are used for theranostic purposes (Wu et al. 2015; Keefe et al. 2010).

In addition, aptamers exhibit affinity for molecules such as proteins and nucleic acids of viral origin. Hence, they are used in the diagnosis and treatment of viral infections. Aptamers are useful for the early detection of viral genes, viral proteins, and host infection markers (i.e., antibodies raised against the virus). For example, H5N1 viral infection is detected using hemagglutinin-specific DNA aptamers. Hepatitis C virus infection can also be detected by the recognition of glycoprotein E2 by DNA aptamers. Aptamers help to treat viral infections by conjugating and delivering the drugs to the virus-infected cells, preventing the entry of virions into the cells, and inhibiting viral replication enzymes.

Macugen is the first therapeutic aptamer approved by the U.S. Food and Drug Administration for the treatment of age-related macular degeneration. Moreover, DNA aptamers are used to treat influenza viruses H5N1 and H9N2. RNA aptamers are used to treat the infections caused by hepatitis B and C viruses, SCV, HMCV, and Ebola. Infections caused by human immunodeficiency virus, hepatitis B virus, hepatitis C virus, severe acute respiratory syndrome, H5N1 avian influenza, and Ebola can be diagnosed and treated by using aptamers (Wandtke et al. 2015).

2.2.5 Small Targeting Molecules

Apart from the major targeting ligands discussed previously, some small molecules such as transferrin, lectin and folates have also been reported to exhibit targeting abilities.

Transferrin is an iron-binding protein with a molecular weight of 80 kDa. Receptors to transferrin are overexpressed in cancer cells due to their enhanced iron requirement as compared to normal cells. Hence, transferrin can easily be attracted

towards the cancer cell surface along with chemotherapeutic drugs, cytotoxic proteins, or cytotoxic enzymes. For example, transferrin is useful for targeting CD71 antigens overexpressed on the cancer cell surface. Transferrin has been reported to deliver adriamycin to HL60 and K562 cells and to mice. The targeted delivery of other drugs such as doxorubicin, ricin A-chain-toxic protein, and therapeutic genes (for gene therapy) have also been reported.

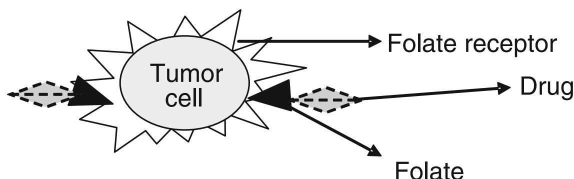
Lectins are proteins found on the surface of certain cell types as receptors, such as galactose-specific lectins and mannose-6-phosphate-specific lectins in the hepatic cells of mammals. Another lectin, the mannan-binding lectin, is distributed on the surface of immune cells. Although these lectins have their own functions in the liver and immune system, they may be exploited for drug delivery. Similar to lectin, carbohydrate residues also exist as cell surface molecules, such as glycoproteins and glycolipids. Lectins have high affinity for carbohydrate residues. Taken together, carbohydrates can provide a port of entry for lectinized drugs or drug–nanoparticle conjugates. In the same way, carbohydrate-based therapeutic molecules can be targeted into cells via endogenous ligands of the cells. Hence, the ligand-carbohydrate interaction can be used in the drug delivery application in two ways (Kaszuba and Jones 1998).

There are two strategies to target lectin:

(i) In direct lectin targeting, the nanoparticle-bound drug or the free drug itself is conjugated to a carbohydrate residue (forming a glyconanoparticle or glycosylated drug). The carbohydrate residue, in turn, is allowed to target cell surface lectins to achieve better efficacy of the drug. For example, intraperitoneal administration of a mannan-methotrexate conjugate in leukemic mice showed enhanced anti-tumor potential compared with free methotrexate (Budzynska et al. 2007).
(ii) In reverse lectin targeting, the nanoparticle is conjugated to a lectin (i.e., the lectinized nanoparticles), which is allowed to target cell surface carbohydrate residues. Lectinized gliadan nanoparticles are a useful system for the delivery of acetohydroxamic acid to inhibit *Helicobacter pylori*. Lectinized liposomes are used for targeting alveolar type II epithelial cells. Lectin-modified solid lipid nanoparticles (SLNs) are used in the delivery of insulin (Gupta et al. 2009).

Folate-mediated drug delivery is a good example of vitamin-aided targeting. Folate receptors are overexpressed in cancer cells. Folate, a vitamin B9 molecule, can be conjugated to the drug or the nanoparticle-conjugated drug and targeted toward the cancer cells (Fig. 2.5). Its low molecular weight and high affinity for folate receptors have made folate an excellent ligand for the delivery of protein toxins, immune stimulants, chemotherapeutic agents, liposomes, nanoparticles, and imaging agents. Folate is used to inhibit the proliferation of ovarian, brain, head and neck, renal, and breast cancer cells (Hilgenbrink and Low 2005).

Fig. 2.5 Folate-mediated drug targeting



2.3 Imaging Ligands

Imaging ligands or, more commonly, imaging agents are molecules capable of demonstrating internal structure and monitoring a treatment regimen, keeping track of the internalized drug molecules. Usually, imaging molecules are constructed to be a part of nanoparticle construct or sometimes nanoparticles themselves, such as quantum dots. Magnetic nanoparticles (MNPs), fluorescent probes (fluorophores), radioactive tracers, and mixed lanthanide oxide nanoparticles are used for imaging tissues via magnetic resonance imaging (MRI), fluorescence imaging (FI), or positron emission tomography (PET) techniques.

Magnetic nanoparticles (MNPs) are conventionally used as imaging agents through MRI (see Chap. 4). MNPs are widely used for imaging cardiovascular disease, such as atherosclerosis, myocardial injury, and stem cell therapy (Sosnovik et al. 2008).

Fluorodeoxyglucose (FDG) and fluorine-18 (F-18) are used as tracers for imaging cancer cells, especially lung cancer and lymphoma. 11C-Metomidate has been used to image adrenocortical tumors. Fluorescent radiotracers have extensive applications in neuroimaging, such as in monitoring Alzheimer disease as well as in cardiovascular disease such as atherosclerosis. Clinically, bacterial infections can also be identified using PET with the help of [^{18}F] maltose, [^{18}F] maltohexaose, and [^{18}F]2-fluorodeoxysorbitol (FDS). Gadolinium oxysulfide nanoparticles doped with other lanthanides [Eu(3+), Er(3+), Yb(3+)] function as new multimodal nanoplatforms for MRI, X-ray, and photoluminescence imaging in vitro (Osseni et al. 2014).

Dual molecular imaging provides us with more information about diseased cells and finds immense use in diagnosis. MRI and FI on a single modality would be an ideal dual imaging system for biomedical diagnosis. A mixed lanthanide oxide nanosystem in which one lanthanide exhibits MRI capability and the other exhibits FI capability would give a successful diagnosis. Dy, Ho, Gd, Tb, and Er are MRI candidates, whereas Eu and Tb are fluorescent candidates. Hence, the mixed ultrasmall nanoparticles Dy/Eu, Ho/Eu, and Ho/Tb are dual imaging modalities. The advantages of mixed lanthanum oxide T2 MRI–FI agents include the following, as proven in vitro (DU145 cells, a PC3 human prostate cancer cell line) and in vivo (mouse) (Xu et al. 2013):

- Facile synthesis condition
- Stable and robust
- Controllable composition in terms of the lanthanide oxides

- High r_2 values and magnetization
- High negative contrast enhancement
- Highly resolved confocal image
- Feasibility of intravenous administration into the mammalian model.

2.4 Biocompatibility Ligands

The biocompatibility of a material is its ability to exist in harmony with the living system without producing adverse side effects. In the context of nanotherapy, biocompatibility is a crucial factor when implants and drug carriers are used for treatment. Inflammation, fibrosis, thrombosis, and infection are some of the adverse effects associated with implantable medical chips, which are mediated via protein adsorption, cell adhesion, and tissue adhesion. Hence, biocompatibility should be enhanced by modifying the surface chemistry of the implant. The biocompatibility of a medical device such as an implant or a drug carrier, can be enhanced when functionalized with suitable molecules—biocompatibility ligands—to minimize the protein adsorption and host-cell adhesion effects. This approach showed success in vitro, but more investigation is needed to validate its biocompatibility in vivo. For example, the biocompatibility of synthetic polymers, such as polyvinyl chloride, can be enhanced using collagen (Lungu et al. 1997). Other methods of increasing surface biocompatibility (Thevenot et al. 2008) include the following:

- Enhancing hydrophobicity to decrease protein adsorption
- Altering surface charges
- Forming flat, chemically homogenous, and well-defined surfaces by activating the bulk material surface, grafting with the polymer, and fabricating self-assembled monolayers (SAMs). SAMs are suitable for gold- and silver-coated surfaces only
- Using the plasma modification method. Gases generate highly excited atomic, molecular, ionic, and radical species called "plasma" upon irradiation with radiofrequency, microwave, or electrons from a hot filament discharge. Plasma modification of an implant or biomedical materials such as metals, polymers, and ceramics results in a uniform surface, which is very essential for tissue engineering and artificial organs
- Adding functional groups as biocompatible ligands. For example, carboxyl (–COOH), hydroxyl (–OH), amino (–NH_2), and methyl (–CH_3) groups are capable of influencing the interaction of protein and cells with the surface.

2.5 Anti-immunogenic Ligands

The biological system (the host) may consider the drug carriers or the implants to be foreign material, resulting in an immune response that would impact the desired effect of the biomedical constituents. This should be prevented by a suitable molecule capable of evading the attack by immune cells. Polyethylene glycol (PEG) is a suitable anti-immunogenic ligand capable of evading the immune response. PEG was reported to decrease the immune response by reducing interleukin-2 and tumor necrosis factor-α secretion from lymphocytes (Jang et al. 2003). PEG is useful for the escape of nanoparticles from RES and endosomes during gene delivery. The final section of this book discusses the application of Si RNA delivery in treating multi-drug resistance cancer, viral infections, and genetic diseases. In spite of these applications, Si RNA delivery has certain demerits: First, they are easily cleared by the RES after systemic administration. Second, they are trapped and subjected to enzymatic hydrolysis in endosomes and lysosomes. This results in decreased accumulation of the therapeutic molecule in the tumor. A PEG graft can overcome these disadvantages to a certain extent (Guo and Huang 2011).

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Chapter 3
Biomarkers

Biomarkers are accurately quantifiable indicators that are capable of reflecting normal biological processes, pathogenic processes, incidence or outcome of a disease, pharmacologic responses to a therapeutic intervention, or the adverse effects of a desired new drug. Thus, the biomarkers may be broadly classified into four types:

- Diagnostic biomarkers (which reveal the abnormality)
- Prognostic biomarkers (which predict the course and/or severity of the disease)
- Pharmacodynamic biomarkers (which monitor whether a therapy will work or is working as this marker is altered during the therapeutic recovery)
- Toxicity biomarkers (which monitor the toxic effect elicited in the body due to the disease or the drug itself).

A single parameter may either be an all-type biomarker or a single-type biomarker.

The indicators may be any one of the following:

- A specific gene variant
- Patterns of gene expression
- Levels of a particular protein in body fluids
- Concentration of a compound
- Activity of an enzyme in the body fluid or in the tissue
- Concentration of a metabolic product.

Biomarkers play a vital task in improving the drug development process as well as in the larger biomedical research enterprise. The U.S. Food and Drug Administration (FDA) is promoting biomarker values in basic and clinical research. Thus, in nanomedicine, there is a need for the evaluation of the diagnostic and therapeutic potential of nanoparticles or the toxic potential of nanotechnology-based drugs during the drug development process. This would help in identifying the therapeutic properties of the test compound, assessing risk, and understanding

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Table 3.1 Different types of diagnostic markers

Marker type	Detectable parameters	Example	Disease/diseased condition
Biochemical	Oxidative damage markers	TBARS	Cancer/toxicity
	Antioxidant markers	Reduced glutathione, catalase	Nephritis
	Cytokines	Interleukins	Pulmonary disorder/inflammation
	Cell viability marker	Formazon	Cell death/cytotoxicity
Genetical	DNA damage	DNA fragmentation	Cardiovascular disease/toxicity
Histopathological	Fibrosis, granuloma	Pulmonary fibrosis	Lung inflammation, hepatitis

the mode of action of the nanoparticles (Strimbu and Tavel 2010). Generally, there are three major types of diagnostic marker details of which are summarized in Table 3.1:

- Biochemical markers
- Genetic markers
- Histopathological markers.

3.1 Biochemical Markers

Biochemical parameters are biological or biochemical constituents that are detectable in tissue homogenates, or membrane isolates in body fluids such as cerebrospinal fluid, and urine samples. For testing a drug inclusive of nanomedicines, in vitro markers can be identified before proceeding to in vivo experiments. Biochemical changes associated with most of the diseased states and environmental pollution-induced abnormalities are hematological changes, oxidative stress, and inflammation. These are probably due to toxicity elicited directly or indirectly in the body, such as cancer, diabetes, Alzheimer disease, myocardial infarction, cardiovascular disease, chemical toxicity, pesticide toxicity, exhaust particle-induced toxicity, and nanotoxicity. Each disease is characterized by a confirmatory marker, which is highly specific. The specificity is determined by the nature of the disease (e.g., cancer-specific markers, autoimmune disease markers, HIV markers), the organ that is diseased (e.g., cardiac markers, hepatic markers, gonadal markers, renal markers), or the markers produced by the disease-causing agent (i.e. infectious disease; e.g., viral or bacterial or parasitic markers). Hence, the marker should correctly be fixed. Some of the biochemical markers are discussed in this chapter.

3.1.1 Cardiac Markers

Biochemical markers for the early detection of myocardial injury in the blood include unbound free fatty acids, ischemia-modified albumin, C-reactive protein, white blood cell count, soluble CD40 ligand, myeloperoxidase, monocyte chemo-attractant protein-1 (MCP-1), and whole blood choline (Panteghini 2004).

3.1.2 Cytokines

Cytokines are low-molecular-weight proteins that attract the white blood cells towards the injured or diseased tissue. They are markers of infectious diseases, cancer, and inflammatory disorders. Proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-la and IL-1β, IL-12, interferon [IFN]-γ, and possibly IL-6) are capable of inducing inflammation during infection or cancer. Anti-inflammatory cytokines control the inflammation process and suppress the immune response. Examples of anti-inflammatory cytokines are interleukin (IL)-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13 (Gogos et al. 1999).

3.1.3 Cancer Markers

Cancer markers are molecules that are elevated in body fluids or tissues of patients with cancer or in animals with induced cancer. These markers are highly specific for cancer and are absent in the normal cells, thus ensuring specificity. Most of the cancer markers are proteins and are organ specific. Some examples of cancer markers are given in Table 3.2.

Table 3.2 Examples of diagnosable cancer markers

Marker	Cancer type
Alpha fetoprotein (AFP)	Liver cancer
Herceptin	Breast cancer
CA19-9	Pancreatic cancer, colorectal cancer
Calcitonin	Carcinoma of the thyroid
Carcinoembryonic antigen (CEA)	Gastric and colorectal carcinoma
Chromogranin A	Neuroendocrine cancer

3.1.4 Cell Adhesion Molecules (CAMs)

Cell adhesion molecules (CAMs) are proteins that extend from the intracellular compartment to the extracellular compartment by spanning through the membrane. They are mostly receptor molecules responsible for the adhesion of the cell to a neighbor or to the extracellular matrix. The families of CAMs are the selectins, the immunoglobulin (Ig) superfamily, the integrins, and the cadherins. Some of the adhesion molecules are found in soluble form in the circulation. The levels of adhesion molecules can be used as biochemical markers (Devasena 2010). A few examples are claudin 7, α-catenin, and β-catenin adhesion molecules, which are reliable biomarkers for prostate cancer (Morgan et al. 2013). The two members of the immunoglobulin gene superfamilies intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) promote the adhesion of leukocytes to the vascular endothelium. They are markers for atherosclerosis (Ballantyne and Entman 2002).

3.1.5 Enzymes

Enzymes are a colloidal, high-molecular-weight, nondialyzable, denaturable, structurally diverse group of proteins. In a diseased state or during cell damage, enzymes leak out into body fluids. Assays of these enzymes in the blood can therefore be used to diagnose disease or damage. Some significant diagnostic enzymes and the disease diagnosed are shown in Table 3.3 (Devasena 2012). Some of the enzymes possess antioxidant functions; their values are discussed as oxidative stress biomarkers in the section 3.1.7.

3.1.6 Hematological Biomarkers

Hematological parameters are indicative of blood loss, abnormalities in the erythropoiesis or erythrocyte degradation, acute and chronic infections, allergies, and problems with blood clotting. Detectable hematological parameters include erythrocyte count or red blood cell (RBC) count, hemoglobin content, and mean red cell volume (MCV), which provides information about anemia (a common condition that occurs when the body has insufficient red blood cells). The erythrocyte sedimentation rate (ESR) is a valuable marker for infection because it is increased during infection (Devasena 1999). The leucocyte count or the white blood cell (WBC) count and its differential count are useful indicators of inflammation, especially related to pulmonary allergy and immune response (Prakash 2015).

Blood acetylcholinesterase and butyrylcholinesterase are used as biomarkers for monitoring the toxicity induced by the suspected agents (Safi et al. 2010). Hence,

Table 3.3 Some diagnostic enzymes and diseases diagnosed

Enzyme	Disease diagnosed
Choline esterase	Paralysis
Alkaline phosphatase	Prostrate carcinoma
Pancreatic amylase	Pancreatitis
Aspartate transaminase	Acute hepatitis
Alkaline phosphatase, gamma glutamyl transferase	Kidney damage
Creatine kinase	Skeletal muscle damage
Hydroxy butyrate dehydrogenase, lactate dehydrogenase	Heart disease
Leucine aminopeptidase	Hepatobiliary disease
Transaminase	Chronic hepatitis
Pepsin	Gastric mucosal damage
Creatine phosphokinase, aspartate transaminase, lactate dehydrogenase	Myocardial infarction
Mitochondrial enzymes	Tissue necrosis

they can be good biomarkers for nanotoxicity studies. Thrombocyte (platelet) counts are useful to monitor the severity of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). They are also used to distinguish acute infection from active disease (Willeke et al. 2015).

3.1.7 Oxidative Stress Markers

Reactive oxygen species (ROS) are highly reactive and unstable free radicals with at least one unpaired electron, Example: superoxide anion (O_2^-), hydroxyl radical (OH^-), and singlet oxygen (O_2*). ROS are capable of inducing oxidative damage to the cell membranes, consequently binding to vital biological macromolecules such as proteins, carbohydrates, lipids, and DNA. This results in the formation of thiobarbituric acid reactive substances (TBARS). However, antioxidants could interact with the ROS, become oxidized, and prevent oxidative damage to the cell. Thus, antioxidants function as reducing agents. However, an imbalance in the ROS: antioxidant ratio (which is called oxidative stress) occurs during disease conditions. Hence, oxidative stress is reported to play a major role in the development of many diseases and toxicity (Devasena et al. 1999, 2001, 2003, 2005). Hence, the measurement of oxidative stress indicators and antioxidant status could be of immense use in the diagnosis of the associated disease and organ toxicity, as well as in assessing the response of the body to the treatment (Devasena et al. 2002; Devasena and Venugopal 2007). This concept is equally applicable to nanoparticle-induced toxicity and nanoparticle-based therapy or treatment (Devasena and Rajasekar 2014).

Markers of oxidative stress include the following:

- Malondialdehyde (also called TBARS)
- Antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST)
- Nonenzymatic antioxidants, such as reduced glutathione (GSH), vitamin C, vitamin A, α-tocopherol, and ceruloplasmin
- Total protein content of the blood and tissue of interest
- Organ-specific enzymes, proteins, and hormones (Table 3.4)
- Inflammatory markers such as cytokines (interleukins) and fibrotic markers such as interferons
- In vitro markers, such as indicators of cytotoxicity and apoptotic changes (Francis et al. 2014).

In general, a spectrophotometer or a colorimeter (see Chap. 4) and an optical microscope will be sufficient to do these tests.

3.2 Genetic Markers

Genetic markers (genetic molecular markers) are a gene, DNA sequence, or gene product that displays abnormal changes, such as single nucleotide polymorphism, minisatellites, DNA fragmentation, and DNA laddering. These are mainly used in the diagnosis of genetic disorders and toxicity. This method requires electrophoresis apparatus, an optical microscope, and a polymerase chain reaction (PCR) machine.

Genetic markers are classified as follows:

3.2.1 Single Locus Markers

- SNP (single nucleotide polymorphism): an SNP marker refers to a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position
- STS (sequence tagged site)
- SSR (simple sequence repeat) or microsatellite
- RFLPD (randomly amplified polymorphic DNA).

3.2.2 Multiple Loci Markers

- DNA fingerprinting
- RAPD (randomly amplified polymorphic DNA)
- ADFLP (amplified fragment length polymorphic DNA).

