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CHAPTER

Inorganic nanoflotillas as engineered particles for drug and gene delivery

14

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14.1 INTRODUCTION

The cellular machinery involves special taskforces comprising of enzymes, hormones, cofactors, and signaling cascadic armamentariums. The cellular gateways made up of proteins for the influx and efflux of special guests, such as metal ions or molecules, have always been a matter of admiration for biologists. Moreover, metals have formed their legacy as an integrated constituent of the structural and functional unit of the cell and organism as a whole. Metals act as cofactors in specific proteins, leading to its proper functioning, conjugate with substrates, thus creating a key to bind the enzymatic lock and catalyze electron exchange reactions for effective production of the energy currency of the cell. They sometimes take the responsibility for maintaining the electrical potential for either generating action potentials (neuronal cells) or energy production (other cells). At times, they can also catalyze DNA replication, transcription, or translation processes. Their role in signal transduction explains how and why a cell survives in an organism. Moreover, they play an active role in energy transduction too. As nature has not skipped the contributions of metal in the design of living bodies, then how can we be callous enough to neglect them? Such a perfect amalgamation of inorganic material into organic matrices has led to the generation of a perfect life. Deficiencies in the availability of metals in the body cause relative disorders, which can sometimes be life-threatening. Hence, inorganics and their nanoparticles, which have found their applications both in the field of diagnosis as well as therapeutics, have chiseled an everlasting impression on the scientific world. A study on the mesmerizing effects of such nanobeacons can make us understand their actual significance in the theranostic universe and can then guide

Engineering of Nanobiomaterials. DOI: http://dx.doi.org/10.1016/B978-0-323-41532-3.00014-2

us to follow a regulated path towards solving many health issues. The unresolved mystery, why nature prefers organic−inorganic hybrids, is something which deserves an answer. After unlocking this ambiguity, we can then respond to the most favorite question of a scientist, "What is life?"

Nanotechnology deals with particles which possess at least one dimension in the range of 1−100 nm and can then be exploited for a special purpose or applications such as bioimaging, biosensing, drug/gene delivery, hyperthermia, photothermal therapy, photoacoustic imaging, and a neverending list of other uses. The self-assembly of metal ions in such a way that they stick together to form nanoparticles, is a biomimetic approach, a lesson learnt from nature itself. Physiologically, most essential elements such as zinc, iron, cobalt, manganese, magnesium, and chromium have proved that metals are always a matter of interest for the survival of an organism. Hence, scientists have opened the torch of reconnaissance for the exploration of biocompatible agents from the periodic table, so that they can be exploited in the field of nanomedicine (Sengupta et al., 2014).

Metal nanoparticles have long been documented (Prakash, 1997) as bhasmas since 1500 BC as per Charak Samhita (Prakash, 1997). This alternative form of medicine was an integrated part of Ayurveda. Bhasmas are the biologically synthesized nanoparticles, which are ground, sieved, triturated, and pelletized for therapeutic purposes (Sarkar and Chaudhary, 2010). Siddha Nagarjuna has been designated as the Father of Indian Alchemy and Rasa Shastra. Metals and their alloys exploited for the purpose of Ayurvedic medicines are gold, silver, copper, lead, tin, zinc, and iron. Furthermore, mercury and sulfur are used to transform metals into bhasma (Prakash, 1997). This has intrigued the present scientific world to explore such inorganic nanoparticles since they possess a plethora of different characteristics, which includes wide availability, good biocompatibility, high functionality, synaphic drug/gene-delivery capacity and regulated drug/gene-release efficiency. Inorganic nanoparticles have attracted connoisseurs of material and medical science at different frontiers of biomedicine, pharmaceuticals, optics, and catalysis. In this journey towards comprehension of biomedical applications of inorganic nanoflotillas, we would first elaborate different properties of drug/gene-delivery vessels. A variety of methods and their mechanisms for synthesis of snorganic nanoparticles such as carbon nanotubes, gold nanoparticles, gold nanorods, gold nanocages, silica nanoparticles, and iron oxide nanoparticles, will also be discussed.

This voyage will reach towards its horizon from understanding the nanoparticle-mediated biosensing, surface modification, noncovalent drug/gene delivery, biomolecular attachment, drug/gene release kinetics in physiological milieu, driving forces for delivery and release, such as light and magnetic field. Figure 14.1 shows a schematic view of nanomaterials, which possess multifunctional characteristics of diagnostics, therapeutic mechanisms, and targeting. Nanoparticles interact with fluorescent dyes or biomarkers for the detection of miscreant cells via targeting ligands and consequently lead to the release of peptides, drug/genes, DNA, or siRNA (interference RNA).

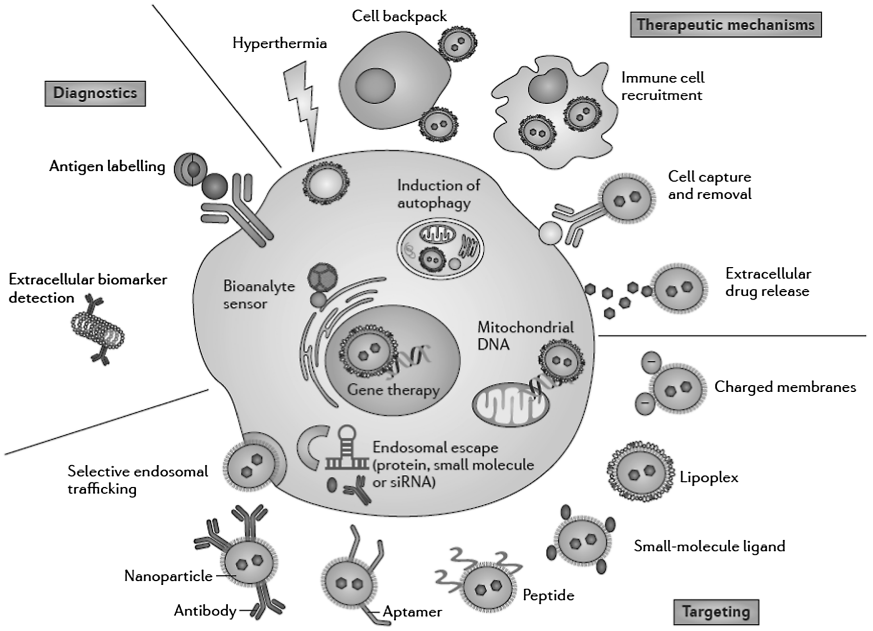

FIGURE 14.1 Nanomaterial strategies from the point-of-view of the cell.