Table 3.4 Organ-specific diagnostic markers

Organ	Organ-specific enzymes/proteins	Example
Liver	Alanine transaminase (ALT), aspartate transaminase (AST)	Hepatic inflammation
	Alkaline phosphatase (ALP)	Liver and biliary tract disease
	Prothrombin	Liver dysfunction/injury
Lung	Anaplastic lymphoma kinase (ALK), carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), cytokeratin 19 (CYFRA-21-1), alpha-fetoprotein, carbohydrate antigen-125 (CA-125), carbohydrate antigen-19.9 (CA-19.9), ferritin	Lung cell proliferation and tumor Lung cancer
Heart	Cardiac troponins T	Myocardial infarction
	Myocardial muscle creatine kinase (CK-MB)	Myocardial injury
	Myoglobin	Early myocardial infarction
	B-type natriuretic peptide (BNP)	Severe heart damage
Pancreas	DEAD-box protein 48	Pancreatic ductal adenocarcinoma (PDAC)
	Calreticulin, vimentin, RNase 1, kininogen-1, anti-thrombin-III, haptoglobin-related protein	Pancreatic cancer
Kidney	Cystatin C and β-trace protein	Renal dysfunction
	Podocin, nephrine, podocalyxin	Abnormal renal glomerular damage
	Urinary 8-hydroxydeoxy guanosine	Renal oxidative stress
	Urinary tenascin and TIMP-1	Chronic kidney disease
	Asymmetric dimethyl arginine (ADMA)	End-stage renal disorder
Brain	Plasma/CSF Aβ1-peptide	Alzheimer disease
	CSF tau protein	Alzheimer disease
	Dopamine transporter protein	Parkinson disease
	α-synuclein	Parkinson disease
	Blood 8-hydroxyguanosine	Parkinson disease
	Tumor necrosis factor (TNF-α) in CSF	Parkinson disease
	Glutamate in the CSF and blood	Amyotrophic lateral sclerosis
Intestine	Fecal calprotectin	Inflammatory bowel disease
	Fecal lactoferrin	Intestinal inflammation
	Fecal neopterin	Ulcerative colitis
	Adenosine deaminase	Crohn disease
	Serum and urine mopterin	Ulcerative colitis and Crohn disease
	Fecal chitinase 3-like-1	Inflammatory bowel disease

(continued)

Table 3.4 (continued)

Organ	Organ-specific enzymes/proteins	Example
Bone	Bone alkaline phosphatase (BAP), osteocalcin (OC), procollagen type 1 carboxy-terminal propeptide (P1CP)	Diseases associated with bone proliferation
	Carboxy-terminal cross-linked telopeptides of type 1 collagen (CTX), type 1 collagen alpha 1 helicoidal peptide (HELP), pyridinoline (PYD), dickkopf-related protein 1 (DKK1), sclerostin (SCL), bone mineral density	Diseases associated with bone resorption
Muscle	Dystrophin	Myopathy, muscular dystrophy
	Troponin I, carbonic anhydrase 3 CA3, creatine kinase M-type CKM, mitogen-activated protein kinase 12 (MAPK12), alanine aminotransferase 1, myoglobin, fibrinogen, hepatoma-derived growth factor-related protein 2, heparin cofactor 2, proteosome subunit α type-2, cAMP-dependent protein kinase catalytic subunit α, neurogenic locus notch homolog protein 3, disintegrin and metalloproteinase domain-containing protein 9, kunitz-type protease inhibitor 1, tumor necrosis factor receptor superfamily member 19L	Duchenne muscular dystrophy, muscle transformation, muscular atrophy, muscular dystrophy, motor neuron disease, inclusion body myositis, myotonia

The principles of the techniques used for the identification of these markers are described in Chap. 4. To focus on the theme, this chapter is restricted to the biomedical applications of these markers.

3.2.3 Examples of Genetic Markers

(a) ***Genetic markers for liver disease***

Marker genes identified in hepatic cells such as stellate cells, Kupffer cells, and hepatocytes have been used to indicate the presence and development of liver fibrosis (an excessive accumulation of extracellular matrix proteins, such as collagen). For example, a hepatic stellate cell (HSC)-specific marker indicates the self-activation process in the development of fibrosis. Markers of HSCs such as Acta 2, Cryab, Spp1, Prnp, and Pai-1 are upregulated during the initial stage but experience a gradual fall in the next stage. However, other HSC markers such as Gpc3, Lox, and Mgp are not upregulated initially but are raised linearly during fibrogenesis (Takahara et al. 2006). Alcohol dehydrogenase 1C*1 allele is a genetic

marker for alcohol-associated cancer in heavy drinkers. RANTES (a chemokine that is produced by T cells and stimulates mast cells) is expressed at a higher level, thus indicating the involvement of mast cells in fibrosis. Furthermore, the expression profile of hepatocyte-specific marker genes (upregulation of Gck and downregulation of Pck1) is an indicator of the abnormal metabolism of liver fibrosis (Takahara et al. 2006). Human hepatocellular carcinoma (HCC) of different etiology exhibits upregulation of a gene called SPINK1, which codes for a secretory trypsin inhibitor (Marshall et al. 2013).

(b) ***Genetic markers for heart disease***

The dysregulation of gene expression during heart disease is a useful diagnostic markers. Abdominal aortic aneurysm, atherosclerosis, atherothrombosis, calcific aortic valve stenosis, cardiac hypertrophy, and carotid artery atherosclerosis are major heart diseases (see Table 3.4), which are strongly predictable via genetic markers.

Dermatoglyphic phenotypes such as FTP, T-D count, and palm patterns are related to an enhanced risk of coronary heart disease (CHD, characterized by the accumulation of plaque in the coronary artery, which supplies oxygenated blood to the heart). Inflammation plays a pivotal function in the pathogenesis of atherosclerosis; as such, the characterization of genetic markers associated with inflammation is of prime importance. The expression profile of genes coding for

Table 3.5 Some important genetic markers associated with heart disease

Disease	Phenotype	Marker gene
Abdominal aortic aneurysm	Enlargement of the lower part of the aorta, the major blood vessel that supplies blood to the body	Methylenetetrahydrofolate reductase (MTHFR)
Atherosclerosis	Thickening and hardening of the arterial wall and narrowing of the arterial lumen with impaired blood flow	Matrix metalloproteinase 3 (MMP3), also called stromelysin 1; matrix metalloproteinase 13 (MMP13); apolipoprotein E (APO E); methylenetetrahydrofolate reductase (MTHFR); cholesterol ester transfer protein (CETP); angiotensin I converting enzyme 1 (ACE); angiotensin II receptor type 1 (ATIIR1)
Atherothrombosis (angina pectoris)	Imbalance in blood supply and oxygen demand	Thrombin activatable fibrinolysis inhibitor (TAFI)
Calcific aortic valve stenosis	Narrowing of the aortic valve due to calcium deposition	Vitamin D receptor
Cardiac hypertrophy	Narrowing of heart chambers due to thickening of heart muscles	Interleukin 6 (IL-6)
Carotid artery atherosclerosis	Block in the carotid artery (artery supplying oxygenated blood to the head neck)	Matrix metalloproteinase 3 (MMP3)

C-reactive protein, soluble intercellular adhesion molecule-1, interleukin-6, and P-selectin are associated with the progress of cardiovascular disease (CVD, which involves diseased heart or blood vessels) (Raman et al. 2013). Some of the markers related to different types of heart diseases are shown in Table 3.5.

(c) ***Genetic markers for lung disease***

Lung diseases are associated with genetic variations. HLA-DR2 and HLA-DRw53 expressions are elevated in tuberculosis (Pospelov et al. 1996). Chronic beryllium disease (CBD) refers to the accumulation of beryllium-specific CD4+ major histocompatibility complex (MHC) class II restricted T lymphocytes in the lungs. Major histocompatibility complex, class II, DP beta 1 gene, which is also called the HLA-DPB1 gene, is the marker for CBD (Richeldi et al. 1993).

3.3 Histopathological Markers

Histopathological markers are microscopic and nanoscopic changes that are induced in tissues due to disease and some severe biochemical changes. This includes membrane blebbing, abnormal cell adhesion, aggregation, granuloma, fibrosis, proteinosis, histocytosis, necrosis, and hyperplasia. These markers are detectable by optical microscopes or scanning electron microscopes in advanced cases. Several studies, including those from our own laboratory, have emphasized the significance of these markers and the respective instruments in diagnosis and monitoring of treatment efficacy in various disease models, both in vitro and in vivo (Devasena et al. 2005). For histopathological investigations, tissues are perfused with formalin (10 %), dissected, and stored in 10 % formalin. They are later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section, and stained with hematoxylin and eosin (H&E). They can be viewed with an optical or scanning electron microscope, depending on the need. Certain markers linked to tumors, such as cellular skeletons, lymphocyte surface antigens, cytoplasmic markers, oncogene products, and CAMs, can be visualized using immunohistochemical analysis (Kijima et al. 2003).

The imaging of cell and tissue architecture with high resolution is very essential for histopathological analysis. This task is now getting easier due to nanoparticles. Nanoparticles can be used for imaging the tissues and obtaining information about the chemical composition, biodistribution of markers and drugs, morphological and structural discriminations, identification of biomolecules such as proteins, and tracking of cancer cells. Nanoparticle-based imaging can be performed using the following techniques:

- Raman spectroscopy (RS)
- Magnetic resonance imaging (MRI)
- Ultrasonography (US)

- Computed tomography (CT) or positron emission tomography (PET)
- Single-photon emission CT (SPECT)
- Mass spectrometry (MS).

Details of these techniques are presented in Chap. 4.

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Chapter 4
Diagnostic and Therapeutic Techniques

The use of molecular probes for the visualization and investigation of the structure, function, characteristics, and processes of cells in a living organism is called molecular imaging. The probes bind to the target cells or molecules and indicate their presence by giving a quantifiable signal. This signal can be measured with the aid of many imaging techniques (Table 4.1).

Nanoparticle beacons play a major role in molecular imaging and thus have wide applications in diagnosis and therapy due to their unique physical, chemical, magnetic, tunable absorption, and emission properties. In fact, the signals and sensitivity produced by various diagnostic techniques are enhanced by the use of nanoparticles. Many such applications aim to detect cancer in different organs and at different stages. Knowledge of the different techniques used in diagnosis and therapy (Table 4.2) is therefore essential to proceed further with biomedical applications of the functionalized nanoparticles. This section discusses the techniques used for diagnosis and treatment in terms of working principles and applications.

4.1 Diagnostic Techniques Using Nanoparticles

(a) ***Computed tomography imaging (CT imaging or CT scanning)***

In computed tomography (CT) imaging, an X-ray beam is penetrated through the tissue, the beam is attenuated (i.e., weakened), and a plane cross-sectional image (slicing image) is generated with a three-dimensional view (Fig. 4.1). The degree of penetration depends on the density of the tissue, which in turn depends on the attenuation coefficient (i.e, the degree of attenuation of beam by the tissue).

Although CT imaging is an important radiology technique, it requires X-rays of high-dose and iodine-based compounds to produce a high-contrast image. Iodine-based contrast agents have shortcomings, such as a short duration of imaging, quick clearance in the kidneys, nephrotoxicity, and vascular permeation.

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Table 4.1 Imaging techniques enhanced by nanoparticles

Diagnostic techniques	Nanoparticles used
CT imaging	Gold nanoparticles, iron oxide-silicon oxide core shell nanostructures
Photoacoustics imaging	Gold nanostructures, semiconducting polymeric nanoparticles
Colorimetry	Gold nanospheres
Magnetic resonance imaging	SPIONS, polymer-coated SPIONS, $MnFe_2O_4$ nanoparticles
Optical coherence tomography (OCT)	Gold nanoparticles, polypyrrole nanoparticles
Surface-enhanced Raman spectroscopy (SERS)	Gold-silica core-shell structures, carbon nanotubes
Positron emission tomography (PET)	Graphene oxide nanoparticles, liposomes, gold nanoparticles, dendrimers
Single photon emission computed tomography	Iron oxide nanoparticles, polymeric nanomicelles, silver nanoparticles
Fluorescence correlation spectroscopy	Gold nanoparticles

Table 4.2 Therapeutic techniques using nanoparticles

Therapeutic techniques	Nanoparticles used
Photodynamic therapy	Gold nanoparticles, colloid gold, quantum dots, paramagnetic nanoparticles, silica-based materials, polymer-based nanoparticles
Radiotherapy	Gold nanorods and silica
Hyperthermia	Gold nanoparticles, gold-silica nanoshells, gold-gold sulphide nanoparticles, SPIONS
Chemotherapy	Polyacrylic acid-calcium carbonate nanoparticles
Gene therapy	Dendrimers, viral nanoparticles

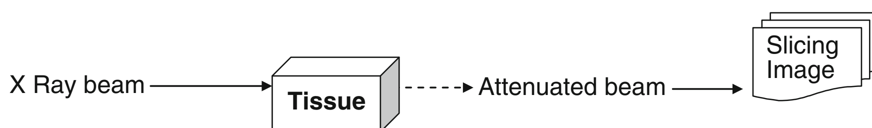

Fig. 4.1 Principles of computed tomography imaging

Hence, there is a need to both reduce the X-ray dose and to obtain high contrast without side effects.

Nanoparticles can effectively fill this gap. They are superior to conventional contrast agents in that they possess high circulatory half-life and specific targeting ability. Gold has a higher atomic number and its X-ray absorption coefficient is also greater than iodine. Gold nanoparticles of 25–50 nm functionalized with PEG on

their surface allow for the reduction of radiation dosage and counteract the shortcomings of conventional iodine-based contrast agents. Core-shell nanostructures with iron oxide or silicon oxide cores and gold shells absorb and scatter near-infrared radiation, thus holding promise as a contrast enhancer for CT as well as MRI imaging. Drug-loaded gold nanoparticles functionalized with RNA aptamers turned out to be a targeted drug delivery and imaging system with higher CT contrast intensity and treatment efficacy (Kim et al. 2009). A gold nanoparticle-doxorubicin conjugate, functionalized with a PSMA-RNA aptamer (i.e., an aptamer complementary to prostate-specific membrane antigen; see Chap. 2 for aptamers) would be an efficient theranostic agent in terms of targeting, image contrast, drug delivery, and treatment efficacy (Kim et al. 2009). PEG-conjugated gold nanoparticles and gum arabic stabilized gold nanoparticles are also useful to enhance the contrast of CT imaging. Nanoparticles have become the next-generation contrast agents for blood pool, in vivo, and in vitro imaging.

(b) ***Photoacoustic imaging***

Photoacoustic imaging (PAI) is a non-invasive imaging technique that works by converting light energy into sound waves via thermal expansion. In principle, the sample to be imaged is irradiated with a pulse of infrared (IR) or near-infrared (NIR) radiation. The sample absorbs the light and gets heated rapidly, resulting in thermal expansion. This heat dissipation generates detectable broadband sound waves (the acoustic waves), which helps in imaging the tissues. The inherent optical properties of gold nanoparticles have made them an ideal exogenous contrast agent for PAI. Gold nanoparticles of different morphologies, such as spheres, rods, shells, prisms, cages, stars, and vesicles, are highly suitable for PAI. This would help in the imaging of cancer, atherosclerotic plaques, and brain plaques, as well as in image-guided therapy. For example, Mallidi et al. (2009) have demonstrated that gold nanoparticles targeted to epidermal growth factor receptor (EGFR) have the ability to enhance the selectivity and sensitivity of multiwavelength photoacoustic imaging of cancer cells. This is due to the plasmon-resonance coupling effect of gold nanoparticles.

Semiconducting polymeric nanoparticles are also useful in photoacoustic imaging due to their high photostability, chemical stability, and infrared absorption. These nanoparticles absorb the light energy from infrared or near-infrared radiation, which gets dissipated into heat energy for generating sound waves. The sound waves in turn are exploitable for PAI. For example, semiconducting polymeric nanoparticles are excellent photoacoustic probes for in vivo real-time imaging of reactive oxygen species (ROS), which are involved in the pathogenesis of many diseases (Pu et al. 2014).

Single-walled carbon nanotubes (SWCNTs) functionalized with RGD peptide (cyclic Arg-Gly-Asp) are capable of producing excellent photoacoustic signals and help in the non-invasive imaging of tumors upon intravenous administration (Zerda et al. 2008).

(c) ***Colorimetric detection***

Colorimetry is the measurement of the color of a solution using a colorimeter. The application of colorimetry in quantifying many biochemical parameters of body fluid has already been discussed (Devasena and Rajagopal 2013). Because gold has tunable optical properties, the change in the color of the gold solution measured using a colorimeter may be used as a signal for biodetection. The distance between the gold nanoparticles (i.e., aggregation or dispersion) will differ in the presence of a detectable targeting molecule. This distance determines the plasmon resonance frequency, which then determines the color of the solution. Oligonucleotide-conjugated gold nanoparticles are used for the detection of *Mycobacterium tuberculum,* which is the causative agent for tuberculosis (Baptista et al. 2006). Gold nanoparticles are also used for the colorimeteric detection of polynucleotides (Elghanian et al. 1997).

(d) ***Magnetic resonance imaging***

Magnetic resonance imaging (MRI) is a technique that uses magnetic fields (usually 1.5–3 T) and radiowaves for imaging the tissues. This technique is also called nuclear magnetic resonance imaging (NMRI) or magnetic resonance tomography. A magnetic field (i.e., energy) of appropriate strength is applied to the area of the body to be scanned.

Superparamagnetic iron oxide nanoparticles (SPIONS) such as γ-Fe_2O_3 (maghemite) or Fe_3O_4 (magnetite) are the best contrast probes for MRI because of their intrinsic biocompatibility and cost-effectiveness. SPIONS have no net magnetization in the absence of an external magnetic field. This would prevent particle aggregation and subsequent nonspecific engulfment by reticular endothelial system, which would otherwise significantly reduce the contrast. SPIONS are usually mixed with the hydrophilic polymers, such as polyvinylpyrrolidone (PVP), polyaniline (PA), dendrimer (DDR), or dextrans. Polymers will coat on the surface of the nanoparticles and serve the following purposes:

- Enhance the dispersibility of the particles
- Prevent the aggregation of the particles
- Provide functional groups of the covalent conjugation of ligands, which are usually the probing molecules or the targeting agents
- Facilitate the encapsulation of drugs.

Strategies used for SPION-based theranostics

(i) *Imaging and therapeutic approach*

Hydrophobic imaging dyes and receptor-targeting ligands can be added to the polymers and the drugs can be encapsulated into the hydrophobic pockets. This would result in a theranostic carrier system with MRI or optical imaging and targeted delivery applications. This is mainly used for imaging and delivering drugs to cancer cells. For example, taxol can be released into cancer cells targeted via folic acid receptors (Santra et al. 2009).

(ii) *Thermoresponsive carrier*

Secondly, amphilic polymers with hydrophilic and hydrophobic qualities can be coated onto magnetic nanoparticles for achieving high drug loading efficacy, high imaging contrast with NIR dyes, and superior hyperthermia effect. Pluronic F127, a non-ionic triblock polymer, is amphiphilic owing to the hydrophobicity of its central polyoxypropylene moiety and the hydrophilicity of its flanking polyethylene moieties. This polymer when coated onto SPIONS along with β-cyclodextrin is ideal for the efficient delivery of the polyphenolic drug curcumin and also for the superior contrast effect (Yallapu et al. 2011).

(iii) *Dual sensitive carriers*

Thirdly, a nanocarrier that is sensitive to both pH and magnetic resonance can be fabricated with the targeting ligand and the drug. This is highly suitable for releasing the drug upon encountering an acidic environment in the cancer cells. The anticancer drug can be coated on magnetic resonance-sensitive nanocrystals using a nano-emulsion method. The drug is then conjugated to a suitable molecule, with which it establishes acid-labile π bond. The outermost part of this system is functionalized with the targeting antibody. This pH-responsive drug delivery system can be targeted and imaged in an acidic environment, as illustrated in Fig. 4.2. A classic example of this strategy was demonstrated by Lim et al. (2011) to target fibroblast cells. Fibroblasts are cells present in the extracellular space, capable of secreting the extracellular matrix. $MnFe_2O_4$ coated with the anticancer drug doxorubicin was encapsulated in α-pyrenyl-ω-carboxyl PEG, which establishes a π-bond with the doxorubicin via the pyrene moiety. The herceptin antibodies capable of targeting the herceptin receptors are loaded on the surface of the nanoplatform. Once the nanostructure is targeted to the cancer cells, the acidic condition breaks the π bond to release the doxorubicin. The cells can be imaged by MRI due to the resonating nature of the nanocrystal.

(iv) *Nonpolymeric grafting*

Non-polymeric moieties with high biocompatibility and biodegradability may be grafted onto the SPIONS. These moieties may function as a binding site for ligands, such as the drug, the targeting agent, and the imaging agent. For example,

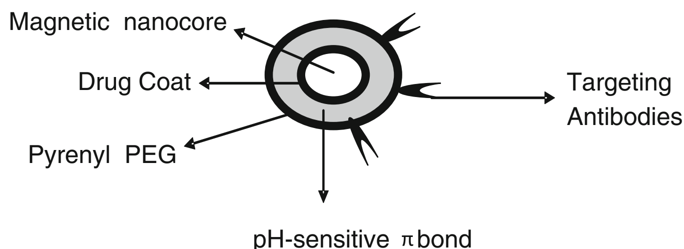

Fig. 4.2 The pH-sensitive nanotheranostic system

methotrexate (MTX) may be delivered when loaded onto SPIONS grafted with *N*-phosphonomethyl iminodiacetic acid (N-POME-IDA) in the presence of a folate-targeting ligand and rhodamine isothiocyanate as an image contrast agent. The acid moiety of the N-POME-IDA will form ester bonding with the methotrexate. The drug can be released by the rupture of a pH-sensitive ester linkage in the acidic tumor cells or magnetic guidance, which enhances the MRI contrast. Paclitaxel coated on magnetic nanoparticles can be delivered after grafting with lectin. Paclitaxel is an anticancer drug that induces cell death by mediating the disintegration of the cytoskeleton (tubulin) and causing cytotoxicity. Lectin (see Chap. 2) can be conjugated to magnetic nanoparticles via ethyl(dimethylamino-propyl) carbodiimide/*N*-hydroxysuccinimide. This nanocarrier possesses a high therapeutic index in vivo (Meng et al. 2010).