The ability to target nanoparticles to cancer cells (secondary targeting) and to influence their uptake into specific cellular compartments (tertiary targeting) is now feasible. This figure summarizes unique targeting, diagnostic and therapeutic mechanisms as they relate to the cancer cell. siRNA, small interfering RNA.

The release is possibly due to stimuli-triggered modus operandi such as pH, hyperthermia, and electromagnetic radiations.

14.2 WHAT ARE THE ESSENTIAL PROPERTIES OF NANOPARTICLES FOR THERAPEUTIC PURPOSES?

Inorganic nanoparticles, due to their fluorescent properties, excellent bioavailability, good biodistribution, proficient biomolecular attachment and detachment processes, have led to material scientists exploiting them from diagnostics to therapeutics. These nanoparticles must possess a plethora of clinically important properties, which can make them efficient nanosystems for interaction with biomolecular interfaces. They must be highly stable to circumvent the hydrodynamic fluid pressure and surface charges to prevent their agglomeration and interact with physiological milieu. The nanoparticles that are involved in biomedical applications possess the following capabilities.

14.2.1 NANOPARTICLES AS SCAFFOLDS

Metallic nanoparticles act like a platform or scaffold for assemblage of multifunctional structures. They are colloidal suspensions, soluble in complex environments such as blood and tissues. They can be efficiently utilized for a plethora of applications such as sensing, imaging, and targeting of nanoparticles inside the physiological system. This proficiency is enhanced by surface orchestration, ligand binding, and slight alterations in the composition of the nanoparticle assembly (Shenhar and Rotello, 2003; De et al., 2008; Saha et al., 2011). Surface orchestrations, such as polymers, lipids, peptides, and DNA on the inorganic nanoparticles help them to be stable due to both electrostatic and steric effects. Ligands, such as antibodies and enzymes bound to the nanoparticles can tether surface receptors or substrates on the cellular exterior. Inorganic nanoparticles can be doped by different metals so that there is a physiologically desirable change in their properties, which helps them to maintain stability, biocompatibility, effective biodistribution, and clearance capabilities. When the stability of nanoparticles are been ensured they are used for functionalization with drug/genes, fluorescent dyes, targeting ligands, so that they act as an efficient theranostic agent (Boal and Rotello, 2000; Ghosh et al., 2008a).

14.2.2 SURFACE AREA OF NANOPARTICLES

The large surface area of nanoparticles is a critical factor which allows different surface orchestrations, such as drug/gene, peptides, and DNA, leading to its multifaceted functions of biosensing, imaging, and synaphic delivery of the drug/genes and gene. Moreover, surface modification by lipids, polymeric coatings act like a stealth carrier circumventing the problem of reticuloendothelial clearance. However, the basic requirement is to have a staunch and solid inorganic core which can give support for functionalization as well as allowing biomolecules to interact along with the synaphic ligand. Such a paradigm scaffold helps in eliminating major problems arising due to the unfolding and rearrangements taking place when present in biological systems, thus leading to enhanced control of the physical chemistry of the surface.

14.2.3 SIZE MATTERS!

Nanoparticles en route to their target in biological systems have to confront many barriers such as the host immune system, hemodynamic shear force, oxygen tension, interstitial fluid pressure (IFP), and extracellular matrix. The transportation of a circulating nanoparticle is possible due to main reasons:

1. Applied convective forces
2. Brownian motion (Decuzzi et al., 2006).

The extravasation of nanoparticles is the resultant of the rate of fluid flow as well as filtration through the capillary, that completely depends on the hydrostatic pressure gradient (HPG). HPG is nothing but the vascular pressure and IFP difference. In the case of solid tumors, IFP is very high as compared to normal tumors due to leaky vasculature and diminished lymphatic drainage. Reduced blood flow and enhanced IFP lead to inhibition of transendothelial migration of nanoparticles. Hence, increased nanoparticle size requires higher hemodynamic shear force to circumvent the higher IFP in tumors. This can be possible either by increasing the blood pressure transiently for enhanced drug/gene delivery or inducing hyperthermia for increased blood flow. Moreover, the most important step even before transvascular transport is their margination, that is, the radial drift which dictates the transportation of a moving nanoparticle towards the blood vessel walls. The size of the nanoparticle is important enough when they have to interact with the vascular bed and have meaningful interactions with them. Margination is especially not favored when the nanoparticles are transported in the tumor vasculature via convective means (Lee et al., 2009; Gavze and Shapiro, 1998; Gentile et al., 2008). Moreover, the transportation of larger spherical nanoparticles is purely driven by convection, resulting in a greater difficulty in breaking away and moving towards the vessel wall. In contrast, smaller nanoparticles show a relatively higher diffusion rate, thus allowing them to travel laterally in the blood vessel easily. This has been reported by a study performed on liposomes which showed that liposomes of size 65 nm possess 3.4 times more margination rate as compared to a 130-nm liposome (Toy et al., 2011).

14.2.4 SHAPE OF A NANOPARTICLE

Shape plays a pivotal role in margination of the nanoparticles towards the blood vessel walls. In contrast to spherical nanoparticles, oblate-shaped particles experience torques which cause tumbling and rotations, that lead to lateral drift of nanoparticles towards the blood vessel walls (Lee et al., 2009; Gavze and Shapiro, 1998; Gentile et al., 2008). The cellular membranous interface interacting with nanoparticles is dominated by the shape of the nanoparticles. Moreover, the reticuloendothelial clearance of the nanoparticles is also dictated by the shapè of the nanoparticles. There are reports which say that oblate-shaped nanoparticles are more favorable for circulation due to their lower uptake by macrophages (Chithrani et al., 2006; Sharma et al., 2010; Geng et al., 2007; Champion and Mitragotri, 2006). This leads to an increment in the blood residence time of nanoparticles, thus increasing the probability of nanoparticles reaching their destination without being cleared by the immune system. The immune system itself acts as a major obstacle which needs to be circumvented either by capping the nanoparticles using stealth carriers or altering the shape of the nanoparticles. Moreover, entry of nanoparticles inside normal and cancerous cells is also dominated by its geometry and strongly depends on the avidity of the nanoparticles. Geometrically enhanced targeting is essential and can efficiently counterbalance

hemodynamic forces, thus shedding off the nanoparticles from the endothelium (Decuzzi and Ferrari, 2006).