(e) ***Optical coherence tomography***

Optical coherence tomography (OCT) is a non-invasive, clinically useful, imaging technique used to record cross-sectional subsurface images of biological tissue with micrometer scale resolution. This technique involves the passage of NIR light into the tissues and depth-wise measurement of its reflections based on interference, which is mapped into a cross-sectional image with micrometer resolution. The contrast of the OCT image can be enhanced by the tunable properties of some nanoparticles, thus leading to better diagnostic applications. Gold nanoshells, gold nanorings, and gold-capped nanoroses are useful for contrast enhancement in OCT due to their selective accumulation in tumor tissues, thus providing a better tool for tumor imaging (Conde et al. 2012). OCT coupled with gold nanoparticles (formed in situ) results in contrast enhancement. This is used in dentistry for the nondestructive imaging of dentin structures (Braz et al. 2012). Further, polypyrrole nanoparticles can function as an NIR absorbing contrast agent, which are useful in early-stage cancer diagnosis (Au et al. 2011).

(f) ***Surface-enhanced Raman spectroscopy***

When a beam of light (from a Toronto lamp or a helium-neon laser) is incident on a molecule without a permanent dipole moment, the molecule vibrates, undergoes a change in polarizability, and scatters the light. The frequency of the scattered light differs from that of the incident light. This phenomenon of frequency shift is called the Raman effect; the spectrum of the scattered light is called the Raman spectrum; and the difference in frequency is called the Raman frequency, which is characteristic of the vibrating functional group. The intensity of the Raman line is proportional to the change in polarizability. The spectrum is characteristic of the target compound, such as a peptide, metabolic product, or nucleotide. However, the imaging of deeper tissues using the Raman effect needs a signal enhancer, which is a plasmonic substrate. This signal-enhancing version of Raman spectroscopy is referred as surface-enhanced Raman spectroscopy (SERS), which results in signal stability and high sensitivity when compared to conventional fluorescence. Gold-silica core–shell nanostructures, gold nanorods, gold nanospheres, gold

roughened spheroids, and carbon nanotubes are used in SERS. SERS is used for the non-invasive imaging of biomarkers associated with specific diseases, as well as for the imaging of tumors of the ovary, brain, and pancreatic adenocarcinoma.

(g) ***Positron emission tomography***

Positron emitters are radioactive isotopes that decay and emit positrons, which annihilates with the electrons of the neighboring atom. This results in the generation of gamma rays. Positron emitters are of two types: short-lived positron emitters and long-lived positron emitters. Short-lived positron emitters, such as ^{11}C ($t_{1/2}$ = 20 min), ^{15}O ($t_{1/2}$ = 2 min), ^{18}F($t_{1/2}$ = 109.7 min), ^{68}Ga ($t_{1/2}$ = 67.7 min), ^{64}Cu ($t_{1/2}$= 12.7 h), and ^{76}Br ($t_{1/2}$ = 16.2 h), have half-lives from several minutes to hours. Long-lived emitters, such as ^{89}Zr and ^{124}I, have half-lives of 3.2 and 4.2 days, respectively. Nanoparticle-positron emitter conjugates are used for the investigation of diagnostic imaging and for pharmacokinetics and pharmacodynamics investigations in vivo. For example, graphene oxide, liposomes, gold nanoparticles, metal oxide nanoparticles, nanomicelles and dendrimers are used in the PET imaging of different organs, especially for tumor and cardiovascular imaging (Liu and Welch 2012).

(h) ***Single-photon emission computed tomography***

Single-photon emission computed tomography (SPECT) uses gamma photon–emitters to produce two-dimensional images, which can be reconstructed to three-dimensional images. This is used for the imaging of cancer, as well as organs such as the heart and liver. Similar to PET, high-resolution anatomical information can be obtained in SPECT when the photon emitters are conjugated to nanoparticles. For example, technetium-labeled iron oxide nanoparticles are used for imaging breast cancer and malignant melanoma. PEGylated polymeric nanomicelles conjugated to indium radioisotope is useful for the imaging of prostate cancer cells (Zhang et al. 2011). Iodine-125-labeled silver nanoparticles are used to enhance the imaging resolution of the liver and spleen using SPECT (Chrastina and Schnitzer 2010).

(i) ***Fluorescence correlation spectroscopy***

Fluorescence correlation spectroscopy (FCS) is widely used to examine the dynamics of biomolecules and nanoparticles in living cells. FCS measures the fluorescence intensity fluctuation upon continuous irradiation of the molecules or nanoparticles. The sample is irradiated and the fluorescence intensity fluctuates due to the physical and chemical changes taking place in the sample. The fluctuations in the fluorescence intensity are determined using the temporal autocorrelation. FCS gives information about the diffusion coefficient and the average concentration of the molecules when taken up by the cells. It is also used for the determination of DNA sequences. Nanoparticles used for biomedical applications can be analyzed for their mobility, their association and dissociation with the biomolecules, and their self-aggregation.

One of the important applications of FCS is in thrombin assay (Xu et al. 2014). Thrombin's major role in blood coagulation and its ability to induce vasoconstriction are strongly related to the incidence of cardiovascular diseases. Hence, thrombin assay is very crucial for health and disease. In this scenario, FCS is useful for the sensitive detection of thrombin through the determination of the diffusion time of gold nanoparticles with a sensitivity of 0.5 nm. In principle, gold nanoparticles labelled with two different aptamers interact with the thrombin molecules in the solution and assemble into dimers and oligomers, resulting in an enhanced diffusion time in the detection volume, as reflected by the FCS.

In addition, FCS is also useful in the investigation of two important factors related to biomedicine: drug-DNA interaction and the density of drug-bound macromolecules.

(j) ***Sundry techniques***

IT-AFM-mediated nanomechanical profiling is useful in the clinical diagnosis of breast cancer with translational significance (Plodinec 2012). Cryo-TEM is used for the analysis of cubosome phases, which is used in ocular drug delivery.

4.2 Therapeutic Techniques Using Nanoparticles

(a) ***Photodynamic therapy***

From Chap. 3, it is clear that reactive oxygen species (ROS) are capable of interfering with cell membrane fluidity and forms adduct, not only with the membrane but also with vital macromolecules such as proteins and DNA, thus damaging the whole cell. Photodynamic therapy (PDT) exploits this process of ROS-induced cell destruction by killing cancer cells in the presence of light-sensitive molecules called photosensitizers. The stepwise protocol of PDT is as follows:

- Light-sensitive nanoparticles are targeted to the vicinity of the tumor cells using a targeting ligand (see Chap. 2).
- The particles are excited with light rays of suitable wavelengths.
- The particles absorb the light energy and pass onto the surrounding oxygen, resulting in the production of ROS.
- ROS bind to the tumor cells and induce oxidative stress and damage, finally killing them.

Traditional photosensitizers were irradiated with ultraviolet light, but without penetration into deeper tissues. Since then, nanoparticles have become better photosensitizers because they can absorb near-infrared light and penetrate deeply into the tissues. However, parental administration of a photosensitizer and its local irradiation is difficult. Also, it is difficult to treat metastatic tumors by this method (Wilson 2000).

(b) ***Radiation therapy***

Radiation therapy (also called radiotherapy) is the process of controlling the growth of tumor cells by passing ionizing radiation, such as X-rays. The morbidity-to-recovery ratio is higher in radiotherapy. However, this can be evaded by selectively targeting the radiosensitizer to the tumor cells without affecting the healthy tissues. This would not only prevent the morbidity of the healthy tissues but also perk up the liberation of the targeted dose. In this regard, nanoparticles can be used to mediate radiotherapy. The nanoparticles should be functionalized with a targeting ligand, which may complement the cancer cell surface receptors. Folic acid is one such targeting ligand capable of targeting these receptors, which are overexpressed on the surface of tumor cells. Huang et al. (2011) revealed the role of folic acid ligands in tumor targeting. Gold nanorods decorated with silica and functionalized with folic acid can be used for radiotherapy and plasmonic photothermal imaging of gastric cancer cells (Kwatra et al. 2013).

(c) ***Hyperthermia***

In humans, hyperthermia is defined as a temperature greater than 37.5–38.3 °C (99.5–100.9 °F). Exposure of cancer cells to a higher temperature (i.e., greater than 42 °C) in order to damage the cellular proteins and other vital molecules and kill the tumor cells is called hyperthermia or hyperthermal therapy, or thermotherapy. Cellular hyperthermic effects are as follows:

- Loss of cytoskeletal structural integrity
- Altered membrane fluidity
- Membrane blebbing
- Denaturation of cellular and circulatory proteins
- Impairment of replication and transcription processes
- Programmed cell death (apoptosis).

Irradiation can be carried out using microwave, near-infrared light, radiofrequency, or ultrasound radiation using a suitable probe to produce heat in the case of whole body exposure. Thermal chambers and hot water blankets may also be used to generate heat. The temperature and the duration of exposure should be critically followed in this type of treatment.

There are three types of hyperthermia:

(i) Plasmonic photothermal therapy
(ii) Radiofrequency-induced ablation
(iii) Magnetic fluid hyperthermia.

Plasmonic photothermal therapy involves the use of gold nanoparticles and their irradiation using NIR radiation. A diverse range of gold-based nanoparticles such as gold–silica nanoshells, gold nanorods, gold colloidal nanospheres, NIR-tunable gold nanocages, gold–gold sulfide nanoparticles, and hollow gold nanoshells are used for this purpose. NIR laser radiation is perfectly suitable for gold-mediated thermal ablation in vivo. This is because the absorption coefficient of the body's

chromophores (blood hemoglobin and water) is lesser in the NIR region (650–900 nm) as compared to the visible region (400–600 nm). This would result in minimal off-target damage.

Light is incident upon the particles to activate them. The resulting electron–phonon interactions dissipate heat, thereby inducing irreversible thermal ablation of the tumor cells. Interestingly, a pulsed laser such as the femtosecond-pulsed NIR laser is better than a continuous-wave laser in terms of power consumption and therapeutic efficacy. The reason is that a pulsed-laser flow has lapses between the pulses, which increases the electron-phonon relaxation time, thereby leading to more efficient photothermal conversion. Although NIR radiation is effective, the penetration depth of NIR is low, thus restricting its application to directly accessible solid tumors and skin tumors.

RF-induced ablation involves the use of magnetic nanoparticles, where hyperthermia can be coupled with MRI. Because magnetic nanoparticles were targeted for imaging the cancer cells, radiofrequency (RF) pulses can be passed. RF not only produces images for detection, but it also can be converted into heat to kill cancer cells. This process is called RF ablation or RF-induced hyperthermia. Because the penetration depth of the RF wave is greater than that of NIR wave, RF can be used to kill deeper tumors unlike NIR. Nonetheless, the process of RF ablation involves the insertion of electrodes into the body to deliver RF to the target site, making it an invasive method.

In magnetic fluid hyperthermia (MFH), superparamagnetic iron oxide nanoparticles were used in the form of suspension or fluid. The fluid is allowed to interact with an AC magnetic field. Within the nanoparticles, the magnetic energy gets converted to internal energy, which is eventually converted into thermal energy via Brownian and Néel relaxations. The advantages of MFH include the following:

- SPIONS convert magnetic energy into thermal energy at a lower field strength.
- SPIONS are biocompatible.
- It is highly efficient in treating deeper tumors because the alternating magnetic fields are not susceptible to attenuation by off-target tissues.
- MFH can simultaneously be used as a contrast agent for in situ imaging and diagnosis (Kennedy et al. 2010).

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Chapter 5
Nanotoxicity and Risk Assessment

The fields of research, medicine, materials science, metals, food, and electrical and electronics could not resist the production and use of nanoparticles because of their unique mechanical, physical, and chemical properties. Thus, nanotechnology plays a leading role in cutting-edge research and technology. Because nanoparticles are unique, their interactions with biological components such as cells and their vital molecules are also unique when compared to their bulk counterparts. The high surface-to-volume ratio and the size of the nanoparticles help them to gain entry into the environment and the living system, including human beings via different routes of portal entry (Fig. 5.1):

- Inhalation
- Ingestion
- Dermal
- Intravenous (during medical applications).

Nanotoxicity is the degree of the negative effect of a nanoparticle toward a living organism upon intentional or occupational exposure to that particle. Toxicity assessment should be done to nanoparticles intended for biomedical use, as well to nanoparticles that are likely to cause biohazards. Humans are exposed to nanoparticles from various sources (Fig. 5.1), including industries, laboratories, biomedical applications, environmental/atmospheric sources, and occupational sources.

Inhalation is a major route of exposure. The inhalation of nanoparticles results in the diffusion of nanoparticles from the nasal mucosa to the lungs through the respiratory tract. These particles are internalized into the epithelial and endothelial cells of the lungs via different processes, such as phagocytosis, endocytosis, and subsequent transcytosis. Finally, the particles enter into the lymph and the blood. From the blood, the particles can gain entry into vital organs such as the heart, liver, kidney, and sometimes brain. The particles can also be translocated to lymph nodes and the spleen. The pharmacokinetics of the nanoparticles generally depends on their surface chemistry and in vivo modifications. We have commented elsewhere

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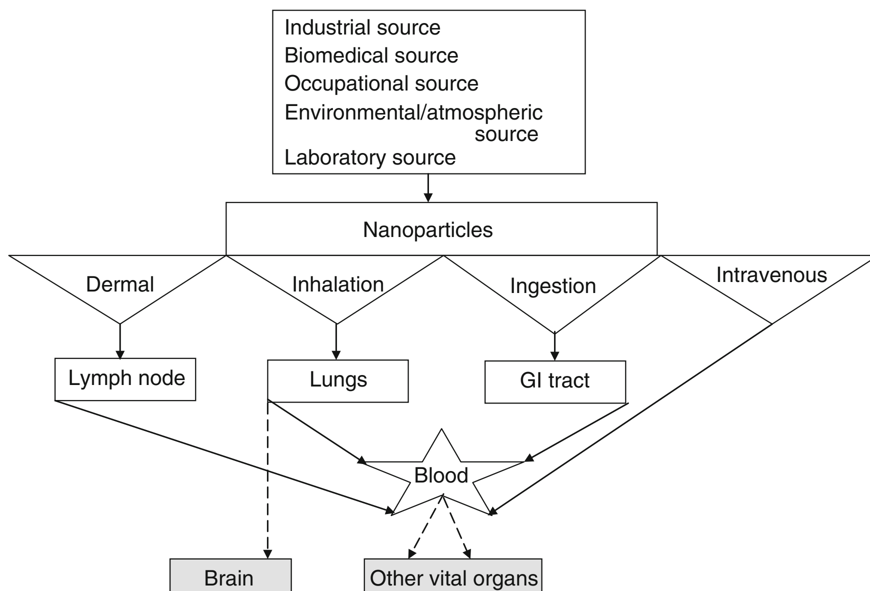

Fig. 5.1 Sources and routes of exposure of nanoparticles to biological systems

on nanotoxicity and its impact on biological systems (Devasena and Francis 2014, 2015). Mostly, nanoparticles liberated into the atmosphere enter into the airway through breathing, thus paving the way for inhalation toxicity upon continuous exposure.

The ingestion of nanoparticles refers to their entry from the buccal cavity (oral route) into the digestive system. This is facilitated by the intake of solid or liquid food, or even water and medicine. Nanoparticles entering into the gastrointestinal tract are mostly eliminated from the body via feces. However, some of them may be engulfed by the gut mucosal cells, thus gaining access into the systemic organs (Jani et al. 1990, 1994); they are primarily targeted to the liver. The probability of ingestional toxicity is less than inhalation toxicity.

Dermal exposure to nanoparticles is mostly due to the repeated application of sunscreen lotions and other cosmetics formulated with titania or zinc oxide nanoparticles. Damaged skin is even worse because it is readily permeable to the nanoparticles, thus providing access to the dermis layer. The rich supply of blood and lymphatic vessels and predominant distribution of immune cells (the tissue macrophages and dendritic cells) enhance the biodistribution of the nanoparticles, especially to neuromuscular junctions.

Intravenous exposure is possible when nanoparticles are administered through veins for some biomedical applications. Usually, an intravenous route of administration is preferred to enhance the bioavailability of poorly aqueous soluble drugs. In such cases, the effect of nanoparticles on systemic parameters such as hemolysis, hematological changes, and biochemical changes should be analyzed as toxicity

indices. A toxicity assessment of intravenously administered nanoparticles in mammals is very essential to approve the drug for human use (Francis et al. 2015a, b).

Any route of entry could allow the nanoparticles to gain entry into remote organs by one or more of the following strategies: transferring into the deeper alveoli in the lungs, moving into the bloodstream via the blood-air barrier, or crossing the blood-brain barrier to reach the brain. Cell vision (i.e., the interactions between nanomaterials and cells of animals, humans, and plants) are remarkably complex. The physiochemical properties and interactions with biomolecules and the cell surface determine the toxicity of nanoparticles in a biological system. Carbon and metallic nanomaterials are among the most widely used types of engineered nanomaterials. Among carbon nanomaterials, the production of carbon nanotubes (CNTs) is greatly increasing.

Toxicity can be assessed in vitro by checking the viability of the cells using the MTT assay. This assay is based on the principle that viable cells have reactive succinate dehydrogenase enzyme capable of converting the yellow compound MTT into a purple formazon, the intensity of which is proportional to the number of viable cells. Therefore, the cell lines can be treated with the test nanomaterial and the color change can be observed. The formation of purple formazon indicates the nontoxic nature and biocompatibility of the nanomaterial (Francis et al. 2011; Suganya and Devasena 2015). Using cell lines, the IC_{50} value of any nanoparticle can be determined. IC_{50} is the minimal inhibitory concentration that is capable of inducing 50 % cell death. From this value, the toxic and the therapeutic dose can be fixed for any nanoparticles of biomedical use. Erythrocytes can also be used as a reliable model to assess the hemolytic activity of nanoparticles intended for intravenous administration (Francis et al. 2015a, b). However, toxicity testing in vivo models such as rats, mice, or zebrafish is more valid for advanced studies. Depending upon the nature of the nanomaterial, the route of entry into the test animals should be standardized and the toxicity studies should be done in two ways: acute and chronic.

Acute toxicity refers to the adverse effect of the test material upon single exposure, with the effect being manipulated within 14 days of exposure. Chronic toxicity is an adverse effect caused by repeated exposure to lower levels of the test materials for a longer period of time (several months). All studies should be done per Organisation for Economic Co-operation and Development (OECD) guidelines. The animals can be intentionally exposed to the test nanomaterial at a dose less than that of its LD_{50} value. The animals can be sacrificed after the experimental duration and the organs analyzed for certain biomarkers of toxicity and histopathological changes.

Toxicity can primarily result in inflammation, oxidative stress, and antioxidant imbalance—all leading to cell and tissue damage. Tissue damage then results in the following biochemical changes, one or more of which can be used as biomarkers (see Chap. 3) in addition to histopathological changes:

- Protein denaturation and protein leakage from the cells
- Loss of cell membrane integrity and fluidity

- Peroxidation of lipids leading to the formation and accumulation of malondialdehyde
- DNA damage and DNA fragmentation
- Loss of homeostasis of cytosolic and mitochondrial enzymes
- Decreased antioxidant enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase
- Decreased levels of nonenzymatic antioxidants such as glutathione, ascorbic acid, and total thiol content
- Enhancement of the level of pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha, TNF-α) and pro-fibrotic cytokines (e.g., interleukin-4, IL-4).

Some of the histopathological changes indicative of toxicity are listed in Table 5.1.

The toxicity of various nanoparticles is discussed in the following sections.

Table 5.1 Histopathological markers of nanotoxicity

Organs	Nanomaterials	Histopathological observations	References
Lung	MWCNT	Deposition in airways, alveolar lumen and interstitium, alveolar macrophages with intracytoplasmic MWCNTs, bi-, multi- and or anucleated alveolar macrophages, alveolar histiocytosis, alveolar proteinosis, acute pulmonary inflammation, mononuclear inflammatory cell infiltration, interstitial fibrosis, epithelioid granulomas	Francis et al. (2015a, b)
	CeO_2	CeO_2 nanoparticle-laden neutrophils on the bronchiolar epithelial lining, severe acute inflammatory reaction in alveoli, migration of neutrophils towards the bronchiolar lumen, multifocal granulomas, CeO_2 nanoparticles in the tracheobronchial lymph nodes, necrosis of alveolar epithelium, infiltration of mononuclear cells in alveolar and bronchiolar spaces, fibrosis, granuloma, cytoplasmic vacuolation, alveolar proteinosis	Srinivas et al. (2011), Aalapati et al. (2014)
Kidneys	CeO_2	Cytoplasmic vacuolation, tubular necrosis	Aalapati et al. (2014)
	ZnO	Intratubular protein deposition, vascular congestion	Ben-Slama et al. (2015)
Liver	CeO_2	Nanoparticle-laden Kupffer cells, hepatocytomegaly with multiple nuclei, nanoparticle-laden endothelial cells, cytoplasmic vacuolation	Aalapati et al. (2014)
	ZnO	Sinusoidal congestion, nanoparticle deposition and inflammatory response, hepatocellular necrosis	Ben-Slama et al. (2015)

5.1 CNT Toxicity

The global production of CNTs exceeds several thousand tons per year, thus increasing exposure to the environment, including humans and the ecosystem. MWCNTs showed time-dependent pulmonary toxicity, even after a single nose-only inhalation, as evident by intermittent sacrifice on days 1, 7, and 14 post-exposure in a rat model. MWCNTs induce inflammation, fibrosis, and granuloma characterized by progressive elevation of TNF-alpha and IL-4. Histopathological studies revealed the distribution of MWCNT in lungs and trachea bronchiolar lymph nodes (TBLN). Therefore, there is an urgent need to explore its repeated dose toxicity and validate an appropriate antitoxic candidate (Francis et al. 2015a, b).