Evasion of nanoparticles, especially by macrophages, is possible by two most critical parameters, size and shape. Spherical-shaped nanoparticles are responsible for an enhanced rate of macrophage uptake. When six distinct classes of nanoparticles of different shapes were evaluated, based on contact angle parameter, particle internalization velocity can also be deduced. Particles which possess high aspect ratios (~ 20) and if they are aligned with the long axis parallel to the cell membrane, it will internalize very slowly as compared to the particles which are aligned with their short axis parallel to the cell membrane. There is a discrepancy between the high rate of attachment of nanoparticles and their internalization rate, in the sense that its not necessary that the those particles which possess a high rate of attachment will also have very high internalization. Previous reports have found that prolate ellipsoids having a major axis of $0.35-2\,\mu m$ and minor axis of $0.2-2\,\mu m$ had both the highest attachment rate and slow internalization rate as compared to spherical particles of radius $0.26-1.8\,\mu m$ and oblate ellipsoidal nanoparticles having a major axis of $0.35-2.5\,\mu m$ and a minor axis of $0.2-2\,\mu m$ (Sharma et al., 2010). Moreover, the rate of phagocytosis of nanoparticles depends on a particular geometry as well as it being purely organ-dependent. Once this process of phagocytosis is evaded by the nanoparticles, the next most important task is to escape the blood vessel and traverse it, once it reaches the target site. The margination of nanoparticles is dominated by their translational and rotational motion as well as buoyancy, viscous drag, Reynolds number, gravity, van der Waals forces of interactions, electrostatic double layer, and steric repulsive interactions. Spherical nanoparticles follow streamline flow during their travel to balance these forces (Gavze and Shapiro, 1997; Decuzzi et al., 2005). In contrast to spherical nanoparticles, rod-shaped particles, based on their angle of orientation, face lateral drift in the blood circulation. This drift occurs due to variable drag forces and torques experienced by the rods under blood flow. These dominant forces are responsible for their ability to marginate (Gavze and Shapiro, 1997; Park and Butler, 2010). Furthermore, discoidal shapes ($AR = 0.5$), hemispheres, and ellipsoidal nanoparticles ($AR = 0.5$) also possess higher drift velocities as compared to spheres (Lee et al., 2009). However, out of ellipsoids, hemispheres and disks, only discoidal particles distinctly move in a highly oscillatory trajectories leading to enhanced interaction with the vessel wall (Figure 14.2). As the hemodynamic shear forces are decreased, the spherical nanoparticles marginate almost twofold. Shape readily enhances the process of margination; nanorods of $AR \sim 2$ exhibited sevenfold more accumulation than nanospheres under the same shear forces. Disks marginate more readily as compared to rods. High hemodynamic shear forces are also involved in lower adhesion rates of nanoparticles, since high shear rates dislodge the adhered particles and prevent any particle margination since it is extremely difficult for nanoparticles to circumvent the problem of rapid flows.

Geometry of the vessel is also an important criterion to evaluate margination of nanoparticles (Figure 14.3). At the bifurcation point, particles are more prone

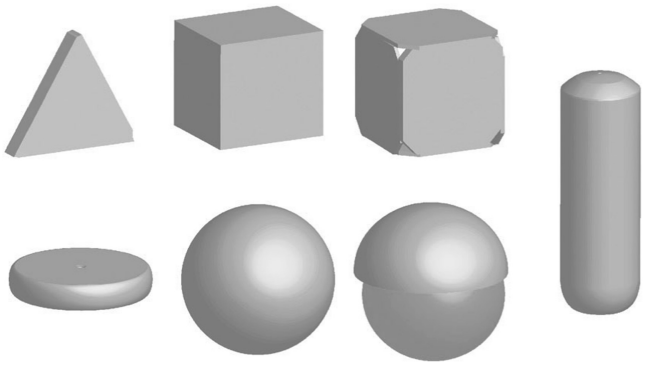

FIGURE 14.2 Different shapes available for NPs.

Triangle prisms, cubes, truncated cages, plates, spheres, shells (using a SiO_2 core), and nanorods.

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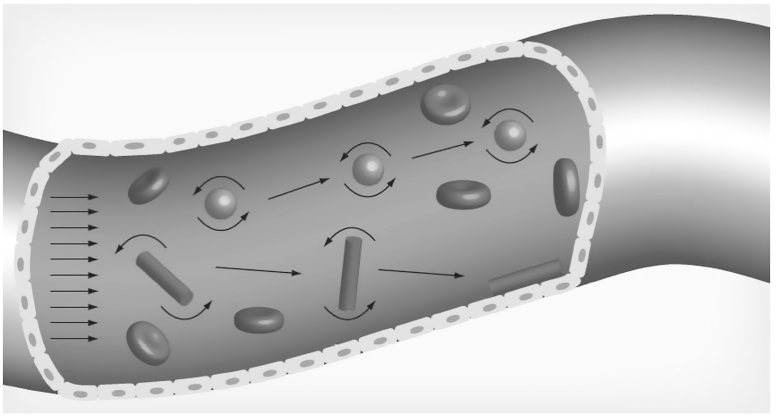

FIGURE 14.3 Effect of shape on nanoparticle margination.

Spherical nanoparticles tend to remain in the center of the flow. Variable forces and torques exerted on rods under flow allow them to marginate and drift towards the vessel wall, where they are able to bind to wall receptors or extravasate through gaps between cells of the endothelium.

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to get deposited rather than straight vessels (Doshi et al., 2010). The shape of the nanoparticle further enhances the deposition rate at the vessel junction, for instance, spheres display a lower ratio of bifurcation to straight vessel wall attachment than the ellipsoidal disks. Moreover, oblate shapes exhibit more advantages towards complex vessel geometries.

14.2.5 OPTICAL PROPERTIES

Biomedical scientists working on inorganic nanoparticles exploit their optical properties for sensing (Murphy, 2002; Barone et al., 2005; Jain et al., 2008; Buecker et al., 2008; Chang et al., 2010) and imaging purposes (Jana, 2011; Huang et al., 2010). There is a very narrow window of light which allows penetration through the tissue, but often, this narrow window is responsible for effective excitation of organic fluorophores, the use of NPs poses a dynamic opportunity for the exploitation of this spectral regime. Both semiconductors and metallic nanoparticles exhibit optical properties. In the case of semiconductors, the physical confinement of the material at nanoscale leads to specific optical properties. This concept of treating excitation of electrons as a "particle in a box" is known as quantum confinement effect (Alivisatos, 1996). Any alteration in the size or shape of a nanoparticle leads to changes in its electronic excitation behavior. This change in the absorption correspondingly causes a shift in its photoluminescence (PL), thus these nanoparticles can be tuned based on their desired applications as shown in Figure 14.4.

Figure 14.4 shows a CdSe quantum dot series which exhibits different emission wavelengths due to alterations in size. They can be exploited in biomedical applications since quantum confinement is material-specific (Michalet et al., 2005; Chuang et al., 2010).