5.2 Silica and Titania Nanoparticles

Silica (SiO_2) and titania nanoparticles (TiO_2) are used in food additives while zinc oxide nanoparticles (ZnO) are used in food packaging. All of these particles are known to elicit cytotoxicity in human intestinal Caco-2 cells with an enhancement in lactate dehydrogenase release. SiO_2 and TiO_2 nanoparticles are capable of inducing significant DNA damage in vitro (Gerloff et al. 2009).

Silica nanowires have many biological applications and exhibit rapid cellular entry. Hence, their toxicity needs to be justified. Silica nanowires are toxic to epithelial cell lines at concentrations greater than 190 mg/ml. The toxicity is mediated via necrosis. However, silica nanoparticles are less toxic than their one-dimensional counterpart (Adili et al. 2008).

5.3 Fullerene

Fullerene is used in drug delivery, cosmetics, and health care products. In addition, it is used during the manufacture of catalysts, lubricants, and sporting goods. Therefore, knowledge of fullerene toxicity is very essential. Fullerene is lipophilic; it is found to accumulate in cell membranes and induce lipid peroxidation. In the presence of light, fullerene is known to induce cell death by DNA fragmentation, cytotoxicity, and autophagy. In human vascular endothelial cells, 100 μg of fullerene was found to be toxic and induced cytotoxicity, vacuolation, and accumulation of polyubiquitinated proteins. At a dose of 600 mg/kg body weight, fullerene was found to induce lipid peroxidation (Yamago et al. 1995; Pap et al. 2008).

5.4 Quantum Dots

From Chap. 1, it is clear that semiconductor nanocrystalline quantum dots and their conjugates have plenty of biomedical applications, such as targeted drug delivery, gene delivery, and imaging. All of these applications should be used only after assessing the toxicity of quantum dots. The toxicity of quantum dots is not consistent because it depends on the following factors:

- Nature of the constituting element
- Method of synthesis
- Nature of the surface ligand or the conjugate
- Physiochemical properties
- Size
- Surface charge
- Concentration administered
- Oxidative, photolytic, and mechanical stability.

Hence, the toxicity assessment of quantum dots is a broad area. Cadmium (Cd) and selenium (Se) are the common elements used in the quantum dot core. Many of the toxicity studies were done using these quantum dots, which are capable of inducing acute and chronic toxicity in vertebrate models. These quantum dots, like any other nanoparticle, are internalized into the cells via endocytosis. Cadmium is known to generate reactive oxygen species and elicit lipid peroxidation; it also impairs the function of mitochondria and nuclei, ultimately leading to cell death. Cadmium has a half-life of 15–20 years in humans, leading to bioaccumulation; during bioaccumulation, it is systemically distributed to many organs, especially the liver and kidney. These elements are also capable of crossing the blood-brain barrier and placenta (Hardman 2006).

5.5 Manganese Nanoparticles

The role of manganese in inducing neurotoxicity associated with impaired motor and psychological skills is well established. The industrial applications of manganese nanoparticles are increasing. As a result, toxicity studies on manganese nanoparticles deserves consideration. Manganese nanoparticles are known to deplete the neurotransmitter dopamine, as well its metabolite dihydroxyphenylacetic acid and homovanillic acid in cell lines. Mn nanoparticles are likely to cross the blood-brain barrier and promote neurotoxicity (Hussain et al. 2006).

Awareness of nanotoxicology in terms of mechanisms of action, biocompatibility, biodegradability, and the biological fate of any target nanomaterial is an urgent need. A parallel move towards the exploration of a proper tool to ameliorate the toxicity is also essential. However, there is still a long way to go ahead before we fill the gap.

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Chapter 6
Applications of Select Nanomaterials

The diagnostic and therapeutic applications of nanomaterials are numerous. This chapter presents select examples of the biomedical applications of the following nanomaterials:

1. Dendrimers
2. Antibodies
3. Lipid-based nanomaterials
4. Viral nanoparticles
5. Nanomaterial-based devices

6.1 Dendrimers

Dendrimers are hyperbranched core shell nanostructures ranging in size between 10 and 100 nm. Dendrimers were invented by Don Tomalia in 1980. The structural components of a dendrimer are as follows (Fig. 6.1):

- Central core
- Dendrites or branches arising from the core
- Surface functional groups
- Internal cavities called voids.

A dendrimer has a central core surrounded by several generations (layers) terminating in an outermost polyvalent generation. Each generation acts as a shell. The weight and the properties of the dendrimer changes with the addition of every shell. The outer groups can be tuned to carry reactive chemical groups, antibodies (Abs), DNAs, drug molecules, or any desired ligands. Dendrimers can wiggle into the cell membrane and be distributed within the cells. The rigid three-dimensional structure of dendrimers is responsible for various unique and valuable physical, chemical,

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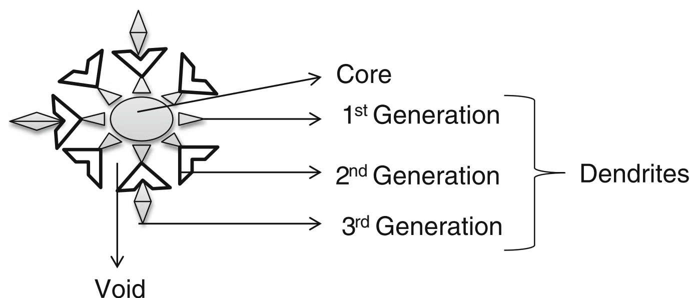


Fig. 6.1 Structure of a dendrimer

and biological properties (Table 6.1), making them excellent tools for biomedical applications, including drug delivery (Klajnert and Bryszewska 2001).

The synthesis of dendrimers is generally expensive and time consuming, restricting the number of commercially available dendrimers. Some common dendrimers are:

- PAMAM-poly-(amidoamine)
- DAB-diaminobutane
- Phosphorus PMMH (phenoxymethyl methylhydrazone)
- Bis-MPA (2,2-bishydroxymethyl-propanoic acid).

Dendrimers can be constructed in two ways (Fig. 6.2). The first strategy is a divergent approach where the reactive core is extended with branches towards the periphery in the form of layered growth. This method is suitable for the production of bulk quantities of dendrimers. In the second method, a convergent approach begins from the surface or the periphery of the dendrimer, projecting inward on the addition of monomeric units. In both the cases, the attachment of complementary groups occurs via click chemistry (Abassi et al. 2014).

6.1.1 Dendrimers in Therapeutic Applications

Gold (Au) and silver (Ag) nanoparticles have received immense scientific and technological interest because of their extensive applications in biology and catalysis. Therefore, it is important to understand the delivery system for these nanoparticles; dendrimers can act as a host for accommodating these nanoparticles. Platinum, palladium, and cobalt can also be encapsulated in dendrimers. Some of the applications of dendrimer-encapsulated nanoparticles (DENs) are shown in Table 6.2. DENs are illustrated in Fig. 6.3; their preparation protocol is schematized in Fig. 6.4 (Bradshaw et al. 2005). The amine-terminated PAMAM dendrimer is usually used for entrapping nanoparticles. However, these dendrimers have high

Table 6.1 Unique properties of dendrimers

Properties	Contributing factor
Ordered architecture	Several generations
Monodispersity	Uniformity
High loading capacity	Cavities
High shear resistance	Three-dimensional structure
Low immunogenicity	Biocompatible groups that could be added to the outer generation
Polyvalency	Tunable functional groups in outer generations
Flexible charge and solubility	Tunable functional groups in outer generations
Flexible binding property	Polyvalency
Large active surface area	Rigid construction
Film forming property	Solubility and monodispersity

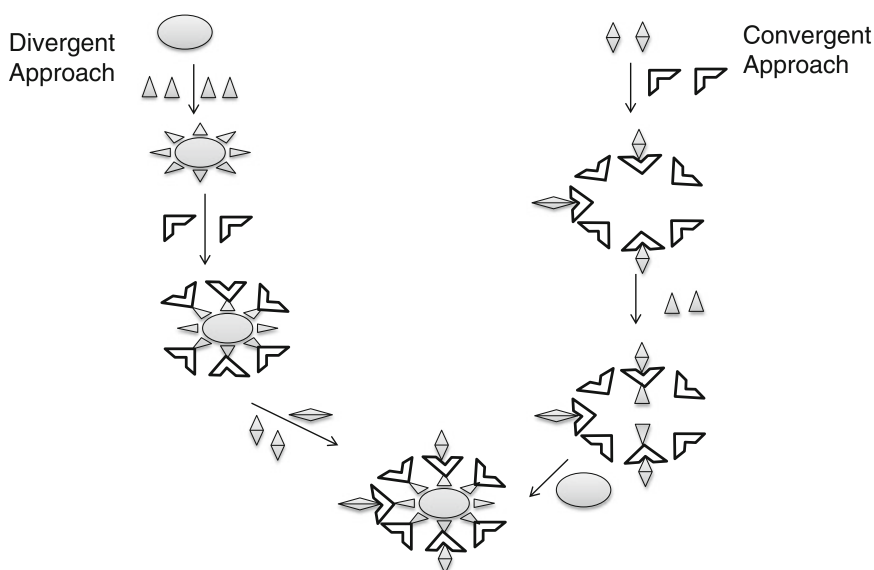


Fig. 6.2 Two strategies for the synthesis of dendrimers

cytotoxicity, low biocompatibility, and nonspecific membrane binding due to the amine surface on the dendrimer, which limits their biological applications. This problem can be circumvented by preparing biocompatible nontoxic DENs via surface modification. The usual surface modification process involves decreasing the surface charge of the amino function to neutral by acetic anhydride–mediated amidation or glycidol-mediated hydroxylation.

Table 6.2 Applications of dendrimer encapsulated nanoparticles

Nanoparticles	Applications
Ag	Antimicrobial agent Used in sensor design
Au	Functionalized Au nanoparticles are used in cancer treatment Used in improving the efficiency of anti-HIV drugs Used for identifying different classes of bacteria
Co	Used for heating applications (hyperthermia)
Pd	Catalyst

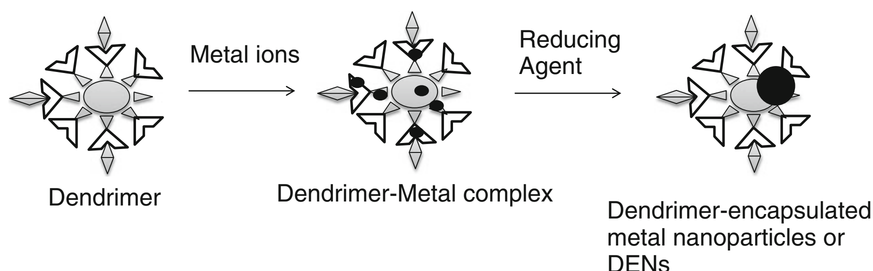


Fig. 6.3 Dendrimer-encapsulated nanoparticles

6.1.2 Nanoscale Containers

In dendrimers, the crowding effect and the surface density increases with an increase in the number of generations. The generation that results in the complete shielding of the core portion of the dendrimer due to the steric crowding effect is called the "De Gennes dense packing limit". This limit is specific for each type of dendrimer. For example, in a PAMAM dendrimer, the eighth generation causes steric crowding and the shielding effect. Thus, G8 is the De Gennes dense packing limit for PAMAM. G4, G5, and G6 PAMAM dendrimers have nanoscale container properties, whereas G7 and G10 PAMAM dendrimers display nanoscaffold properties.

There are four forms of dendritic nanocontainers (Torchilin 2006):

- Dendritic micelles
- Dendritic boxes
- Dendrophanes
- Dendrocleft.

(a) ***Dendritic micelles***

These amphiphilic molecules contain a polar head and nonpolar tail (Fig. 6.5a). In aqueous media, they assemble into a core shell structure with a polar head on the

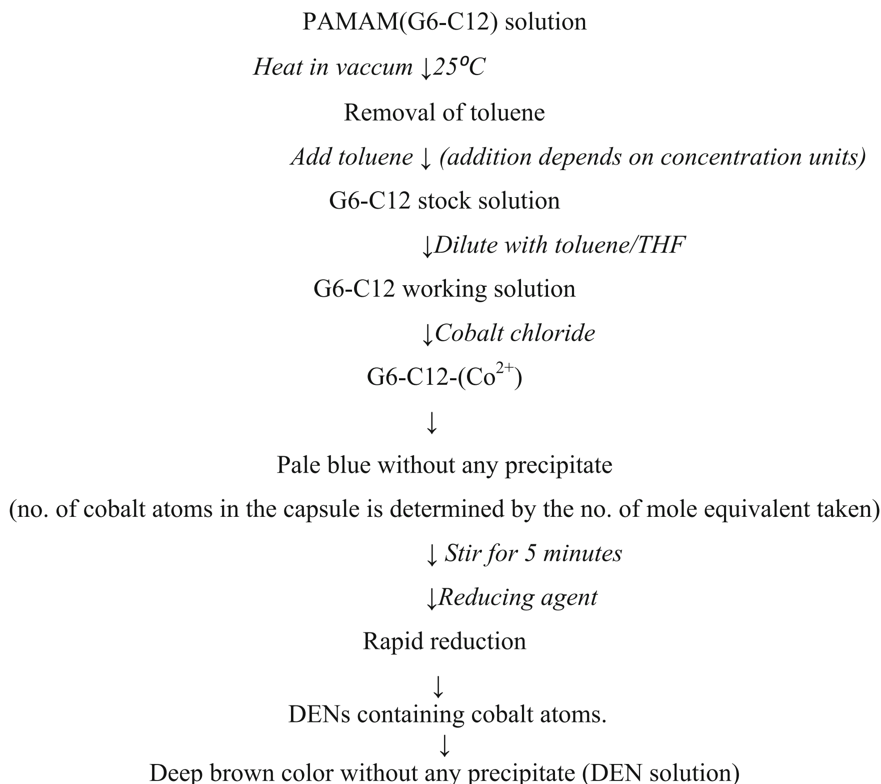


Fig. 6.4 Schematic for the preparation of DENs using dendrimer and cobalt

surface and non-polar tail in the core, forming a structure called a regular micelle (Fig. 6.5b). However in non-polar solvents, the polar head assembles at the center and the non-polar tail is projected on the surface to form reverse micelles (Fig. 6.5c). Regular micelles are good carriers for the transport of non-polar drugs in an aqueous medium. Reverse micelles effectively carry polar drugs in a nonpolar medium. PAMAM dendrimers functionalized with sodium carboxylate (so-called sodium carboxylated PAMAM) or with alkylene chains mimic regular micelles. These two dendritic carriers can accommodate hydrophobic drug molecules. Dendrimers with aryl groups and less polar groups mimic inverse micelles and carry hydrophilic drugs.

(b) ***Dendritic box***

G5 polypropylene imine (PPI) dendrimers surface modified with BOC (t-Butoxycarbonyl) protected amino acids result in surface shells with dense hydrogen bonds. This structure has a cargo space or cavity with encapsulation properties; it is called a dendritic box.

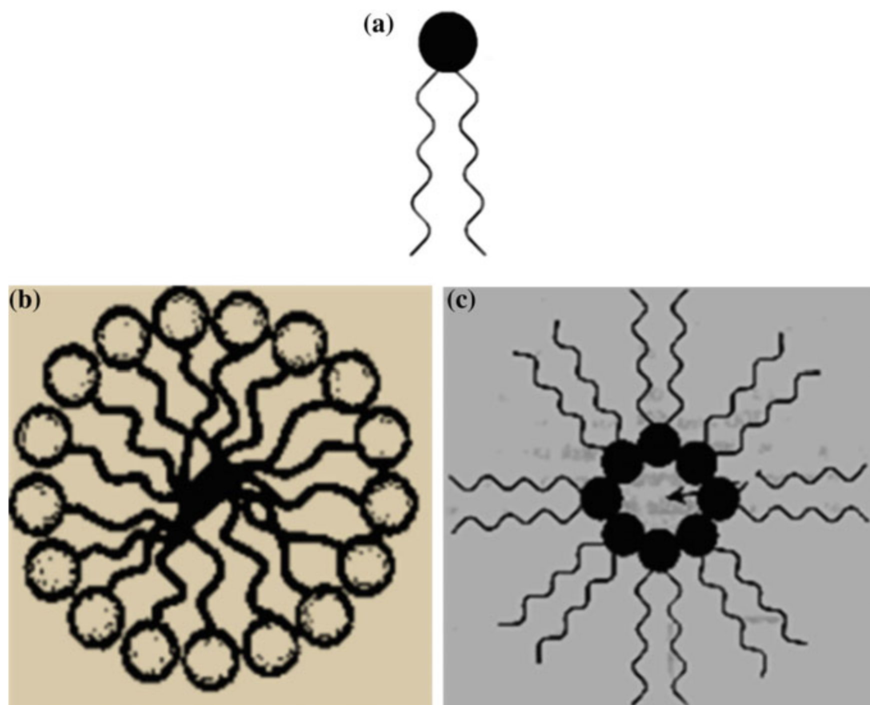


Fig. 6.5 Structure of micelles

(c) ***Dendrophanes***

Dendrimers with cyclophane cores are called dendrophanes or dendritic cyclophanes, which are capable of delivering steroidal drugs and aromatic drugs.

(d) ***Dendritic cleft***

PMAM dendrimers modified with TRIS [tri(hydroxyl methyl)-amino methane] exhibit hydrophilic clefts (dendroclefts). They are capable of accommodating carboxylic aromatic antibacterial compounds, which can be delivered when pH is decreased. Dendrimers with porphyrin cores are also used to design dendritic clefts.

Cell entry of the drug-loaded nanocontainers can be studied using techniques such as ultraviolet-visible spectroscopy, Confocal microscopy, electron microscopy, and atomic force microscopy after labeling with the targeting and the imaging ligand (see Chap. 2 for ligands). The entry may occur via nonspecific absorptive endocytosis, fluid phase endocytosis, or specific receptor-mediated endocytosis. The speed of cell entry depends on the net charge on the surface of the container, with cationic being the fastest, anionic the slowest, and neutral being intermediate in speed. This can be represented simply as follows: cationic surface > neutral surface > anionic surface.

Table 6.3 Drugs possibly delivered by nanocontainers

Drug name	Function
Cisplatin	Antitumor activity
Silver salts	Antimicrobial activity
Adriyamycin	Antitumor activity
Methotrexate	Antitumor activity
5-fluorouracil	Antitumor activity
Etoposide	Antitumor activity
Ibuprofen	Anti-inflammatory activity
Indomethacin	Anti-inflammatory activity
Nifedipine	Calcium channel blocking agent
Paclitaxel	Anticancer activity
Methylprednisolone	Anti-inflammatory activity
Doxorubicin	Cancer chemotherapy
Camptothecin	Anticancer activity

A range of drugs that can be delivered by nanocontainers (Cheng 2012) are discussed in this section and summarized in Table 6.3.

(a) ***Cisplatin***

Cisplatin is a platinum-based chemotherapy drug used to treat various types of cancers, including sarcomas, some carcinomas, lymphomas, and germ cell tumors. This drug functions in vivo by binding to and causing crosslinking of DNA, which ultimately triggers apoptosis (programmed cell death). Cisplatin is loaded to G3 or G4 PMAM dendrimers or N-(2-hydroxypropyl)methacrylamide dendrimers in order to be delivered into tumor grafts in a mouse model. Cisplatin in dendrimers has more antitumor efficiency than free cisplatin.

(b) ***Silver salts***

Two types of dendrimers are used to load silver salts and are used for antimicrobial functions.

- G4-PMAM dendrimers with an ethylene diamine (EDA) core and tris-OH surface
- G5-PAMAM with an EDA core and carrboxylate surface.

Dendrimers are treated with silver acetate, forming a complex that is more active against *Staphylococcus aureus, Pseudomonas aureginosa,* and *Escherichia coli* when compared to conventional silver-based drugs.

(c) ***Adriamycin, methotrexate, and 5-fluorouracil***

G3 and G4 PAMAM dendrimers with PEGylated surfaces are used for the delivery of adriamycin (Doxorubicin), methotrexate (an antifolate drug used in the treatment of cancer and autoimmune diseases), and 5-fluorouracil (a pyrimidine analog that is used as a drug in the treatment of cancer). The length of a PEG chain determines the

encapsulation efficiency. The longer the PEG chain length, the higher is the encapsulation efficiency and the slower is the drug release.

(d) ***Etoposide***

G2 PMAM dendrimers with PEG coating are used to enhance the chemotherapeutic efficacy of etoposide phosphate, an inhibitor of topoisomerase II (trade names: Eposin, Etopophos, Vepesid). It is used in the treatment of Ewing's sarcoma (cancer in bone and soft tissues), lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme.

(e) ***Mefenamic acid and diclofenac***

A triblock dendrimer containing citric acid-PEG-citric acid is used for the controlled and effective delivery of mefenamic acid and diclofenac (an antianalgesic), which could be helpful in the treatment of dysmenorrhea.

(f) ***Ibuprofen***

Ibuprofen (a nonsteroidal anti-inflammatory drug, NSAID) encapsulated in G3 and G4 PAMAM dendrimers having surface hydroxyl groups is able to suppress the expression of mRNA encoding cyclooxygenase-2 (Cox2), thereby suppressing inflammation.

(g) ***Indomethacin***

When indomethacin is encapsulated in G6 PAMAM dendrimers with an amino group or hydroxyl group on the surface, the solubility is enhanced. Hence, biocompatibility is also enhanced. This would be an effective treatment for fever, pain, stiffness, and swelling.

(h) ***Nifedipine***

Nifedipine is a dihydropyridinecalcium channel blocker. Its main uses are as an antianginal (especially in Prinzmetal's angina) and antihypertensive drug. It is sold under the brand names Adalat, Nifedical, and Procardia. G0-G3 PAMAM dendrimers with an EDA core and amino surface or with an EDA core and ester surface are used to enhance the solubility and bioavailability of nifedipine. The efficacy of the drug is ranked in the following order: PAMAM with ester surface > PAMAM with amine surface > free drug.

(i) ***Paclitaxel***

Polyglycerol dendrimers are used to conjugate Paclitaxel, which is a poorly soluble drug. The conjugate possesses higher solubility and bioavailability than the free drug. The conjugate functions as an effective mitotic inhibitor and helps in the treatment of metastatic breast cancer, ovarian cancer, and Kaposi's sarcoma (a tumor caused by human herpesvirus 8, HHV8).

(j) ***Methylprednisolone***

Methylprednisolone is a synthetic glucocorticoid drug. A G4-PMAM dendrimer with a free hydroxyl group or polyol group is linked to the drug via a succinic acid or glutaric acid spacer. This results in sustained drug delivery for effective treatment of arthritisacute bronchitis and long-term management of autoimmune diseases, most notably systemic lupus erythematosus and skin inflammation and irritation. The efficiency of drug delivery is determined by the spacer used. The efficiency is of the order: Dendrimer with succinic acid >>> dendrimers with glutaric acid >> free methyl prednisolone.

(k) ***5-ASA***

PAMAM is conjugated to 5-acetyl salicylic acid (5-ASA) via an azo bond mediated by spacers, such as para amino hippuric acid or para amino benzoic acid. The azo bond is specifically cleaved by bacterial flora in the colon. This is useful for colon-specific delivery of 5-ASA.

6.1.3 Dendrimers in Gene Transfection

The gene transfection process plays a pivotal role in the transfer of a therapeutic gene and helps in gene therapy for many genetic disorders (Cheng 2012). Some of the chief diseases currently being investigated for gene therapy include the following:

- Cystic fibrosis
- Sickle cell anemia
- Hemophilia
- Muscular dystrophy
- Cancer.

Cyclic core dendrimers composed of 1,4,7,10-tetra-azo-cyclodecane are ideal candidates for gene transfection. The core is allowed to react with methyl acrylate by a reaction called Michael addition. Then, the reaction mixture is treated with ethylene diamine for an amidation reaction. This results in the production of a tetravalent PAMAM dendrimer. This PAMAM dendrimer is acylated using acetic anhydride to generate dendrimers with high solubility (Fig. 6.6).

The gene construct created for transfection contains four adjoined segments:

- A gene obtained from the CV-1 cell line (cultured from the kidney cells of African Green monkeys)
- Lac Z gene coding for β-galactosidase
- Luciferase gene coding for luciferase, which catalyzes a photoluminescent reaction
- Green fluorescent protein (GFP) gene encoding GFP.

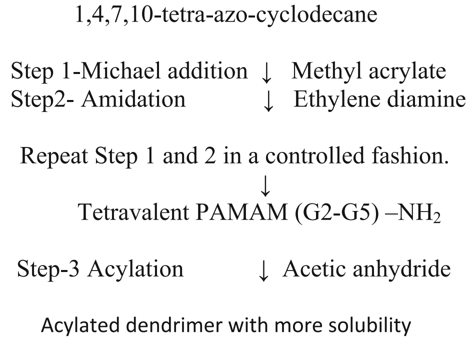


Fig. 6.6 Preparation of dendrimers for gene transfection

Coupling of the gene construct to the dendrimer is usually carried out at a pH of 7 at room temperature with incubation for 30 min to 1 h. Dendrimers confer a positive charge and the DNA backbone of the gene will confer a negative charge to establish an ionic bond, resulting in the formation of a dendrimer–DNA complex (DDC). The process of transferring a foreign gene/DNA (here, the DDC) into a mammalian host cell is called transfection. According to previous reports, scientists have used human small intestinal cancer metastatic ascities cells (HICMA cells) as a host for testing gene transfection. HICMA cells can be incubated with the DCC and transfected. The transfected cells are recombinant cells. Cells that do not acquire the foreign gene are called non-recombinant cells. Recombinant cells can be recognized by testing for the presence of foreign gene segments. The following procedures are done for identification:

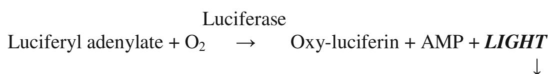
- Analysis of GFP by fluorescent in situ hybridization.
- Analysis of Lac Z gene activity
- Analysis of Luciferase activity.

Green fluorescent protein gene, if expressed in the host cell, can result in fluorescence in the cytoplasm. Fluorescence can be detected by fluorescent in situ hybridization (FISH); thereby, the role of dendrimer in gene transfection can be confirmed.

The Lac Z gene codes for an enzyme called β-galactosidase. Thus, the presence of this enzyme activity indicates the presence of a foreign gene in the cell, which in turn confirms the gene transfection ability of the dendrimer. β-galactosidase activity is determined by adding its substrate X_gal (chemically called bromo-chloro-indolylgalactopyranoside) to the cell. The enzyme react with the substrate to form 5-bromo-4-chloro-3-hydroxy indole; this is oxidized into 5,5′- dibromo-4,4′-dichloro indigo, which forms an insoluble blue color. Thus, the presence of a blue-colored cell colony indicates successful gene transfection. White-colored colonies correspond to non-recombinant host cells. This technique is called color selection or blue-white selection.

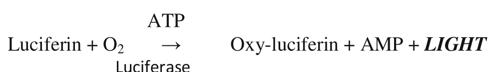
Similar to Lac Z gene, the luciferase gene expression is identified by using the reaction catalyzed by the enzyme luciferase. When the substrate luciferin is added,

luciferase catalyzes a luminescent reaction, during which light is emitted. The reaction is represented as follows:



- Confirms gene transfection
- Confirms presence of recombinant cells

Both steps of the reaction given above can be condensed as follows:



Some advantages of using dendrimers for gene transfection are:

- Dendrimers increase the transfection efficiency. They increase the number of cells transfected per minute to the total number of cells.
- Dendrimers are less toxic to cells.
- Dendrimers are biodegraded into amines, which is nutritive to the cells.
- Dendrimers protect the gene from nuclease attack by the HICMA cells.
- Dendrimer-mediated transfection is superior to viral-mediated gene delivery.

6.1.4 Dendrimers in Diagnostic Applications

Although dendrimers have several therapeutic applications, their potential diagnostic value also deserves consideration. Dendrimer-based contrast agents could help in imaging of cancer cells (Majoros 2006).

Dendrimers have the ability for selective accumulation and induce an EPR effect. Therefore, they can be used to conjugate imaging agents. Gadolinium-conjugated dendrimers can help in the imaging of kidney, liver, and tumor cells (Sampathkumar and Yarema 2007).

Gold nanodiscs functionalized with thiol-terminated PAMAM dendrimers and thereafter linked to amoxicilloyl groups have been used for sensing specific immunoglobulin (Ig) E produced against this antibiotic during an allergy outbreak. This sensor is capable of giving an excellent limit of detection (LoD) of 0.6 ng/mL (i.e., 0.25 kU/L) by immunofluorescence assay. This is a fast, reliable, highly sensitive, and label-free analysis (Soler et al. 2015).

6.2 Antibodies

Antibodies are glycoproteins produced by immune cells (B-lymphocytes) made of fragment antigen binding (Fab) and fragment crystallisable (FC). They possess highly specific binding moieties used for recognizing and attaching to their complementary molecules, called antigens with high affinity. Antibodies obtained from a single- ancestor-derived B cells are highly specific against a single type of epitope; they are referred as monoclonal antibodies (MAbs). In the field of nanomedicine, MAbs are used for the diagnosis of specific antigens, which are markers of specific diseases. The method of disease diagnosis using antibodies is called nanoimmunodiagnosis. The technology that makes use of the antigen-antibody binding concept for disease diagnosis is called nanoimmunotechnology. This technology may use a reaction mixture capable of giving a detectable signal, either optical or electrochemical. Sometimes, the detection system may be a chip or a glass slide, which may be fabricated into a sensor for producing a detectable signal on encountering the biomarkers of a specific disease. Apart from diagnostic ability, antibodies are also superior carriers used to deliver drugs into the central nervous system (CNS) because they easily traverse through the endothelial cells and cross the blood–brain barrier (BBB).

Rituximab was the first monoclonal antibody used to treat cancer. It is specific to CD20 antigen clusters, which are overexpressed on the surface of leukemic cells. Other examples of therapeutic MAbs are ofatumumab, veltuzumab, ocrelizumab, milatuzumab, gemtuzumab, ozogamicin, brentuximab, trastuzumab, inotuzumab, and lorvotuzumab (Firer et al. 2012). Diseases that can be diagnosed using MAbs are summarized in Table 6.4 and discussed in this section.

6.2.1 *Blood–Brain Barrier*

The BBB is a physical membranous partition of endothelial cells that separates the blood circulation and the cerebrospinal fluid (CSF) in the brain. It is impermeable to

Table 6.4 Immunodiagnosis of diseases using antibodies

Antibodies used	Detectable signal	Disease diagnosed
OX-26	Radioactivity	CNS disorder
MAb against food pathogen	Impedence	Food poisoning
MAb against cardiac troponin	Fluorescence	Heart diseases
MAb against prostate-specific antigen (PSA)	Fluorescence or scattered light	Prostate cancer
MAb against alpha feto protein	Fluorescence or scattered light	Hepatocellular carcinoma
MAbs against tau proteins	Color change and scattered light	Alzheimer's disease
Cetuximab	Optical	Epidermal cancer

the passage of many larger hydrophilic molecules (usually greater than 500 Da) from the blood to the CSF. However, the BBB allows the passage of small lipophilic molecules such as O_2, steroid hormones, CO_2, glucose, ethanol, and some amino acids. Most drugs are greater than the molecular cut off of 500 Da, thus preventing them from crossing the BBB. In addition, the P-glycoprotein efflux system of brain cells also prevents the accessibility of drugs to the brain due to backflow. However, this problem can be overcome by using antibodies as carriers because they easily enter into the brain via receptor-mediated transcytosis across the BBB. The antibody-drug conjugate can be delivered to the target by any one of the following three mechanisms (Torchilin 2006):

- Avidin–biotin mechanism
- Fusion protein method
- Trojan horse liposome mediated transport process.

In the avidin–biotin mechanism, the drug is conjugated to biotin and the antibody is conjugated to streptavidin. The two conjugates are then linked through biotin–avidin bioaffinity. The entire combination is targeted to the receptor of the cells present in the BBB. In the fusion protein method, the therapeutic agent and the antibody are linked by means of a linker protein. In the liposomal process, the drug is encapsulated within the liposome and the surface of the liposome is functionalized with the antibodies specific for the receptor. In all three methods, the antibodies interact with the receptors and are internalized to deliver the drug.

OX-26 antibodies are suitable for the delivery of methotrexate into the brain (Friden et al. 1991). OX-26 is complementary to the extracellular epitope of the transferrin receptor. OX-26 is generated from murine immunized with a transferrin receptor from rats using hybridoma technology. OX-26 can act as a nanocarrier for small drug molecules, neuroactive peptides, proteins, and methotrexate (MTX). A radiolabeled antibody-drug conjugate is used for immunohistochemical analysis and distribution studies in capillaries and brain parenchymal fractions. The activity of OX-26-(MTX) in the brain capillary fraction increases initially and decreases over time, dropping down after 24 h. Activity in the parencymal fraction shows a linear rise in activity, suggesting the time-dependent migration of anti-transferrin receptor antibody (OX-26) across the BBB. The activity can be realized using radiolabeled carbon.

6.2.2 Nanoimmunosensors

Immunosensors (ISs) are miniaturized devices used to enable the detection of the targets (the antigens) by means of immune reaction; they provide qualitative and quantitative signals. Sometimes, the sensor may be in the form of a reaction mixture that produces the signal. In principle, biological specificity between Ag and Ab results in an Ag–Ab complex or immune complex. This complex produces a

variation in optical properties, electric charge, mass, or heat, which can be detected directly or indirectly by a variety of transducers. The protocol by which the sensor recognizes and signals the target is called an immunoassay.

6.2.3 Types of Immunosensors

Immunosensors are divided into two categories: labeled and label-free.

Labels such as enzymes [e.g. glucose oxidase, horseradish peroxidase (HRP), galactosidase, alkaline phosphatase], nanoparticles, and fluorescent or electro-chemiluminescent probes are used as sensor components to quantify the target. A label-free immunosensor produces signals when the target analyte binds to the antibody in the absence of labels. Some examples of diagnostic nanoimmunosensors are discussed in the following sections.

1. Diagnosis of food pathogens using titania-based label-free sensors

Foodborne pathogens such as *Listeria monocytogenes* (LM), *Salmonella typhimurium, S. aureus, Campylobacter jejuni, Clostridium perifringens, Yersinia enterocolitica,* and *E. coli* are conventionally detected by cell cultures, microscopy, biochemical tests, luminescence assay, polymerase chain reactions, and enzyme-linked immunosorbent assays. These methods are labor intensive and time consuming. Immunosensors that detect the pathogen based on an antibody-antigen affinity reaction circumvent the disadvantages of conventional methods by providing high specificity, sensitivity, versatility, and speed. Wang et al. developed a titania-based nanosensor for the detection of LM, which is discussed here (Wang et al. 2008).

Semiconductive nanowires and nanotubes are used to fabricate nanoimmunosensors due to their unique properties and their ultrasensitivity. For example, titania is a very good choice for sensing pathogens for the following reasons:

- TiO_2 nanowire and nanotubes have band gaps between 1.8 and 4.1 eV, which are not available for other nanowires
- Easy-to-fabricate sensors using titania nanowire
- Large specific surface area
- Good chemical and photochemical stability
- Negligible protein denaturation.

The principle behind this sensor is schematically shown in Fig. 6.7.

The fabrication and operation of the impedence sensor are outlined in the following sections.

Fabrication of TiO_2 nanowire

Nanowires of titanium dioxide (around 80 nm in diameter, usually less than 100 nm) is prepared in a Teflon-lined autoclave container by means of a hydrothermal reaction.

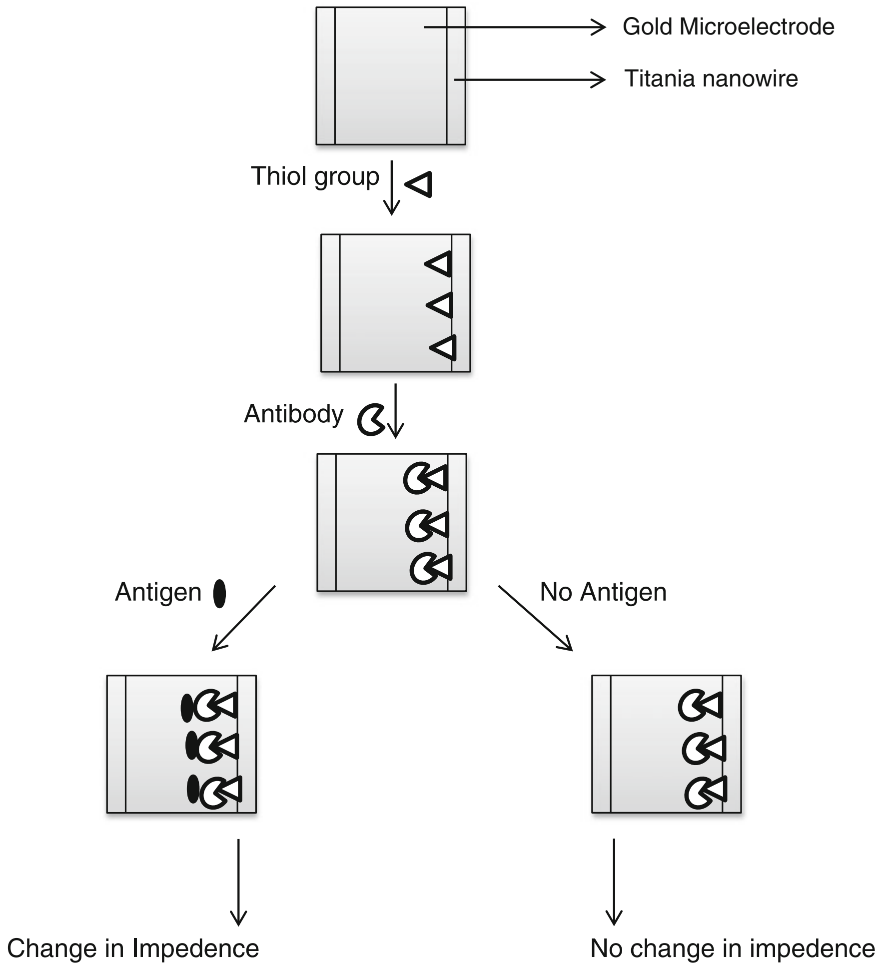


Fig. 6.7 Detection of food pathogen by a titania nanowire-based immunosensor

Connection of nanowires with gold microelectrodes
A nanowire is placed on the surface glass substrate under optical microscopy and sputtered with gold, forming a gold microelectrode. The gold microelectrode crossed with a nanowire bundle can be compared with an electrode without the bundle (negative control).
Blocking the gold surface with thiol to enable specific binding of Ab to the wire
The microelectrode is covered with ethanol, rinsed with water, and then incubated with 99 % 2-methyl-2-peopanethiol for 20 min in a hood. and then rerinsed with ethanol and water. The blocking helps the specific binding of Abs to wire.

Adding MAb for LM to the wire
An MAb for the target antigen (i.e., the pathogenic organism or bacteria) is dropped to the nanowire and incubated for 2 h. The impedence is measured (as Imp1) by applying AC potential of 5 mV across the wire (frequency range of 1 Hz to 1 MHz).
Addition of antigen solution to the MAb
The test sample (suspected to contain the pathogenic antigen) is dropped into the chip and the impedence is measured as Imp2. Simultaneously, mannitol buffer without any microbial cells is used as a negative control sample and the impedence is measured as Imp3. In parallel, a culture of the organism can also be dropped and impedence measured as a standard value, Imp4. Usually,

- Imp1 = Imp3.
- Imp2 = Imp3 indicates the absence of LM antigen in the test.
- Imp2 ≥ Imp4 indicates the presence of LM antigen.

2. Fluorescent-based troponin sensor

Troponin is a complex of three regulatory proteins that is integral to muscle contraction in skeletal and cardiac muscle, but not smooth muscle. Cardiac troponin T (cTnT) is a protein released into the bloodstream upon heart muscle damage; testing for it is done to diagnose heart attacks (myocardial infarctions). cTnT can be detected by sandwich immunoassay, where two fluorophore-labelled antibodies that are complementary to two different epitopes of the same antigen are involved in the production of signal. Gold nanoparticles are used in this type of detection.

The first antibody (the capture antibody) is attached to gold nanoparticles of approximately 20 nm; the second antibody (the detection antibody) is labeled with fluorescent dye. On adding the antigen, the two antibodies will bind to their two different epitopes. The gold nanoparticles quench the fluorescence of the dye with efficiencies as high as 95 % (this is due to fluorescent resonance energy transfer). As a result, there would be a significant change in the fluorescence intensity. On the other hand, the intensity would remain constant if the antigen is not sandwiched between the antibodies. That is, the fluorescent intensity remains constant if the suspected antigen is not present. This method is highly suitable for the detection of CTnC antigen and helpful in the diagnosis of heart diseases (Mayilo et al. 2009).

3. Bio-barcode assay

The biobarcode assay is a powerful amplification and detection system for nucleic acids and proteins with ultrasensitivity, greater than the one produced by conventional assays. The assay involves the specific biological interaction of a magnetic particle bearing antibody and a nanoparticle bearing antibody/biobarcode with the target antigen protein in a medium, forming a sandwich. An antibody conjugated to magnetic microbeads (MMB) is called a recognition Ab and one conjugated to a nanoparticle is a detection Ab. The magnetic particle allows the separation of

reacted target molecules from unreacted ones. The nanoparticles aim at amplifying and detecting the target of interest.

The components of a biobarcode assay include the following:

(a) Recognition Ab
(b) Detection Ab
(c) Biobarcode sequence
(d) Biobarcode complement
(e) Target antigen
(f) Nanoparticle
(g) Magnetic microbead (MMB) for binding to recognition antibody
(h) Nanoparticle (NP) bound to detection Ab, bio-barcode sequence.

Typically, MMBs are iron oxide–based particles functionalized with an amine group that links to the recognition antibody via glutaraldehyde as a cross-linking agent. Biobarcode complements and the capture antibodies are both immobilized on the nanoparticle's surface. The antibody that is bound to the nanoparticle recognizes an epitope of the target different from that recognized by MMB in a reaction medium forming a sandwich = *MMB/target/NP*.

The sandwich is separated from the medium and from unreacted material using a magnetic field. The sandwich is then redispersed in pure water and added to the chip containing oligonucleotides, which is complementary to the bar code sequence, thus forming a hybrid. The free end of the hybrid is then allowed to react with oligonucleotide-functionalized gold nanoparticles. Then, the chip is immersed in a photographic solution. As a result, the gold nanoparticles reduce the silver salt in the solution into silver particles, which are detected by light scattering. Alternatively, a fluorophore may also be attached to the biobarcode end and the fluorescence is detected to diagnose the antigen. A biobarcode assay is used to detect femtomolar, attomolar, and zeptomolar concentrations of antigens pertaining to prostate cancer (prostate-specific antigen), hepatocellular carcinoma (alpha feto protein), and gonadal cancer (human chorionic gonadotropin) (Cheng et al. 2006).

4. Nanoimmunodiagnosis of CNS disorders

A central nervous system disease (CNSD) can affect either the spinal cord (myelopathy) or the brain (encephalopathy) of the central nervous system. Because the CNS controls the motor and psychological behavior in the human body, a CNSD may be a fatal illness.