Metallic nanoparticles exhibit a unique phenomenon of surface plasmon resonance (SPR) (Huang et al., 2009; Daniel and Astruc, 2004). SPR is defined as collective oscillation of conduction electrons at the surface of the metal nanoparticles due to excitation by the corresponding resonant wavelength of light. Hence this consequently causes a strong absorption of $10^9\,M^{-1}\,cm^{-1}$ OD for a 40-nm gold nanoparticle and ultrafast electronic relaxation (Jain et al., 2006). The SPR shift towards longer wavelengths is purely dependent on the size and shape of the metal nanoparticles. As the size of the nanoparticle increases, the amount of scattered light also increases as per Mie's theory. Furthermore, variations in shape such as nanorods, nanocages, and nanoplates also results in unique scattering

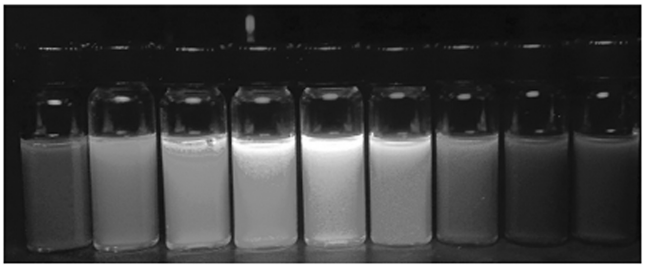

FIGURE 14.4 CdTe/CdSe quantum dot photoluminescence under UV excitation.

The size of the QDs increases from left to right, modulating the emission from 520 nm to the NIR.

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properties. This marriage between unique SPR phenomena and scattering results in the enhancement of scattering properties, which makes them a proficient candidate for biomedical imaging applications.

14.3 PHYSIOLOGICAL BARRIERS TO BE CIRCUMVENTED BY INORGANIC NANOPARTICLES FOR EFFECTIVE DRUG/GENE AND GENE DELIVERY

The present trend of proving whether a nanoparticle is effective in drug/gene or gene delivery, is only through *in situ* experiments. But the real game starts when such particles orchestrated by drug/gene or gene traverse through the physiological complex environments of blood vasculature, lymphatic vessel, tissue microenvironment, and finally the cellular milieu. Moreover, the complexities become worsened when we are dealing with cancer, specifically solid tumors. There are three physiological barriers imposed by solid tumors leading to poor localization of macromolecules and even nanoparticles:

1. Heterogeneous blood supply
2. Increased interstitial pressure and
3. Long distances within the interstitium.

The first physiological barrier hampers the delivery of bloodborne molecules, such as nanoparticles, into well-perfused regions of a tumor. The second barrier diminishes transendothelial migration of macromolecules and nanoparticles in the high interstitial pressure regions, causing radially outward convection in the tumor periphery opposing the inward diffusion. The sluggishly moving particles in tissue acts as a third barrier, which further increases the time required to reach towards the distal regions of the tumor.

The intravenous journey of nanoparticles orchestrated by drugs/genes dictates their efficacy to act as an efficient delivery system. This endeavor of inorganic nanoparticles to travel through the tough vasculature and find their way inside the pathological tissues *in vivo*, has been a matter of study for material scientists. The unresolved mystery is what physical properties manuevre the efficiency of NPso act as a nanomedical tool.

14.3.1 BIOLOGICAL SYSTEMS ARE COMPLEX AT DIFFERENT LEVELS

The giant leap of nanoparticles from the highly controlled environment of a test tube to the cellular milieu to its *in vivo* transportation inside a complex biological system, is an intimidating challenge. The two important complexities in living systems are as follows:

1. Passive transport
2. Active transport.

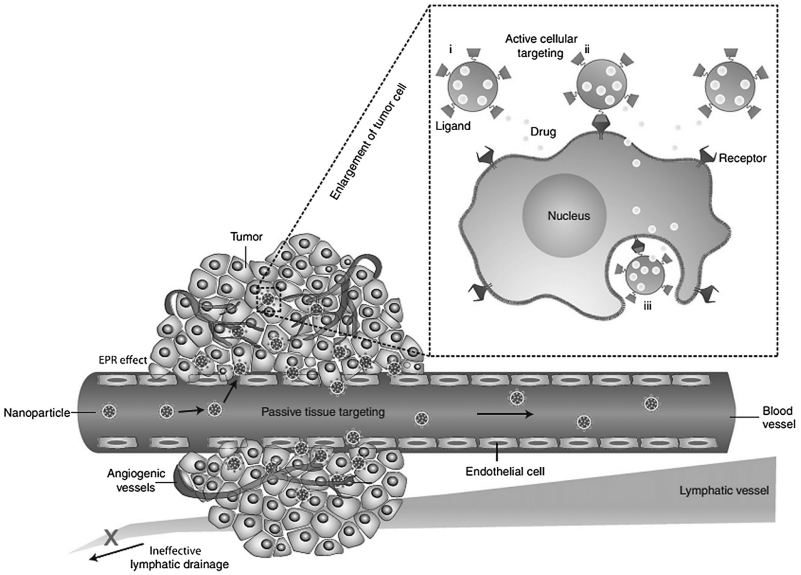

FIGURE 14.5 Illustration of the complex environment NP–drug/gene conjugates experience upon intravenous delivery.

Particles initially flow through the vascular system (red), diffuse through the leaky endothelial walls associated with several diseases (dark gray), and finally reach the targeted cell (enlarged box).

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Passive transport is related to inherent physical properties (diffusion, size, shape, etc.) of the nanoparticles and active transport refers to specific biological processing of nanomaterials (macrophages, enzymatic destruction, etc.) (Moghimi et al., 2001). The movement of nanoparticle conjugates in the living system is divided into three parts: intravenous transport, vasculature, and tissue penetration, as well as cellular interactions (Figure 14.5).

14.3.1.1 Intravenous transportation

The voyage of nanoparticles, especially inorganic nanoflotillas, when injected intravenously or directly to the target is a matter of curiosity amongst biomedical scientists. Once the NP–drug/gene conjugate is dispersed in the complex nonNewtonian fluid, that is, blood, which comprises of high salt concentrations and a plethora of proteins that can interact with NP, this leads to diminished electrostatic repulsive forces caused due to high ionic strength, while protein agglomeration on NP causes a change in both the hemodynamic radius and charge on the particles (Dobrovolskaia et al., 2009). There is a need to sterically shield nanoparticles from the immune system, since they tend to be cleared by the reticuloendothelial system. Hence there are reports in which nanoparticles have been coated by surface ligands

such as PEG and other polymers. Furthermore, physical properties of the nanoparticles have to be considered, such as deformability, surface geometry, surface charge, and anchoring linkers, that affect the body, which also gives a response in the form of an immune response known as complement activation.