The following are some CNSDs of serious concern:

- Alzheimer disease (AD)
- Encephalitis
- Meningitis
- Tropical spastic paraparesis
- Arachnoid cysts
- Huntington disease

- Locked-in syndrome
- Parkinson disease
- Tourette syndrome
- Multiple sclerosis.

AD—the most common form of dementia causes memory loss and other intellectual abilities serious enough to interfere with daily life. AD results from an increase in the production or accumulation of a specific protein tau proteins in the brain, which leads to nerve cell death. Because there is no cure for AD, early diagnosis is crucial with the currently available drug treatments.

Tau proteins stabilize microtubules in the neurons. Defective tau proteins result in the destabilization of microtubules and degeneration of neurons. Furthermore, the tau proteins become hyperphosphorylated, form unusual knots, and are released into the CSF. This is the characteristic feature of AD. Thus, ultrasensitive detection of tau in CSF is of diagnostic use. Tau proteins are actually sensed by an assay mixture containing the nanoparticle and the signal is optical, (i.e., scattered light).

The steps involved in the fabrication and operation of this nanosensor (Neely et al. 2009) are as follows:

- Preparation of an MAb against tau antigen (the antibody is denoted as tau–MAb)
- Coating of Tau–MAb on gold nanoparticles (AuNP) to form Tau–MAb–Aunp
- Mixing of the Tau–MAb–AuNP with tau protein (antigen)
- Differential analysis of Ag–Ab conjugate for diagnosis of Ag.

Initially, the antibody-coated gold nanoparticles are orange. which changes to dark brown and then to reddish brown on adding tau. The change in color is a qualitative representation of the presence/detection of TauAg in the CSF, which is confirmation for AD. As a negative control, a portion of the solution is also treated with BSA (the most abundant protein in CSF sample).

The MAb–Aunp is mixed with different concentrations of tau protein (test). A separate aliquot of the MAb–AuNP is also mixed with BSA (control). The photon scattering intensity drastically increases for the test when compared to the control. Moreover, the TRPS intensity increases with increasing concentration of the antigen, thus providing quantitative information. This assay is highly sensitive and specific for tau protein.

5. Detection of epidermal cancer

Gold nanoparticles are used as a detector system for epidermal growth factor receptor (EGFR). This can be used to diagnose cancers. The fabrication and use of the sensor described by Yang et al. are summarized here (Yang et al. 2008).

The surface of a siliconized glass slide (SGS) is first siliconized by treating with 3-aminopropyltrimethoxysilane at 80 °C for 24 h. After incubation, the aminated SGS is purified with excess water and an ethanol wash. The preparation usually looks colorless, with the presence of amino and silicon groups shown using FTIR.

Gold nanoparticles are immobilized on the surface of the SGS by gentle stirring. The slides can be purified by decantation and further washing with water. The product appears red (with an absorption peak at 520 nm), confirming the immobilization of gold to form Au–SGS.

Next, the Au-SGS is soaked in a solution of cetuximab (CET; a monoclonal antibody raised against EGFR). After 4 h of incubation, the CET binds to gold nanoparticles through its amino group by means of electrophysisorption, forming a sensor (which can be represented as CET–AuNp–SG).

The sensor has a rough surface area, which increases the probability of binding to the cell surface that overexpress the EGFR receptors. For example, A431 cell lines, which have many EGFR receptors, bind with greater affinity to the CET–AuNp–SG. The EGFR-deficient cells (i.e., the negative control; e.g., MCF7 cell lines) do not bind to the sensor. Hence, this sensor is highly specific. The cells can be stained with Hoechst stain in a dark room at 4 °C.

6. High-density lipoprotein (HDL)-like nanoparticles for imaging artherosclerotic plaques

Atherosclerosis is a major cause of morbidity and mortality in industrialized nations and throughout the world. Arthrosclerosis is characterized by the accumulation of harmful lipoproteins and white blood cells (especially macrophages) in the vessel wall, leading to the development of atherosclerotic plaques. Atherosclerotic plaques block the blood vessel, causing many ailments (Table 6.5).

Because of the associated serious health threats, the detection and identification of plaques are essential. The rapid detection and imaging of plaques may help in the timely treatment and monitoring of the efficacy of therapy. The image quality of an MRI scan can be enhanced by the administration of contrast agents into the body (contrast enhancement). The presence of a contrast agent causes a large increase in the water proton relaxation rate, giving better resolution. Clinically approved contrast agents are mainly based on gadolinium (Gd3+) complexes, such as magnevist, prohance, and dotarem. Contrast agents based on gadolinium can also enhance the resolution of MRI images of atherosclerotic plaques.

Currently, HDL-like nanoparticles are used for enhancing the resolution. HDL is a heterogeneous class of plasma lipoproteins that has a hydrophobic core

Table 6.5 Ailments caused by atherosclerotic plaques

Condition caused by the plaques	Symptoms
Angina	Chest pain or discomfort that occurs when an area of the heart muscle does not get enough oxygen-rich blood due to plaques
Myocardial infarction	Interruption in the blood supply to part of the heart, causing some heart cells to die. This is most commonly due to occlusion (blockage) of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (fatty acids) in the wall of an artery
Stroke or cerebrovascular accident (CVA)	Block in the blood supply to the brain and the eventual loss of brain function

surrounded by a monolayer of phospholipids, inesterified cholesterol, and apolipoproteins. The major apolipoprotein is apoA-1.

Some reasons for using HDL as a nanoparticle for MRI include the following:

- HDL-like nanoparticles can easily be fabricated by the reconstitution of selected components.
- The small size of HDL nanoparticles enables them to cross the endothelium and to enter or exit plaques.
- Particles provide a surface for attaching MRI contrast-enhancing materials.
- The protein components of HDL nanoparticles are endogenous and biodegradable and do not trigger immunoreactions.
- They lack atherogenic effects, unlike LDL.

6.2.4 Preparation of HDL-like Nanoparticles

HDL-like nanoparticles can be prepared using one of two methods. In the first method, human plasma is used as a source of HDL. Total HDL-apolipoprotein (apo-HDL) is isolated from human plasma. Apo-HDL is reconstituted by sonication with an excess of a single species of phospholipid choline (POPC) and the contrast agent gadolinium-diethylenetriamine pentaacetic acid bis-stearylamide (Gd-DTPA-bSA). This results in the formation of a disc, called a reconstituted disc or r-disc. The second method uses purified, intact, native HDL, with core triglycerides and cholesteryl esters incubated in the presence of Gd-DTPA-bSA. This disc is called a native incubated disc or ni-disc.

These two types of HDL-like nanoparticles can be conjugated with fluorescent phospholipid analogue, 1,2-dipalmitoyl-sn-glycero-3-phosphoethenolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)(NBD-DPPE), which has $\lambda_{Ex} = 460$ nm and $\lambda_{Em} = 534$ nm. This conjugation is used for confocal fluorescence microscopic imaging and localization of HDL-like nanoparticles in tissue (Frias et al. 2006).

6.2.5 Plaques in an ApoE-/- Model

Apolipoprotein E (ApoE) is essential for the transport and metabolism of lipids. ApoE is encoded by the ApoE gene. Lack of the ApoE gene or a mutation in the apoE gene results in an impairment in the transport and metabolism of lipids, leading to the development of atherosclerotic plaques/lesions. Hence, an apoE-deficient mouse model (apoE-/-) is used for the administration of HDL-like nanoparticles to investigate imaging capability. ApoE-/- mice are fed a high-fat diet to accelerate plaque formation. Wild mice with a normal apoE gene can be used as a negative control. After administration of nanooparticles, MRI reveals the predominant localization of both r-discs and ni-discs in atherosclerotic plaques.

The nanoparticles are used to image both uncomplicated (early) plaques (rich in macrophages) and advanced plaques (rich in cholesterol crystals with very few macrophages) based on differential enhancement rates. Early plaques are enhanced within 1 h as fatty streaks because they are rich in macrophages. Advanced plaques are enhanced after 72 h (Frias et al. 2006).

6.2.6 Gold Nanoparticles

Gold nanoparticles are used in the diagnosis of DNA sequences specific for a particular disease with the help of SERS (see Chap. 4). SERS is a highly sensitive technique used to detect single molecules and gene sequences of pathogenic organisms by a method called three-component sandwich assay (Fig. 6.8; Souza et al. 2006).

The following materials are needed for the assay:

- Gold nanoprobe
- Capture chip
- Target DNA
- Silver enhancement solution
- Buffer solutions
- Enhancement solutions.

The steps are as follows:

- *Formation of the chip and coating of target DNA*: A chip is spotted with the appropriate capture strand of 15 base pairs, which is complementary to the detectable target. Then, a phosphate-buffer solution containing a 30-NT target sequence (100 pM) is added. The entire setup is incubated for 4 h in a humidity chamber at room temperature. After this, the chip is washed with phosphate-buffered saline (PBS) solution to remove nonspecifically bound targets.
- *Formation of gold nanoprobe*: Gold nanoprobes are prepared by functionalizing gold nanoparticles (of approximately 15-nm diameter) using an ONT strand that is prelabeled with Raman dye (e.g., Cy3, tetramethyl rhodamine, Texas red, Cy3.5, Rhodamine 6G, Cy5) and capped with alkylthiol. On an average, a gold nanoparticle of 13-nm diameter can load about 110 ONT strands.
- *Hybridization*: The incubated chip is treated with nanoprobes in PBS solution and incubated for 2 h to enable hybridization between the probe DNA and the target DNA.
- *Washing*: The hybridized chip is washed with PBS to remove chloride ions and nonspecifically bound nanoprobes.
- *Silver enhancement*: Immediately after washing, the chip is treated with a silver enhancement solution for 8 min.
- *Rinsing and drying*: After enhancement, the chip is rinsed with nanopure water and dried with a microarray centrifuge at 2000*g*.

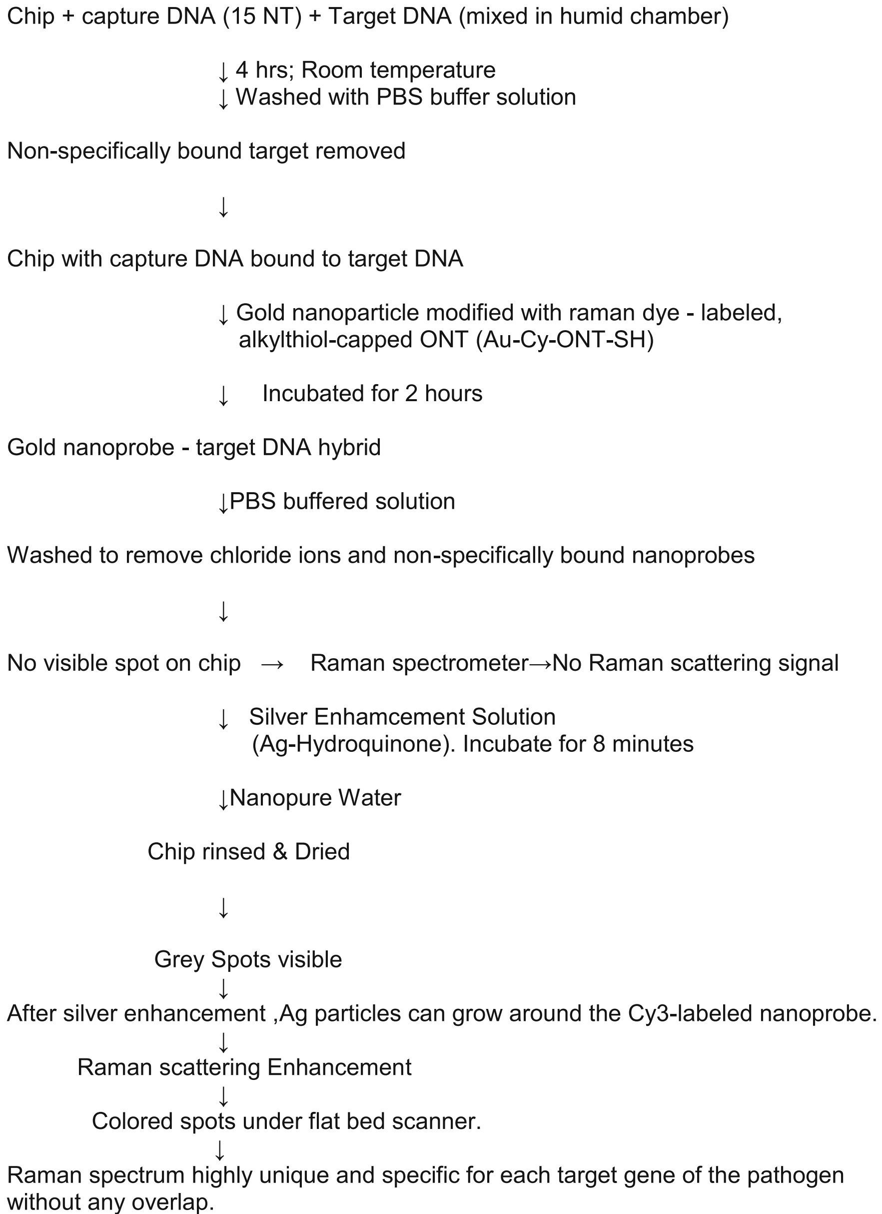


Fig. 6.8 Protocol for diagnosis by SERS

- *Scanning and imaging*: A chip showing a grey spot visible to the naked eye can be imaged with a flatbed scanner and Raman enhancement can be investigated by Raman spectrometer, which shows colored spots depending on the dye used.

6.2.6.1 Applications of Gold Nanoparticles with Enhanced Raman Spectroscopy

SERS is used for the sensitive detection of the following:

- Human immunodeficiency virus
- Ebola virus
- Hepatitis A virus (Va117 polyprotein gene)
- Hepatitis B virus
- Variola virus
- *Bacillus anthracis*.

6.3 Lipid-Based Nanocarriers

Among the range of nanotechnology platforms used for biomedical applications, lipid-based nanoparticles or nanocarriers are appropriate choices for in vivo applications due to their biomimetic composition, easy preparation, and fewer toxic effects. Lipid-based nanoparticles can be fabricated with uniform sizes, shapes, and compositions, which makes them ideal for biomedical applications. Lipid-based nanocarriers are used to formulate vaccines and pharmaceuticals for delivery through topical, oral, pulmonary, or parenteral routes. They are also used as imaging agents for the diagnosis of diseases such as cancer and neurodegenerative disorders. Liposomes, solid-lipid nanoparticles, and cubosomes are examples of lipid-based nanocarriers with successful biomedical applications. Each carrier has its own advantages and method of preparation.

6.3.1 Liposomes

Liposomes were the first lipid carriers introduced for the delivery of drugs, vaccines, and genes for a variety of disorders. They are spherical vesicles that can be produced from natural phospholipids and cholesterol. Phospholipids form bi-layered spheres in aqueous media, during which the lipid molecules self-assemble into liposomes. Nanoliposomes are tiny vesicles that are typically 60- to 80-nm in diameter. The size and morphology of liposomes can be regulated by the method of preparation and lipid composition. Water-soluble drugs are trapped inside the liposomal cavity, whereas fat-soluble drugs are incorporated within the phospholipid bilayer. The lipid bilayer of the liposome can integrate with other bilayers (e.g. cell membrane), thus delivering their cargo (Akbarzadeh et al. 2013).

Nanoliposomes used for therapeutic applications can fall under any one of the following categories (Fig. 6.9):

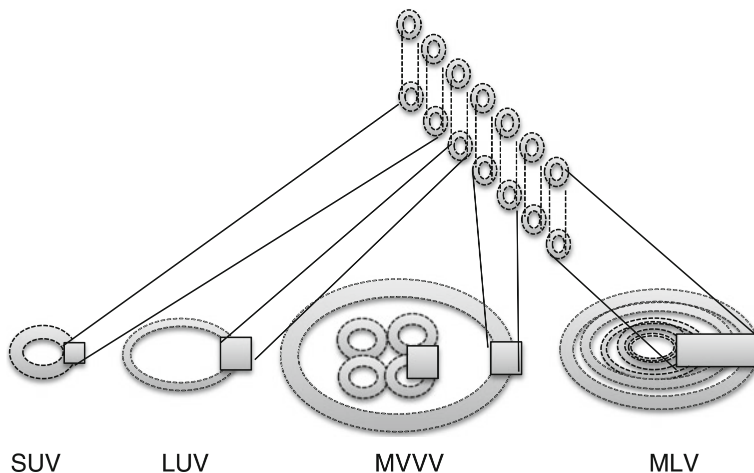


Fig. 6.9 Types of nanoliposomes

- Multilamellar vesicles (MLV) or multilamellar liposomes, composed of spherically concentric multilamellar structures (i.e., with many bilayers)
- Small unilamellar vesicles (SUVs) or unilamellar liposomes, composed of spherical concentric single bilayer structures
- Large unilamellar vesicles (LUVs)
- Multivesicular vesicles (MVV).

The properties of liposomes that make them useful for drug delivery include the following:

- Structural stability on dilution and on circulation in the blood
- Flexibility of the bilayer to uptake and deliver different kinds of molecules
- Ability to entrap both water soluble and hydrophobic substances to improve drug solubility
- Controlled delivery of small molecules, peptides, proteins, or nucleic acids
- Controlled bioavailability of small molecules and peptides following localized administration (e.g. to the lungs, subcutaneous tissue, brain)
- Ability to encapsulate siRNA involved in gene therapy.

6.3.1.1 Stealth Liposomes: The Second-Generation Liposomes

Liposomes can easily be coated by a plasma protein in a process called opsonization. These surface-bound proteins are recognition markers for liposomes to be cleared from the reticuloendothelial system (RES). Thus, the opsonized liposomes will rapidly be cleared by the RES and their circulatory half-life will be a question. In addition to this, the opsonized liposomes are easily destabilized to

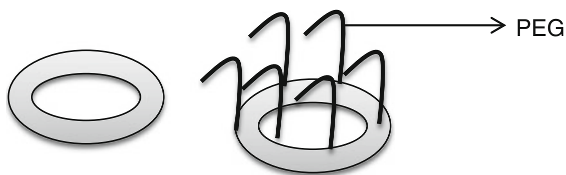


Fig. 6.10 Conventional first-generation liposomes and second-generation stealth liposomes

release the drug in off-target sites, thus reducing the biocompatibility of the drug. To overcome these difficulties, liposomes are functionalized with PEG (see Chap. 2). The PEGylated liposomes are called second-generation liposomes or "stealth liposomes" (Fig. 6.10) because they can escape the engulfment by the macrophages of the RES (Immordino et al. 2006).

6.3.1.2 Applications of Nanoliposomes

The following sections discuss the applications of liposomes in drug delivery, mostly tested in cell cultures and laboratory animals.

Liposomal Anticancer Agents

Liposomes are the first generation of nanosized drug delivery devices approved for the treatment of cancer. Both stealth liposomes and non-stealth liposomes are used for anticancer drug delivery (Hyodo et al. 2013).

Doxil (also called Caelyx) is a formulation containing doxorubicin encapsulated in a stealth liposome. Intravenous administration of Doxil is used to treat Kaposi's sarcoma (a tumor caused by human herpesvirus 8). Two factors that make Doxil a versatile anticancer drug carrier are its passive targeting via extravasation and high drug payload.

Daunoxome is a formulation containing the durg daunorubicin encapsulated in liposomal vesicles of high phase transition temperature lipids, such as distearoyl phosphatidylcholine. This is used to treat Kaposi's sarcoma as well as paclitaxel and cisplatin-resistant ovarian cancer.

Other drugs are also formulated with liposomes. These include 5-fluorouraci l lipid analogue, vincristine, bleomycin, mitozantrone, paclitaxel, valinomycin, and cisplatin.

Thermosensitive liposomes for melanoma

Thermosensitive liposomes release their contents in response to elevated temperatures by extravasation. Egg phosphatidyl choline-based thermosensitive liposomes encapsulating taxol are used for treating melanoma tumors.

Co-delivery of anticancer drugs and Si RNA for small cell lung carcinoma: The third- generation liposome

A very important application of a nanoliposome is its role in the co-delivery of drugs and Si RNA for treating small cell lung carcinoma. Small cell lung cancer (SCLC, previously known as oat cell carcinoma) is considered to be an aggressive type of lung cancer that is likely to be fatal if left unattended. It is mainly caused by tobacco smoking. Metastasis of SCLC occurs rapidly in the mediastinal lymph nodes, liver, bones, kidney, and brain. SCLC is resistant to drug-based treatment and chemotherapy, which consequently leads to the requirement for higher doses of the anticancer drug. This results in adverse side effects in off-target organs and healthy tissues. SCLC is characterized by two types of resistance mechanisms, which are both associated with poor drug delivery and bioavailability (Saad et al. 2008): (1) pump resistance, in which multidrug resistance-associated proteins (MRPs) mediate the efflux of anticancer drugs via the memberane efflux pumps; and (2) nonpump resistance, in which the BCL2 protein activates the anti-apoptotic cellular defense. The resistance mechanisms can be suppressed or inactivated by knocking down the genes coding for the MRPs and the BCL2 proteins. Liposomes can deliver the SiRNA to interfere with the expression of these two genes and also

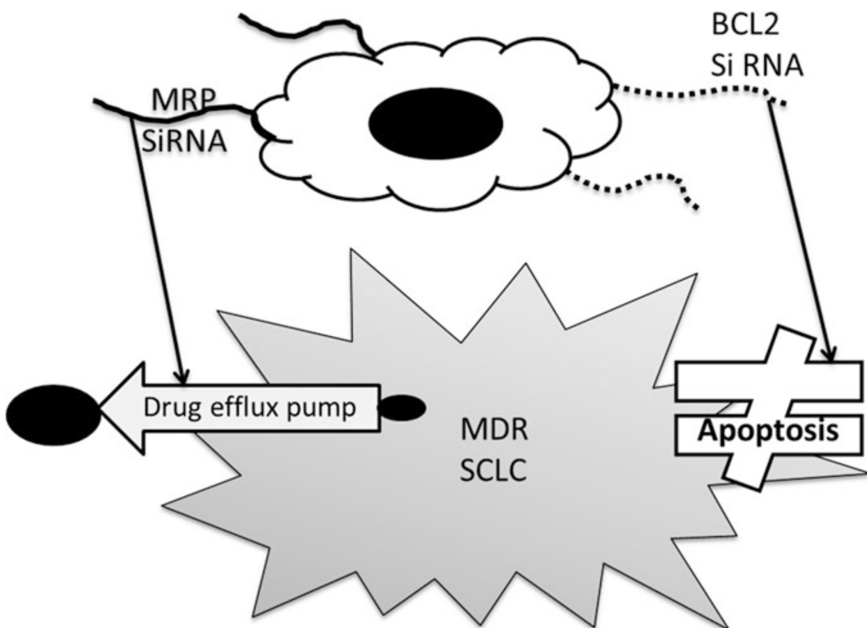


Fig. 6.11 Liposome combo with SiRNA and doxorubicin for treating drug-resistant small cell lung carcinoma

Table 6.6 Anti-infective drugs delivered by liposomes with high efficacy

Name of the drug	Disease treated
Amphotericin B	Systemic fungal infection
Amphotericin B	Leshmaniasis
Hamycin	Systemic *Candida albicans* infection
Rifampicin	Tuberculosis
Isoniazid	Tuberculosis

to co-deliver the drug for achieving full efficacy. Thus, for SCLC, the liposome combo should contain the following components (Fig. 6.11) for effective treatment:

- Cationic liposomes as a carrier
- Doxorubicin as the anticancer agent
- siRNA targeted to interfere with the expression of MRP1 mRNA and to consequently inhibit the pump resistance
- siRNA targeted to BCL2 mRNA to suppress the cellular anti-apoptotic defense (nonpump resistance).

Liposomal Anti-infective Drugs

Liposomes are used to enhance the efficacy and modulate the side effects and toxicity of certain anti-infective drugs (Woudenberg et al. 1994) (Table 6.6).

Some examples of the applications of liposomal drug delivery in the treatment of infectious diseases (Coune 1988) are summarized in the following sections:

Ambisomes for infection
Ambisomes are amphotericin B (antifungal agent) encapsulated in liposomal vesicles. While killing the fungal cells binding to their cell surface sterol, the amphotericin also acts on the cholesterol of mammalian cells and induces renal toxicity. Ambisomes are reported to reduce the toxicity and adverse side effects of unencapsulated amphotericin. Ambisome may also be used to treat drug-resistant leishmaniasis, a parasitic infection of the RES. Because the liposomes are readily taken up by the macrophages and accumulated in the liver and spleen (i.e., the RES), they are useful in the treatment of diseases of the liver and spleen, such as leishmaniasis. Ambisome is superior to antimony, pentamidine, and free amphotericin for treating visceral leishmaniasis.
Hamycin for Candida *infection*
Hamycin is a polyene antibiotic with a similar mechanism of action to amphotericin B. When encapsulated in liposomes, hamycin was reported to improve the survival of mice infected with systemic *C. albicans.*
Liposomes for anti-tuberculosis drugs
Liposomes encapsulated with anti-tuberculosis agents rifampicin or isoniazid and coated with 0-stearoyl amylopectin and polyoxyethylene or monosialoganglioside may be targeted to the lung, which results in modulating the toxicity and improving the efficacy of these drugs.

Vaccine Delivery

Nanoliposomes assembled using phosphotidyl serine can be loaded with immunogens such as microbes (which produce a specific disease), toxins of the microbes, soluble antigens, cytokines, or DNA segments. After this, the vesicles are again trapped in alginate-lysine microcapsules and administered through an intramuscular route. Finally, the immunogens will passively accumulate in the regional lymph nodes. The alginate microcapsules help for the controlled release of the immunogens and for mediating the immunity (Schwendener 2014).

Plasmid DNA vaccine

A plasmid DNA vaccine can be obtained by the expression of a gene construct of the following combination:

(i) The gene encoding the vaccine protein (i.e., the immunogen)
(ii) The promoter sequence (usually sourced from cytomegalovirus or Rous Sarcoma virus) for promoting the m RNA expression
(iii) The polyadenylate sequence at the 3′ end of the DNA for promoting the stability of the expressed mRNA
(iv) The plasminogen activator gene for controlling the secretion of the end product (i.e., the vaccine)

This gene construct is trapped to the liposome vesicle by a mild dehydration-rehydration procedure (Fig. 6.12) and the mixture is freeze-dried. The product is conventionally administered via an intramuscular route because muscle cells are responsible for DNA vaccine uptake and expression. Other possible routes of delivery include intraepidermal, oral, nasal, vaginal, intravenous, intraperitoneal, and subcutaneous (Gregoriadis et al. 2000). Liposomes are used for delivering DNA vaccines for hepatitis A and influenza.

Liposomes have the advantage of simultaneously carrying both hydrophilic and hydrophobic drugs. For example, vesicles prepared by combining a double emulsion method with dynamic high-pressure microfluidization can accomodate both vitamin C and MCFAs with storage stability for two months at 4 °C in a sucrose medium (Yang et al. 2013). A PEGylated nanoliposome attached to thyroid-stimulating hormone helps to target the anticancer drug gemcitabine into thyroid cancer cells in vitro and in vivo. The nanoliposomes showed higher cellular uptake, suggesting potential applications in a broad spectrum of thyroid diseases (Paolino et al. 2014).

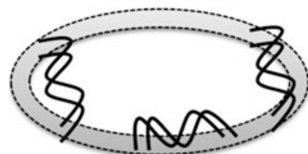


Fig. 6.12 DNA–liposome complex

Diagnostic Applications of Liposomes

Liposomes tagged with the appropriate imaging agent would be of immense use in the diagnosis of plaques and tumors in different organs. For example, the diagnosis of AD can be considered. AD is a neurodegenerative disorder characterized by an excessive accumulation of amyloid peptides in senile plaques. The amyloid peptide can be the recognition moiety for the diagnosis of AD. To recognize this peptide plaque, a signaling molecule is needed.

In this milieu, the fluorescent property of curcumin can be utilized. Curcumin is a polyphenolic compound with two aromatic rings bridged by a seven-carbon skeleton with β-diketone moiety. It has a strong affinity for the amyloid peptide. However, its hydrophobicity limits its dispersibility and bioavailability, thereby hindering its diagnostic use. Nanoliposomes are ideal candidates to overcome this hurdle. Curcumin conjugated to nanoliposomes showed good solubility and dispersity; hence, they could be used for staining the plaques. Fortunately, the conjugate is also endowed with the ability to downregulate the production of the amyloid peptide (Lazar et al. 2012). Efficient targeting is achieved by using nanoliposomes pre-functionalized with an anti-transferrin receptor antibody, which eases the entry of the drug-loaded carrier into the BBB (Mourtas et al. 2014).

6.3.1.3 Solid-Lipid Nanoparticles

Following liposomes, solid-lipid nanoparticles (SLNs, also called lipospheres) were used for drug delivery. An SLN is a spherical colloidal carrier made of simple solid lipids (e.g., triglycerides, tristearin) as a matrix whose surface is made of amphipathic lipids such as phospholipids, bile salts, and poloxamers. The lipid phase remains in a solid state at body temperature and room temperature. The drugs embedded in the solid phase would therefore show lesser mobility, accounting for the controlled release. They can be produced by one of the following methods (Garud et al. 2012; Patidar et al. 2010)

(i) *High-pressure homogenization*: The lipid and the drug are melted at a high temperature, usually greater than the melting point of the lipid. They are then mixed with an aqueous solution of surfactant, stirred, and finally processed in a high-pressure homogenizer (500 bars). This results in the production of a nanoemulsion, which recrystallizes into an SLN when cooled. However, heat-sensitive drugs cannot be formulated by this method. Alternatively, the mixing of the lipid and the drug can be done using liquid nitrogen and a cold surfactant without the use of high temperature.

(ii) *Film ultrasound dispersion*: The lipid and the drug are mixed with an organic solvent. The mixture is then decompressed, rotated, and evaporated to form a lipid film. The lipid film is then dispersed in an aqueous solution to form an emulsion. The emulsion is then sonicated using an ultrasound probe, which could result in the formation of a uniform SLN.

(iii) *Solvent emulsification/evaporation*: The lipids are dissolved in hydrophobic solvents, which are then mixed with an aqueous solvent. Consequently, the solvent mixture is evaporated, which results in the precipitation of lipid nanoparticles (average 25 nm) in the aqueous medium. The excess solvents can be removed from the emulsion by low-pressure evaporation.

(iv) *Microemulsion method*: In this method, fatty acids of low melting point, suitable emulsifiers, and/or coemulsifiers are all mixed with water and stirred at a temperature of 60–70 °C, thus forming a hot emulsion. The emulsion is then dispersed in ice cold water under stirring, which results in the formation of an SLN dispersion.

(v) *Supercritical fluid (SCF) technology*: SCF technology is a novel technology used in the production of an SLN. A SCF is under a pressure and temperature exceeding their respective critical values. Carbon dioxide solution is the best solvent for this method. A SCF has high dissolution capacity and can produce dry particles upon rapid expansion, without the use of organic solvents.

The advantages of SLNs include the following:

- High physical stability
- An ability to safeguard the cargo drugs by preventing their degradation
- Sustained and controlled drug release
- Suitability for multiple routes of administration, such as intravenous, oral, topical, ocular, pulmonary, and rectal
- Low toxicity because they are typically made of biocompatible physiological lipids.

However, SLNs have the following disadvantages:

- An ability to grow and crystallize
- An ability to form gels
- Low drug payload.

Applications of SLNs

Some general applications of SLNs include the efficient delivery of vaccines, genes, peptides, proteins, anti-parasitic, and antituberculosis drugs. SLNs are capable of crossing the BBB and hence can be used for drug delivery to treat neurodegenerative diseases. They also play a pivotal role in ultrasonic drug delivery. Apart from therapeutics, it is also a good carrier for image contrast agents, so it is used in tumor diagnosis. SLNs are promising carriers for proteins, peptides, and antigens of therapeutic value due to their excellent drug stability and ability to prevent the drug from enzymatic cleavage by proteases and peptidases. For example, cyclosporine A, insulin, calcitonin, somatostatin, and protein antigens can be successfully delivered by SLNs (Almeida and Souto 2007).

Most cancers spread from their origin to systemic circulation via the lymphatic system and sentinel lymph nodes. Therefore, the lymphatics and sentinel lymph nodes are targets for drug delivery and diagnosis. SLNs are highly useful for targeting the lymphatic system when administered via duodenal, subcutaneous, and pulmonary routes. SLNs are capable of crossing the duodenal mucosa (i.e., trans-mucosal transport) and getting conveyed to the lymphatics. SLNs show a high degree of tumor uptake. Hence, they can be efficiently used for the delivery of anticancer drugs, such as etoposide, methotrexate, and idarubicin. The SLNs can be tracked if radiolabeled with ^{99m}Tc (Cai et al. 2011).

SLNs containing paramagnetic chelates, such as $[Gd–DTPA(H_2O)]^{2-}$ and $[Gd–DOTA(H_2O)]^{-}$, are capable of enhancing the tissue proton relaxation rate. Hence, they serve as a promising diagnosis tool. SLNs can also be a carrier for radioactive contrast agents to image lung abnormalities. For example, ^{99m}Tc-radiolabeled SLP aerosols can be used for this purpose (Morel et al. 1998).

6.3.1.4 Cubosomes

Cubosomes are bicontinuous cubic liquid crystalline nanoparticles ranging in size from 10 to 500 nm. These particles are viscous and transparent with honeycomb-like morphology. Lipid molecules can be mixed with a suitable surfactant along with the drug molecules, with the mixture dissolved in an organic solvent such as chloroform. The solution can then be volatilized, with the remaining sediment dissolved in aqueous glycerol and subjected to high shear dispersion at 80–100 °C. The dispersion can further be nanotized using high-pressure homogenization at 60 °C to get nanodispersion, which can be filtered to get cubosomes. The cubic phase of the cubosomes has tiny pores that help in controlled drug release. Moreover, cubosomes are capable of solubilizing hydrophilic and hydrophobic drugs. These two reasons have made cubosomes a promising agent in drug delivery applications. They can function as oral, intravenous, and topical delivery systems (Shanmugam and Banerjee 2011).

The advantages of cubosomes include the following:

- High drug loading capacity
- Simple and economical method of preparation with less input of energy
- Biodegradable nature
- Ability to accommodate water-soluble and water-insoluble drugs
- Site-specific release of the cargo drugs
- Higher level of insolubility in the lipid phase, which makes them stable at any degree of dilution
- Higher permeation coefficient during ocular drug delivery
- Higher encapsulation efficiency for lipophilic drugs
- Higher biocompatibility leading to negligible side effects and toxicity
- Excellent bioavailability due to efficient drug releasing capacity.

Cubosomes for Ocular Inflammation

Ocular inflammation occurs due to one or more of the following reasons: surgery for pupil constriction (i.e., intraoperative miosis), postsurgical inflammation, seasonal conjunctivitis, cystoids, macular edema and laser-assisted eye surgery (photorefractive keratectomy). Hence, it is very essential to find an optimal therapy for controlling the inflammation.

Currently, nonsteroidal anti-inflammatory drugs (NSAIDs) are used for ocular inflammation and pain. For example, flurbiprofen (FB), 2-(2-fluorobiphenyl-4-yl) propionic acid, is used as an eye drop. However, it has many disadvantages, such as irritability, low aqueous solubility, transient burning, stinging, lower bioavailability (thus requiring higher doses or repeated doses), and hypersensitivity reactions with itching, reddening, photosensitivity, and keratitis punctata. Therefore, it is of great importance to formulate an NSAID using nanocarriers. Cubosomes can fulfill this need.

A cubosome-based ocular delivery system is an important biomedical application. Cubosome-FB (CFB) has been formulated with superior drug release kinetics, transcorneal permeation, and ocular tolerance. CFB for ophthalmic applications is conventionally synthesized from a glycerol monooleate (GMO)-water mixture via high-pressure emulsification, using poloxamer 407 as a stabilizer. Upon corneal instillation, the lipid component of the CFB functions as a transdermal enhancer, has strong adhesion to its counterpart in the epithelial cells, and facilitates higher drug permeation to the anterior region. Thus, CFB has a higher permeation coefficient than the free drug. Moreover, cubosomes show higher encapsulation efficiency toward NSAIDs, thus shielding the majority of the acidic groups of the drugs from being exposed to the aqueous phase. This results in a significant decrease in irritability.

A cubosome formulation fits very well into the Higuchi square root mode and reveals an appreciable drug release profile in vitro. Due to the decreased irritability, the hydration level of the cornea is maintained and corneal damage is also prevented. In this regard, CFB was documented to show no corneal damage and no clinical signs of damage to the epithelium and the stroma of the eyes in vivo. An unencapsulated drug, on the other hand, shows corneal damage characterized by detachment of the superficial epithelial cells, loss of structural integrity, and an enhanced corneal hydration level. The biomedical applications of cubosomes can further be proven by the increase in the values of pharmacokinetic parameters such as area under the curve, mean residence time and T_{max}. These parameters also account for the enhanced bioavailability of the cubosomal formulation (Han et al. 2010).

In addition to the delivery of anti-inflammatory drugs, cubosomes are also used for the efficient delivery of genes, vaccines, and anticancer drugs (Shanmugam and Banerjee 2011). Interestingly, multifunctional cubosomes were synthesized with excellent theranostic and targeting properties (Caltagirone et al. 2014). Cubosomes can accommodate the anticancer drug camptothecin, the imaging agent squaraine (near-infrared-emitting fluorescent probe) and a folate-conjugated diblock polymer

(pluronic F-108). This could help in the targeting, imaging, and treatment of cancer in vitro, with the potential for in vivo applications as well.

6.4 Viral Nanoparticles

Viral nanoparticles (VNPs) are noninfectious, replication-deficient, monodispersed viruses obtained from hosts such as animal viruses, plant viruses, or bacteriophages. VNPs for biomedical applications can be generated in their original hosts such as plants, bacteria, or mammalian cells such as yeast. However, for human use, VNPs generated from plants are safe and non-toxic because humans are not natural hosts for those viruses.

VNPs are attractive nanotheranostic platforms due to several advantages:

- Natural existence
- Biocompatibility
- Biodegradability
- Easy to engineer into a drug delivery carrier
- Possession of chemically addressable sites on their capsid surface, which increases the possibly to incorporate targeting ligands, imaging agents, genes, and drug molecules
- Capability to conjugate proteins, peptides and other nanoparticles
- Less likely to be pathogenic
- Highly stable/inert and dynamic.

Some VNPs with potential biomedical applications include the following:

- *Brome mosaic virus* (BMV)
- *Cowpea mosaic virus* (CPMV)
- *Red clover necrotic mottle virus*(RCNMV)
- The filamentous phage M13
- *Potato virus X* (PVX)
- *Tobacco mosaic virus* (TMV).

6.4.1 Pharmacokinetics

The pharmacokinetic parameters of VNPs, such as circulatory half-life, cell entry, accumulation, and systemic and renal clearance depend on the surface properties (the charge and the functional groups). For example, bacteriophage Qβ, an icosahedral virus with a diameter of 25 nm, has a positive surface charge and a half-life of >3 h, while CPMV has a net negative surface charge and a half-life of <15 min. The surface charge of any VNP can be tuned by surface

modification with chemical groups or by genetic modification in order to alter the in vivo behavior. Lysine residues of the viral surface proteins are usually subjected to modification. The overall positive charges of the lysine residues contributed by the ε-amino group may be modified to a negative charge by an acetylation process (Steinmetz 2010).

6.4.2 Disadvantages: Nonspecific Interaction and Immunogenicity

Nonspecific biological interactions and primary immune responses are disadvantages of VNPs. These disadvantages can be reduced by surface modifications and a shielding effect, which can be promoted by a suitable ligand. The neutral, hydrophilic, nontoxic, FDA-approved polymer, PEG, may be the best ligand for this purpose. A PEG chain not only reduces immunogenicity and nonspecific interactions, but it also enhances the solubility and stability of VNPs, leading to ample circulatory half-life. Moreover, the limited surface coverage by PEG is sufficient for achieving the goal, thus providing space for accommodating drugs, genes, or imaging and targeting ligands.

CPMV has certain advantages that makes it a perfect carrier, especially for crossing the BBB and targeting the brain parenchyma:

- Excellent bioavailability
- Nontoxic even at high doses (up to 10^{16} particles/kg of body weight)
- Wide biodistribution by entering into numerous organs following intravenous injection
- Presence of lysine and cysteine residues on the surface, which enables the binding of diverse cargo, such as drugs, vaccines, metals and dyes.

6.4.3 Applications

6.4.3.1 VNPs as Imaging Probes and Drug Carriers for Vascular Endothelial Cells

Currently used fluorophores show low endothelial penetration, leading to poor resolution of vascular imaging. However, a biological carrier would show higher affinity and effective penetration for better internalization. This may be helpful for imaging and drug delivery. One such example is CPMV. CPMV is an RNA virus of the picornaviridae superfamily and *Comovirus* genus. It is made of 60 identical asymmetric protein subunits. Each subunit is provided with five accessible lysine residues, thus giving rise to 300 surface sites for binding to ligands.

CPMV has a natural biospecific affinity for the endothelial cells of the mammalian vasculature. Hence, CPMV is a superior, ideal, and natural tool for targeting endothelial cells, which is very essential for tumor cell imaging and therapy and also for getting insight into angiogenesis associated with tumors. This interaction is promoted by a type III intermediate filament protein called vimentin. Vimentin is overexpressed on the surface of endothelial cells of tumor vasculature (Beijnum et al. 2006).

CPMV conjugated to the fluorophore Alexa Fluor 555 (A555-CPMV) shows maximum fluorescence when compared with standard vascular imaging conjugates, such as dextran, lectin, and polystyrene nanoparticles. Hence, it is preferred for the intravital imaging of the microvasculature and the mapping of angiogenesis in tumor cells. A555-CPMV is also characterized by the absence of unfavorable dye-dye or dye-amino acid interactions, which would otherwise result in fluorescence quenching. Concomitantly, the fluorescence intensity correlates with the number of dyes conjugated to the virus particle. Upon intravascular administration, A555-CPMV shows high accumulation in the lumen of the hepatic, renal, cardiac and placental vasculature. Furthermore, A555-CPMV is also capable of probing deeper microvasculature, as evidenced by their accumulation in the yolk sac of chick embryo. CPMV-A555 accumulates in the perinuclear compartments of vascular endothelial cells. It also exists in association with Golgi apparatus, lysosomal-associated membrane proteins, and macrophage vesicles. Because the PEG-conjugated CPMV is not internalized into the endothelial cells, it can be used to visualize blood volume and blood flow. Moreover, the A555-CPMV conjugate is proven to be nontoxic (Lewis et al. 2006).

6.4.3.2 CPMVs as Carriers for Delivering Cargo into the Central Nervous System

The BBB is a tight junction of endothelial cells containing transmembrane proteins, such as claudins, occludins, and junctional adhesion molecules. Multidrug-resistant proteins such as P-glycoprotein and ATP-binding cassette transporters are expressed in the BBB endothelial cells, which prevents the entry of cargo such as drugs and metabolites into the brain parenchyma. This would be a main hindrance to the delivery of drugs targeted to the treatment of CNS disorders. Moreover, drug carriers such as inorganic nanoparticles and polymers and even baculovirus show limited bioavailability in the context of CNS drug delivery.