Intravenous transportation of nanoparticle−drug/gene conjugate is through the bulk vascular system. The conjugate moves via vascular propulsion throughout the body, while the half-life of polymer-coated gold nanoparticles can be on the order of tens of hours. This propulsion is regulated by the reticuloendothelial clearance mechanism and filtration of lymph nodes, kidneys, liver, etc. It has been reported that kidney can clear off particles of hydrodynamic diameter of 5.5 nm, while larger particles are cleared by other filtration modus operandi (Lipka et al., 2010; Choi et al., 2007, 2009; Alexis et al., 2008).

14.3.1.2 Dissemination into tissue and vasculature

NP−drug/gene conjugates traverse through normal vasculature without any hindrance due to nonstickiness as well as high negative charge on the endothelial cells. Moreover the capillary vessels are nonleaky and intact, which allows their movement without any obstacle (Moghimi et al., 2001). But in a cancerous tissue, especially in solid tumors, there are two mechanisms which are exploited by biomedical scientists, leaky vasculature as well as enhanced permeation and retention (Maeda et al., 2000). In solid tumors, due to irregularities in the development of endothelial cells, the gap between them is more than the normal vasculature. This leads to enhanced permeability of NP in such milieu. Furthermore, the retention capacity of such conjugates is also increased in such an environment due to ineffective fluid removal from the tissue and enhanced accumulation of molecules in them. Maeda explains that this process is considered to be the gold standard for solid tumor therapy, and observes that there would not be any favorable partitioning in solid tumor tissue below a finite molecular weight of approximately 80 kDa (Maeda, 2001). The obstacles experienced by NP−drug/gene conjugate delivery is a huge challenge, when they transit from obstruction-free vasculature to anisotropic and dense matrix. This was confirmed by imaging quantum dot movement from the normal vasculature to solid tumor, that gets diffused into the extracellular matrix (Stylianopoulos et al., 2010). Further confirmation was done by using gelatin/QD hybrids ($\sim$100 nm), which accumulate and are successively degraded by excess matrix metalloproteinases, thus releasing encapsulated QDs ($\sim$10 nm, d_h). This proves that larger particles face more obstructions while tarversing through the tumor as compared to smaller particles (Wong et al., 2011). Especially, intermediate hydrodynamic diameters of size 20−80 nm have shown that diffusion of such particles is comparatively more hindered than particles with $d_h < 40$ nm that can traverse from the vascular system to the tumor interstitial space (Perrault et al., 2009).

14.3.1.3 Cellular transport

After the transendothelial migration of nanoparticles from the vascular system to the interstitial space, they need to traverse through the intracellular milieu of cells

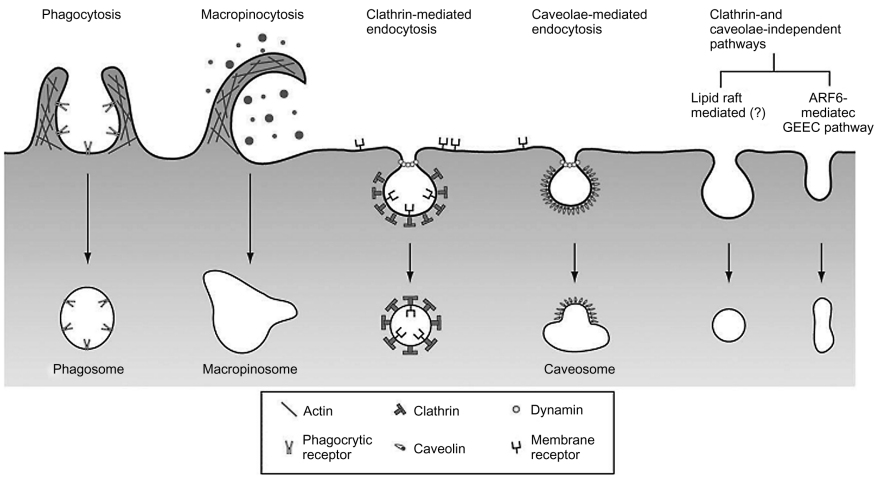

FIGURE 14.6 Pathways of entry into the cell.

An increasing number of endocytic pathways are being defined, each mechanistically distinct and highly regulated at the molecular level. These pathways facilitate cellular signaling and cargo transport. Controlling the route of nanoparticle uptake is important for both mediating their intracellular fate as well as their biological response.

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(Figure 14.6). The intracellular regime of cells is separated from the extracellular region by a staunch boundary known as the plasma membrane. This membrane is flooded by proteins, carbohydrates, and lipids. Nanoparticles travel through the receptors or get diffused via the lipid bilayer. Certain ligands functionalized on the surface of nanoparticles help in synaphic delivery of particles conjugated with drugs or genes inside the cells via receptor mediation.

Nanoparticles or engineered particles are ingested by curious cells by two different mechanisms.

1. Phagocytosis

   Phagocytosis is a process in which materials upto 10 μm in diameter are ingested and the professional workhorses are the reticuloendothelial system such as macrophages and dendritic cells. The cascadic pathway involves three distinct steps: recognition by opsonins in the bloodstream, tethering of the opsonized particles by the macrophages, as well as phagocytosing of the nanoparticles. Opsonization involves tagging foreign particles by proteins called opsonins, so that macrophages can recognize the tag and engulf them. Mostly opsonins are immunoglobulins, complement proteins, blood serum proteins, etc. which, on conjugation with particles, bind certain scavenger receptors or Fc receptors or complement receptors on macrophages. Receptor–ligand interaction is the inception of a signaling cascade caused by

the Rho-family GTPases, which stimulates actin assemblage, leading to the formation of pseudopodia which zippers around the nanoparticles and finally engulfs it. Such a phagosome acts like a cargo and moves through the cytoplasm. Furthermore, when actin depolymerizes from the phagosome, the vacuolar membrane of the phagosome comes into contact with the early endosomes and then fuses with it. There are sequential fusion and fission events causing the early endosomes to mature. This then fuses with late endosomes and finally with lysosomes, thus forming a phagolysosome. The rate of such reactions after ingestion of particles is something around half an hour to several hours.

2. Nonphagocytic endocytosis

Nonphagocytic endocytosis or pinocytosis or cellular drinking mechanism is performed by virtually all the cell types which involve ingestion of submicron particles of smaller size (in the range of 5−10 nm). Unlike phagocytosis, which is limited to specialized cells, other endocytic pathways occur by four main modus operandi:

a. Clathrin-mediated endocytosis (CME)

This receptor-mediated CME is important for drug delivery since the drug-loaded nanoparticles interact via specialized ligands with the receptors on the surface of the cells, which become metabolized into the lysosomes, causing drugs to be released from it for further action. The specialized ligands assigned for the receptor-mediated endocytosis include low-density lipoprotein (LDL), transferrin, and epidermal growth factor (EGF) (Kanaseki and Kadota, 1969). The vesicle is formed during CME, and the process involves assemblage of clathrin triskelions, that is, three clathrin heavy chains into a polygonal lattice, thus leading to the deformation of the plasma membrane to form a coated pit. The structure of the clathrin lattice is similar to a basket which coates the membrane and at the neck of the coated pit, dynamin is rendered with the job of membrane fission. This causes release of the clathrin-coated vesicle in the cytosol. Furthermore, after uncoating such vesicles the clathrin heavy chains can be recycled for coating other membrane pits. The vesicle of an average size of 100−120 nm can deliver its cargo to early endosomes, that are acidified by ATP-dependent proton pumps (pH ∼ 6). At this stage most of the receptor-ligands get dissociated to be recycled for another round of delivery. Such early endosomes are matured into late endosomes whose pH is ∼5, which fuses with prelysosomal vesicles that contain acid hydrolases, thus creating a hostile environment for complete release of the internalized cargo (Mukherjee et al., 1997; Bareford and Swaan, 2007; Conner and Schmid, 2003).

b. Caveolae-mediated endocytosis

This type of endocytosis is considered to be a highly controlled process as compared to the clathrin-mediated one. This process is instigated by the cargo itself (Bareford and Swaan, 2007; Conner and Schmid, 2003).

Nanoparticles which interact with the cell surface via receptor−ligand binding, move along the plasma membrane towards caveolae invaginations. The fission event of such a caveolae from the plasma membrane, which is caused by GTPase dynamin, leads to the formation of cytosolic caveolar vesicle. This pathway helps nanoparticles to bypass the degradation route posed by lysosomes, where the drugs such as peptides and proteins, as well as nucleic acids that are sensitive to enzymes. In short, caveolae-mediated endocytosis has much lower uptake kinetics as compared to CME. The ligands responsible for such a type of endocytosis are folic acid, albumin, and cholesterol.

c. Macropinocytosis
This clathrin-independent pathway (Figure 14.7) occurs in many different types of cells including macrophages. This involves actin-mediated membrane pseudopodia formation, but such protrusions do not zipper up with the ligand-coated nanoparticle.

They collapse and finally fuse with the plasma membrane, thus generating large endocytic vesicles called macropinosomes that range in size from 1 to 5 μm. These macropinosomes, once inside the cells, acidify and shrink, or may fuse with lysosomes, while releasing and recycling their contents onto the surface. This type of endocytosis does not show any selectivity, but rather is responsible for uptake of nanoparticle−drug conjugates (Mukherjee et al., 1997; Conner and Schmid, 2003; Swanson and Watts, 1995; Racoosin and Swanson, 1992).

d. Other clathrin- and caveolae-independent endocytosis
There are a plethora of clathrin- and caveolae-independent endocytic pathways which involve cholesterol-rich microdomains, which are also called lipid rafts that have a diameter of 40−50 nm. There are many other pathways which have been proposed recently. Hence the comprehension of drug delivery using nanoparticles is still in its infancy.

14.3.2 *IN VIVO* PHYSICAL PARAMETERS FOR DRUG/GENE DELIVERY

The physical parameters for NP-mediated drug delivery are bulk flow transport and electrostatics that dictates the efficiency of an optimized delivery system at each and every stage *in vivo*. The complete comprehension of such parameters of nanoparticles only helps us to apprehend the biological transportation of drugs or genes inside the cells.

14.3.2.1 Bulk flow transport

The circulatory system has a great impact on the transportation of nanoparticles via a bulk flow transport mechanism. This mechanical pumping leads to the mobility of blood throughout the body resulting in the systematic flow and sweeping of NP−drug conjugates. Hemodynamic shear rates are proportional to the difference in the arterial and venous pressures. The shear rates are inversely

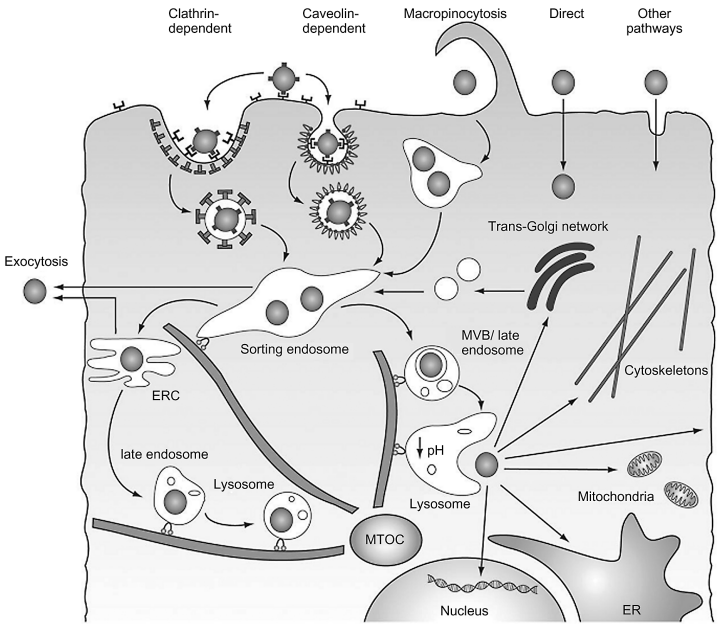

FIGURE 14.7 Intracellular transport of nanoparticles.

After internalization via one or more of the endocytic pathways, nanoparticles are trafficked along the endolysosomal network within vesicles with the help of motor proteins and cytoskeletal structures. Vesicles can transport their contents into sorting endosomes, or excrete/recycle them back to the cell surface using the plasma membrane. Alternatively, endosomes can mature into lysosomes via luminal acidification and recruitment of degradative enzymes, which target the vesicle contents for degradation. In order to access cytoplasmic or nuclear targets, nanoparticles must be capable of escaping from the endolysosomal network as well as traverse through the crowded cytoplasm. Abbreviations: ERC, endocytic recycling compartment; ER, endoplastic recticulum; MTOC, microtubule-organizing center; MVB, multivesicular bodies.

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proportional to the resistances offered by viscosity and geometries of connective tissues. The equation for the blood flow is as follows:

$$Q = \frac{\Delta P}{FR}$$

where FR is equal to ηZ, and η is the viscous resistance, and Z is the geometrical resistance (Jain and Stylianopoulos, 2010).