CPMVs show excellent interaction and permeability through the BBB in vitro (e.g., bEND.3 cells an in vitro BBB model) and in vivo (e.g., mice intracerebrally infected with the neurotrophic mouse hepatitis virus, which is a common model of CNS inflammation and infection). In vitro, CPMV easily binds to endothelial cells with perinuclear distribution of endocytic vesicles confirming their entry into the cerebral vasculature; this was proven using an in vitro model via immunohistochemical staining with anti-CPMV-antibodies.

In vivo, CPMV-A555 conjugate shows robust accumulation in the damaged region of the BBB, thus showing feasibility for targeting various other cell types of the CNS during inflammation. CPMV targets inflammatory lesions infiltrated with macrophages and microglial cells (Shriver et al. 2009).

6.5 Nanomaterial-Based Biomedical Devices

6.5.1 Micropumps

Micropumps are miniaturized devices with parts for actuating fluids. They are useful in drug delivery. There are two types of micropumps, based on the method of actuation:

(a) Mechanical micropumps, which consist of moving parts that are actuated via a piezo effect, electrostatic effect, or thermopneumatic effect
(b) Nonmechanical micropumps, which have immovable parts; the fluid is actuated by an electroosmotic effect, electrochemical effect, or ultrasonic waves.

6.5.1.1 Components of Micropumps

The device contains a motherboard, mounting base, or base chip, over which the following components are fabricated (Nisar et al. 2008; Woias 2005):

- Reaction chamber
- Mixer
- Detector
- Heater
- Actuator
- Valves
- Interconnectors or microchannels (for interconnecting all the above components)
- Electronic system, for data processing and control.

The following are the criteria for an ideal micropump:

- Disposable
- Consumes low power
- Highly miniaturized
- Portable and light
- Follows sanitary conditions when used for medical and drug delivery purposes
- Can be coupled with sensor and cantilevers.

Micropumps have a fluid channel, pump chamber, brass membrane, and piezoceramic material arranged one above the other. High voltage is applied to the piezoceramic material to deform the brass membrane. This results in a downstroke movement so that the fluid is squeezed out from the pumping chamber to the channel. On decreasing the voltage (i.e., when a low voltage is applied), the deformation of the brass membrane results in an upstroke movement, so that the fluid is sucked into the chamber. The device can pump with a speed of 100 cycles/s.

Maillefer et al. (1999) defined criteria for a high-performance micropump (HPMP):

- Low cost
- High performance
- Disposable drug delivery system
- Linear and accurate pumping characteristics for flow rate up to 2 ml/h
- Insensitivity to external conditions such as pressure, temperature, viscosity
- Double limiter
- Reproducible stroke volume
- Pulsatile flow rate
- Low leakage
- Accuracy ±5 %
- Longevity of the device (at least few weeks)
- Drug compatibility (the materials in contact with the drug should be biocompatible), which is ascertained as follows:
  - No damage or corrosion to the device
  - No damage to the pharmaceutically active ingredient (PAI)
  - No release of toxic products in the drug.

The technology used to fabricate HPMP includes SOI technology, silicon DRIE, and sacrificial etch. These technologies have several advantages over the more traditional silicon bulk micromachining techniques, such as optimal design, process simplification, well-defined mechanical characteristics, and compactness.

6.5.1.2 Applications and Advantages of a Silicon-Based HPMP

An important application of an HPMP is the subcutaneous injection of insulin for diabetic patients. The high level of miniaturization helps patients to carry the pump directly taped onto the skin together with an injector using a soft needle.

Advantages of a silicon-based HPMP include the following:

- Operation via remote control device through secured radiofrequency communication
- Accurate and reliable
- Manufacturing cost that is compatible with a single-use application
- Improved safety

- Good resolution
- Programmability
- Autonomy
- Fully disposable pump
- Suitable pump for holding different types of drugs and their delivery at a low flow rate
- Useful for micro total analysis systems (μ-TAS) or bio-MEMS.

Micropumps are used as an automatic water immersion system (called a water immersion microdispenser) in experiments involving aqueous environments, such as the following:

- Live-cell experiments at 37 °C
- Long-term live-cell experiments
- Screening
- Well plate acquisition.

6.5.2 *Microneedles*

Microneedles are microfabricated needles of silicon, metal, or biodegradable polymers like PMMA or PLGA, which are arrayed on a miniaturized roll or on chips, thus forming a patch. They are mainly used for the delivery of proteins, vaccines, and DNAs through a transdermal route. They can be used for the painless delivery of drugs in bolus mode and sustained mode.

There are two approaches for the use of microneedles (Lee et al. 2008).

(i) *Pretreatment approach or permeabilization approach*: In this approach, the skin is pretreated with the microneedles (usually made from silicon or metal) and permeabilized. Subsequently, a transdermal patch is attached to the skin for extended drug delivery via the permeations.
(ii) *Dissolution approach*: In this approach, the biodegradable microneedle of polylactic-co-glycolic acid is prepared and the drug is coated on the needle. The needle is inserted into the skin and subsequently the drug or the needle itself is dissolved. This may result in bolus or controlled release of the drug.

The criteria for a microneedle are as follows:

- Capable of encapsulating the drug within a biocompatible and mechanically robust material around the drug
- Can facilitate bolus or sustained release of drug in a controlled fashion
- Biodegradable
- Ease of self-administration

- No biohazardous waste
- No permanent scar or pits on the skin
- No sun sensitivity
- No pigmentation, wrinkles, or stretches.

The fabrication and dissolution kinetics of transdermally injectable microneedles are as follows:

(a) Pyramidal or conical microneedle master molds can be prepared in an SU-8 photo resist.
(b) A master microneedle structure of polydimethylsiloxane (PDMS) is prepared using the master mold and then sputter coated with gold nanoparticles (100 nm).
(c) The polymer used for encapsulating or accommodating the drugs is made into a hydrogel, which functions as a matrix.
(d) The drug is suspended in the matrix and transferred to the PDMS mold, placed in a conical centrifuge tube, and centrifuged in a 45° angled rotor at 3000*g* (37 °C) for up to 2 h. As a result, the cavity of the PDS microneedle mold is filled with 100–300 mg of the hydrogel. The hydrogel is then dried. This is the base hydrogel layer.
(e) In the next step, the drug can be added as a part of the needle itself or as a separate backing layer. To be a part of the needle, the drug is mixed with a little (about 10 mg) of the hydrogel, poured over the base layer, and dried. Subsequently, a pure hydrogel layer (called the backing layer) is cast over the hybrid layer and dried. To be a part of the surface of the microneedle (as a separate backing layer), a second coating of pure hydrogel layer is avoided.
(f) Finally, the microneedle is inserted into the skin and the dissolution kinetics (i.e., the dissolution of the microneedles with time) can be recorded. The dissolution kinetics depend on the nature and the solubility of the polymer. For example, amylopectin has slower dissolution kinetics and polyvinylpyrolide has faster dissolution kinetics. Usually, the dissolution begins after 10–15 s. After 1 min and 15 min, the needles dissolve down to one-half to one-third of their height. The needles dissolve fully at 1 h.

6.5.2.1 Applications of Microneedles

We developed a nanoparticle system with chitosan for the transdermal delivery of insulin. Chitosan and tripolyphosphate (TPP) were used to prepare the insulin-loaded chitosan nanoparticles based on an ionotropic gelation method and characterized using a Zeta-sizer Nano ZS, scanning electron microscopy, and an optical microscope. A transdermal drug delivery system of the formulated insulin-chitosan nanoparticles was prepared using a solvent casting method. The results indicated that the nanoparticles were in the size range of 465 and 661 nm and exhibited quasicircular structure with better encapsulation efficiency.

Controlled-release transdermal patches of insulin–chitosan nanoparticles were prepared using the polymer combinations HPMC, PVP K30, and PEG 400, with Tween 80 as a plasticizer. The release rate of the drug through patches increased simultaneously as the concentration of the hydrophilic polymer was increased (Sadhasivam et al. 2015).

Other applications of microneedles include the painless delivery of drugs through the skin; the delivery of a vaccine against the H1N1 virus, which is superior to intramuscular injection; and the delivery of protein-based drugs without affecting their integrity.

6.5.3 Implantable Microchip Devices for Drug Delivery

Implantable microchips are biodegradable and biocompatible chips with the following amenities:

- A cavity in the form of a reservoir or tank for accommodating the cargo drugs
- A hole or opening in the cavity wall for releasing the drug upon some biochemical or external stimulus
- A stimulus-sensitive valve to function as a controllable element for the release of the drug.

Based upon the stimulus that acts upon the valve, the microchip can be classified as one of three types (Meng and Hoang 2012): a biochemo-responsive microchip, a thermo-responsive microchip, or a pressure-responsive microchip.

In the biochemo-responsive type, the channel is filled with the drug. These valves are made of a biodegradable polymer that is constructed to be specifically sensitive to the extreme levels of the target blood biochemical, such as sugars, proteins, peptides, or enzymes. When the level of the blood components becomes abnormal, the valves undergo physical changes and contract, forming free space and subsequently allowing the liberation of the drug. For example, insulin can be liberated to control blood sugar levels.

Thermo-responsive release is another approach for implant-based drug delivery. In this strategy, a miniaturized biocompatible perforated reservoir is allowed to hold the drug. Microvalves made of temperature-sensitive hydrogel are attached to the tank for regulating drug release through the pores. Using a radiofrequency oscillator, a magnetic field is generated and the reservoir is activated. The frequency of the magnetic field is allowed to be in equilibrium with the resonance frequency of the tank. As a result, the reservoir is heated up and triggers the thermosensitive hydrogel valve leading to drug release through the perforations.

In the third type, the microvalve is attached to a silicon torsion spring. Using a suitable power source, pressure is generated on the torsion spring to actuate it for releasing the drug.

6.5.4 Nanorobots

A nanorobot is a hypothetical microscopic device or molecular machine designed to perform a task with precision at the nanometer scale. The amount of nanorobots to be injected into the body for the treatment of disease is called the therapeutic dose. The therapeutic dose depends on the severity of the disease. A single therapeutic dose has billions or trillions of nanorobots (Freitas 2005). The criteria for a viable nanorobot are as follows:

- An ability to navigate through the blood vessels of the complicated circulatory system
- Room to accommodate cargo such as medicine and miniaturized tools used for the performance of the robot
- Capacity to be liberated from the host once the desired function is executed
- Susceptible for remote regulation by a physician.

6.5.4.1 Tools or Components of Nanorobots

Carbon (usually in the form of diamond, diamondoid and fullerene) is the principal building block of the nanorobot. To execute biomedical applications (e.g., drug delivery) or disease treatment (e.g., degrading the cancer cells, crushing the stones in organs such as the kidney or joints), the nanorobot should be equipped with some tools, as shown in Table 6.7.

A hollow space or cavity inside the nanorobot, called the medical cleft, is used to hold small doses of medicine or chemicals used as medicine or chemotherapeutic agent. It can also accommodate immunosuppressive drugs to prevent the immunogenic reaction within the host. Probes, knives and chisels can be designed to grab and physically break down the materials, such as clots in the blood vessels or plaques in the organs or stones plaque. A source of fine-tunable microwaves or ultrasonic waves would help a nanorobot with the thermal killing of proliferating cancer cells. A nanorobot equipped with a pair of electrodes would generate electric

Table 6.7 Essential tools of nanorobots with biomedical values

Tool	Function
Cleft for medicine	To hold medicine and chemicals
Probes, knives, and chisels	To grab, break and crush clots, stones, or plaques
Microwave emitters and ultrasignal generators	To kill cancer cells
Electrodes	To generate electric current for killing cancer cells
Laser	To vaporize and burn away plaques and cancer cells

current to kill the cancer cells. A miniaturized laser source could aid the robot in dissolving away harmful material, such as arterial plaques, cancerous cells, and blood clots.

6.5.4.2 Nanorobot Locomotion

In order for a nanorobot to move around the bloodstream without damaging the host, it should be fabricated with locomotive tools. These tools falls under three categories:

(a) Appendages, which are tiny magnetic projections fine tunable by an external magnetic field. As a result, the projection will vibrate and push the device further.
(b) Miniaturized pumps, which might use blood plasma force to push the device forward.
(c) Vibrating membranes, which upon repeated tightening and relaxing force would generate thrust for locomotion.

6.5.4.3 Power for the Navigation of Nanorobots

Nanorobots should receive power for navigating in the body. Power can be supplied from three sources: the host, an onboard source, or an external source. Internal power can be produced if the nanorobot has electrodes, which could form a battery using the electrolytes found in blood. Alternatively, the robot should store a chemical for inducing reactions in the blood to produce energy. Another option is deriving power from the patient's body heat energy. Onboard capacitors can be miniaturized within the nanorobot to supply power to navigate. External power can be supplied using ultrasonic waves, which hits the piezoelectric membrane of the device, where the waves are converted to electric energy. Alternatively, some crystals capable of converting mechanical force into electrical energy can be attached to the device itself. Sometimes, a magnetic field can also be used to give power for navigation.

6.5.4.4 Tracking of Nanorobots

An external tracking system makes use of an ultrasonic signal, magnetic resonance imaging (MRI), or radioactive dye. In the ultrasonic signaling method, the ultrasonic waves are passed through the body, which in turn reflect back and are detected using ultrasonic sensors. In the MRI method, a magnetized device is used, in which the magnetic field is detected using specialized software on an MRI machine. In the radioactive dye method, the nanorobot is traced using radioactive dye. The dye can be traced to create a complex three-dimensional image. In

advanced cases, nanorobots might have a miniature television camera to enable the scientist to steer and watch the device in the presence of a live video.

6.5.4.5 Applications of Nanorobots

Some potential applications of nanorobots are as follows:

- Treating arteriosclerosis
- Treating gout
- Breaking blood clots
- Treating kidney stones
- Treating hemophilia
- Treating skin disease
- Microbicidal activity
- Killing cancer cells
- Parasite removal
- Cleaning wounds
- Surgery
- Tele medicine
- Detection of toxic chemicals.

6.5.4.6 Respirocyte—A Classic Example of a Nanorobot

Respirocyte is a classic example to understand the structure, features, and function of a biomedical nanorobot. It is also called the red blood cell (RBC) nanorobot or mechanical red cell because it mimics the natural erythrocytes in carrying out the gas exchange function. This would help in the treatment of anemia, choking, asphyxia, and lung-related disorders. The main criteria of a respirocyte are as follows:

(i) Size value, which should not exceed the diameter of the capillary, even after being fabricated with all the tools and other executive system (5–10 μm)
(ii) Shelf life
(iii) Feasibility for nanapheresis (a technique in which the blood of the host is separated from the respirocyte by neutral buoyancy with the help of centrifugation and returned back to the body without damage).

Structurally, the respirocyte is a hollow sphere of diamondoid with three storage tanks, one for the oxygen, another for carbon dioxide, and one more for water (the water ballast). It is made of an onboard chemomechanical turbine or fuel cell capable of generating power by using the blood glucose molecules and the oxygen. This power is useful for filling the oxygen tank. The gas loading and unloading can

be controlled by onboard pressure sensors, which could monitor the O_2 and CO_2 pressure. An onboard computer (10,000 bit/s) might help in the maintenance of computations (Freitas 1998).

Respirocytes are superior to natural erythrocytes because they can store 1.5 billion oxygen molecules and provide maximum access to the tissues. The therapeutic dose should have 5.3 trillion devices and be capable of being administered via hypodermal injection.

Potential applications of respirocytes include the following:

- Treatment for anemia
- Prevention of sudden infant death syndrome (SIDS) or crib death
- A disease-free blood substitute
- Treatment of lung diseases
- Treatment for Caisson disease
- Cure for flatulence.

6.5.5 Nanopancreas

The islet cells of the pancreas produce a hormone called insulin, which is responsible for the absorption of glucose (sugar) from the blood, thus regulating the blood sugar level. Deficiency of insulin or a defect in the insulin production would raise the blood sugar level. An artificial pancreas (also called a nanopancreas) produced by a micromachining technique has the ability to stimulate insulin production and control the blood sugar level (http://www.zdnet.com/article/nanotechnology-to-end-insulin-injections-for-diabetics). The steps involved in the preparation of a nanopancreas and its mechanism of action are schematized in Fig. 6.13 and are summarized here.

A silicon membrane is punched to form nanopores of 7–10 nm using photolithography. The pancreatic islet cells isolated from mouse is encapsulated in the porous membrane. The pore size is fabricated in such a way that it is permeable to insulin and glucose but impermeable to antibodies that the host may raise against the mouse pancreatic cells. The glucose molecules of the host blood would pass through the pores of the membrane and stimulate the islet cells, thereby leading to the secretion of insulin. Insulin easily passes out through the pores and promotes the absorption of blood glucose.

6.5.5.1 Titanium Oxide Nanoparticles

Hibiscus rosasinesis (commonly called shoeflower) is rich in polyphenolic phytochemicals, such as tannins and phenolic proteins, triterpenoids, 2,3-hexanediol, n-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid, and squalene. These compounds

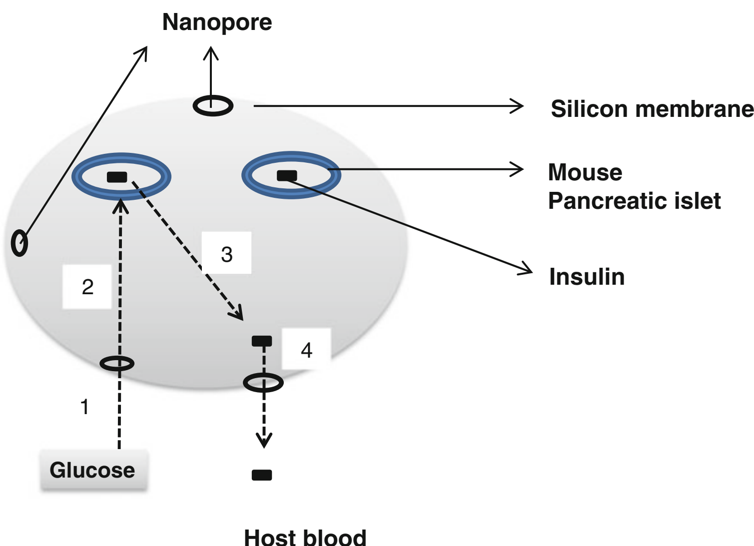


Fig. 6.13 Nanopancreas (*dotted arrows* and *numbers* represent the sequential events in the release of insulin into the blood)

have good antimicrobial, antioxidative, and antiproliferative activity and therefore may be used to treat cancer. The TiO_2 nanoparticles capped and stabilized by phenolic and amine moieties of flower extract have smaller size and more dispersibility than the TiO_2 nanoparticles prepared by a chemical method. Moreover, the flower extract-stabilized TiO_2 nanoparticles exhibit considerable antimicrobial activity against pathogenic bacteria, which is comparable with that of a standard antibiotic. This might be due to its enhanced dispersibility, stability, and surface coatings (Kumar et al. 2014).

6.5.5.2 Magnesium Nanoparticles

The symbiosis between algae and fungi (the lichens) has been used for synthesizing metal nanoparticles because they work as good alternatives for their environmental friendliness, low toxicity, and low processing conditions. Lichen extract reduces magnesium salts into magnesium nanoparticles in an ecofriendly way. The green-synthesized magnesium nanoparticles are around 23 nm and are expected to possess several biomedical applications due to the capping by bioactive components of lichens. Because magnesium is used in bone grafting material for peri-implant defects, green synthesized magnesium nanoparticles will emerge as a potential implant component (Devasena et al. 2014).

6.5.5.3 Silver Nanoparticles

Silver nanorods synthesized through the green route by using the extract of the seeds of *Trigonella foenum graecum* possess immense biomedical values. They exhibit anticancer activity in skin cancer cell lines. Additionally, they are known to possess anti-inflammatory activity against lipopolysaccharide-induced inflammation in skin cell lines. The silver nanorods are stabilized and dispersed due to the constituents of the seeds; they exert their activities by influencing the mRNA expression of interleukins (IL-1, IL-6) and macrophage chemoattractant protein-1 (MCP-1). These phytostabilized silver nanorods may be used in the prevention of skin diseases associated with inflammation. The anti-inflammatory activity of silver nanorods may be attributed to their scaled-down size and the phytochemicals of seeds capped on the surface of the nanoparticles. Because most diseases, including cancer, are associated with inflammation, the silver nanorod is expected to emerge as an effective biomedical and pharmacological candidate (Suganya and Devasena 2015).

6.5.5.4 Chitosan

Polymeric patches loaded with chitosan-encapsulated-insulin and possessing a quasicircular structure are helpful for the controlled release of insulin molecules. Transdermal patches of HPMC, PVP K30, and PEG 400 with Tween 80 as plasticizer accommodate the insulin encapsulated in the chitosan nanoparticles. The release rate of the drug through patches increased simultaneously as the concentration of hydrophilic polymer increased (Sadhasivam et al.2015).

The ophthalmic antibiotic gatifloxacin has several disadvantages, such as rapid tear turnover resulting in precorneal loss and lacrimal drainage. However, encapsulation of the drug within chitosan nanoparticles may be used to increase the half-life period and decrease the dosage of the drug administered. The drug-loaded chitosan nanoparticles showed an increase in sensitivity towards *S. aureus* and *Staphylococcus epidermidis,* with satisfactory drug entrapment efficiency of 84.9 %. This formulation can therefore be used for ocular drug delivery (Ravisankar et al. 2015).

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