The viscous resistance in blood is offered by the viscosity of plasma and the rigidity of red blood cells. Since the cancer cells and WBCs are more rigid than red blood cells, they offer more resistance and even cause transient stasis when present in the vascular system. Hence, tumors exhibit relatively less Fahraeus (this is an effect

which is seen when there is a decrement in the hematocrit of small vessels) and Fahraeus-Lindqvist (this is an effect in which there is a decrement in the blood viscosity of small vessels) effects as compared to normal tissues (Jain, 1988).

Geometric resistance is directly proportional to the vessel length and inversely proportional to vessel diameter to the fourth power. Bulk flow transport is dominated by the vessel diameter. Any alterations in vessel diameter of normal vessels is brought about by smooth muscle cells. Tumor vasculature is constituted by two different types of vessels: one derived from the host vasculature network and and the other derived from angiogenesis.

There are very few instances that there is invasion of arteries and arterioles of the host vascular system by tumor. Instead smooth vessel cells with their contractile and nervous apparatus surround such vessels on receiving physical or chemical stimuli. The overexaggerated rate of tumor vasculature growth leads to defective vessels causing leaky vasculature (Leunig et al., 1992; Yuan et al., 1994; Kamoun et al., 2010; Endrich et al., 1979).

The increased viscous and geometric resistances caused by the vasculature thus can compromise tumor blood flow. Hence, the velocity of RBCs in normal blood vessels is higher than in tumor blood vessels, while the overall perfusion rate which is considered to be blood flow rate per unit volume in normal tissues is higher as compared to tumors. Moreover the blood velocity in tumors is considered to be overall independent of vessel diameter, thus they leave perfused or unperfused regions causing distribution of blood to be highly uneven. Such an unperfused region instigates further hostility in the tumor microenvironment such as less pH, less necrotic tissue, and the partial oxygen pressure is also less. This causes drug resistance and leads to progression in tumors.

The lymphatic drainage sytem is meant to remove excess fluid from the tissues so that there is an overall interstitial fluid balance. This system is collapsed in tumor tissues due to overgrowth of cancer cells, thus causing undue compression of the lymphatic vessel at the center of the tumor. The tumor periphery possesses functional lymphatic vessels which have been rendered the job of ferrying fluid, certain growth factors, as well as cancer cells, thus playing a critical role in tumor metastases.

Thus, the spatial and temporal heterogenous supply of blood, uneven permeability of vessels, and bad lymphatic drainage system leads to the impairment of uniform delivery and efficiency of therapeutic agents in tumors. Thus there is a need to normalize such defective tumor vasculature. There are reports in which nanoparticles are conjugated to drugs along with antiangiogenic factors such as vascular endothelial growth factor and platelet-derived growth factor. Moreover, size, shape, and surface charge of therapeutic nanoparticles play an important role in extravasation and interstitial transportation. Such optimized nanoparticles can be conjugated to antiangiogenic agents for vascular shutdown as well as inhibited expression of intratumoral hypoxia-inducible factor-1-alpha (HIF1α) (overexpression of HIF1α is responsible for an increment in tumor invasiveness and chemotherapeutic resistance) (Sengupta et al., 2005).

14.3.2.2 Electrostatics

The surface charge on the nanoparticles is very critical while considering it to be exploited as a drug-delivery system. This is also important for extravasation and interstitial transport of nanoparticles when moving through tumor vasculature. There are several reports where cationic nanoparticles synaphically target tumor endothelial cells, thus exhibiting a higher vascular permeability as compared to neutral or anionic counterparts (Campbell et al., 2002; Schmitt-Sody et al., 2003; Dellian et al., 2000). In contrast, neutral nanoparticles are capable of faster diffusion and homogeneous distribution inside the tumor interstitial space than cationic and anionic nanoparticles. This is because cationic nanoparticles bind electrostatically negatively charged hyaluronan, while anionic nanoparticles interact with positively charged collagen matrix molecules (Stylianopoulos et al., 2010; Lieleg et al., 2009). However, there is one more obstacle for increased half-life of nanoparticles in the reticuloendothelial system, which is large surface charge (either more negative or more positive). The nanoparticles can be sterically stabilized by modifying them with polyethylene glycol, which renders slightly negative or positive charges. This helps in circumventing the problem of opsonization by serum proteins and then phagocytosis by macrophages.

It has also been proposed that charges present on nanoparticles are also affected by the local electrical field of cells. Even a slight alteration in the cell membrane potentials can generate electric fields in the order of 10^5 mV/mm^{-1}. Furthermore, it has been reported that the proliferation rates of cells are related to the membrane potentials of cells. The proliferating cells possess lower potentials (-10 to -30 mV) while stationary cells possess larger potentials (-70 to -90 mV). This can help in the comprehension of the transportation and extravasation of nanoparticles in the interstitial fluid (Robinson and Messerli, 2003; Glaser, 2000).

14.4 INORGANIC NANOFLOTILLAS AS PROPELLERS FOR DRUG/GENE DELIVERY VIA SURFACE FUNCTIONALIZATION

Inorganic nanovehicles possess size, shape, and surface charge-dependent physicochemical properties. The high surface-to-volume ratio and less surface energies make them suitable to be exploited as an efficient drug/gene-delivery vehicle. Surface functionalization of such inorganic nanoparticles is possible by easy orchestrations with organic materials, biopolymers, peptides, and polymers. Such organic/inorganic nanohybrids are paradigms that have been mimicked from the biological systems. They are considered to be nanoscaffolds for therapeutic and imaging agents, thus acting as a theranostic agent.

14.4.1 GOLD NANOPARTICLES

Gold nanoparticles have been exploited as a theranostic agent as they possess unique capability of binding amine ($-NH_2$) as well as thiol ($-SH$) functional groups (Petros and DeSimone, 2010; Bhattacharya et al., 2011; Kudgus et al., 2011; Arvizo et al., 2012; Doane and Burda, 2013). They act as a paradigm for drug or gene delivery vehicles, since they can synaphically interact with miscreant cells and possess decreased systemic toxicity, improved efficacy, effective biodistribution, and clearance of therapeutics (Li and Huang, 2008). They have attracted biomedical scientists for synthesizing different sizes (in the range of 1−100 nm) and shapes (including spherical, rods, cuboids, core−shell, and many more) (Jadzinsky et al., 2007; Daniel and Astruc, 2003; Tao et al., 2008) of gold nanoparticles. The biomedical applications of such nanoparticles are size- as well as shape-dependent as gold nanorods, gold nanoshells, and other gold nanostructures, when injected in the body can absorb NIR light, since the body tissues possess high transparency towards NIR light. Gold nanostructures aid in drug/gene release as well as photothermal therapy when exposed to NIR. There are different strategies for binding drugs or genes to drug-delivery vehicles, but gold nanoparticles efficiently tether via covalent linking. This is possible either by amino linkage or thiol linkage between gold nanoparticles and therapeutic agents. Smaller-sized gold nanoparticles (5 nm) exhibit large optical absorption due to SPR, while larger nanoparticles (10−100 nm) scatter exorbitantly, but they can absorb light only in the visible region. The biological tissues are less transparent to visible light as compared to near-infrared light, hence gold nanorods and nanoshells are synthesized which possess strong SPR in the near IR region. Gold nanorods are synthesized using a surfactant known as cetyltrimethylammonium bromide (CTAB) as a capping agent. But due to the inherent toxicity of CTAB, several groups are exchanging it with controlled surface chemistries. Gold nanoshells possess a spherical dieletric core having a thin gold layer acting like a shell (Alkilany et al., 2012).

14.4.1.1 Surface functionalization approach of gold nanoparticles

Gold nanoparticles are functionalized via thiol moiety, but this proves to be conditionally advantageous. Thiol groups have the tendency to be exchanged with the solution, which is a limitation, but in certain cases when inside the cells due to high levels of glutathione in the cytoplasm it can also be useful for drug release by means of exchange reaction (Hong et al., 2006). Glutathione partially displaces the thiol group on the surface of nanoparticles, thus causing release of covalently bound drugs. The stability of nanoparticles can be enhanced if the system is made more complex, in the sense that multiple thiols can be grafted onto the single nanoparticle, making it more stable (Li et al., 2002; Rosi et al., 2006). A cyclical disulfide can also be used for multiple binding points in a multidentate fashion with gold nanoparticles increasing the binding energies. Hence exploiting 1,2-dithiane end group, thioctic acid, and *in situ* dithiocarbamate has shown an

efficient way of grafting onto a gold nanoparticle surface (Letsinger et al., 2000; Huff et al., 2007). This refers to thiol groups which are considered to be covalently more stronger as compared to amine groups. In cases where weaker binding forces are needed, such as noncovalent ones (electrostatic, hydrophobic, hydrogen bonding) especially for drug release purposes, nanoparticles can attach or trap molecules via amine groups. If efficient drug release is the desire of a biomedical scientist, then only gold nanoparticles tethered to amines can accomplish this role since bond strength between gold and the amino group is ~ 6 kcal/mol as compared to 47 kcal/mol for thiols (Hoft et al., 2007). Gold nanoparticles are proficient vehicles for delivery of DNA/RNA or drugs for traversing through the cell membrane. The entry of nucleotide inside the cell is restricted due to its negative charge, hence through thiol linkage they can interact with gold nanoparticles and traverse through the membrane via the above-explained mechanisms. There are certain drugs which are toxic for normal cells and cannot enter the miscreant culprits, such as bacteria or cancer cells, due to resistance against them. Gold nanoparticles, nanorods, or nanoshells can tether such drugs and then via a targeted mechanism using antibodies or certain molecules (folic acid, LDL), they can enter inside the cells synaphically. There are many demonstrations where DNA/RNA and drugs have interacted with gold nanostructures (spherical, nanorod, and nanoshell) that can be comprehended as follows.

14.4.1.2 Delivery of siRNA/DNA

Gold nanoparticles are considered to be an efficient system for delivery of either DNA or RNA for gene silencing and consequently for therapeutic purposes. Mirkin et al. demonstrated that a dense layer of ssDNA molecules can coat the surface of citrate stabilized spherical nanoparticles via thiol linkers and hence can be exploited for gene silencing. Though ssDNA possess high negative charge density, the nanoparticle−ssDNA conjugate could not prevent the internalization into the cells. Moreover, the conjugates are protease- and nuclease-resistant. The tethering of complementary DNA to the ssDNA immobilized nanoparticles becomes stronger since there is a staunch dense packing of DNA around the nanoparticles leading to increased efficiency for gene silencing applications (Rosi et al., 2006).

In another instance, instead of regular DNA, chemically modified nucleic acids, known as locked nucleic acids (LNA), were used which have the advantage of being more stable and enhanced complementary DNA binding capacities. They also efficiently tether gold nanoparticles. Hence, tailor-made DNA for desirable functions is the requirement so that we can apprehend and predict the nanoparticle conjugate structures and their functions (Seferos et al., 2007).

The stability of such DNA functionalized gold nanoparticles can be correlated to the salt concentration and curvature of the nanoparticle surface (Hill et al., 2009). DNA packing density decreases as the size of the nanoparticles increases to 60 nm, reaching a plateau near the packing density of ssDNA on a flat substrate. Spherical nanoparticles acted like a paradigm based on which ssDNA attachment on rod-shaped nanoparticles was possible with complete accuracy.

Furthermore, similar to ssDNA, even RNA attachment was possible and was then demonstrated by Mirkin et al. Antifirefly luciferase siRNA was conjugated to gold nanoparticles and their gene knockout potential was noted and compared with the regularly used cationic lipid transfection agents (Giljohann et al., 2009). Nuclease-free synthesis of gold nanoparticles was possible through diethylpyrocarbonate treatment and autoclaving, since RNA is highly susceptible to nucleases. This treatment is possible only for the citrate-capped spherical gold nanoparticles and not for gold nanorods since at high temperatures they tend to reshape into spherical ones. This kind of coupling between gold nanoparticles and siRNA is considered to enhance the stability of such conjugates in serum eightfold as compared to bare siRNA. Such conjugates are resistant to degradation from many physiological RNA cleavers.

There are reports where ssDNA−LNA chimera functionalized gold nanoparticles were internalized for controlling the mRNA levels (Prigodich et al., 2009). Reporter DNA sequence tagged by a fluorescent marker when bound to such oligonucleotide tethered nanoparticles, can detect mRNA when present on its surface. The modus opernadi is that when reporter DNA is in propinquity to gold nanoparticles, the fluorescence is quenched while tethering of complementary mRNA causes desorption of reporter DNA into the solution, thus fluorescence of reporter DNA returns back. The accuracy with which DNA is bound to a gold nanoparticle surface and nucleotide binding chemistry studied in detail helps in the development of theranostic systems. Hence, there is a combination of both covalent and noncovalent linking of nucleotides onto the gold nanoparticles since only the ssDNA strand is covalently attached to gold while reporter DNA is noncovalently attached to it (Prigodich et al., 2009).

Oligonucleotides possess negative charge on the surface and this is the fact that bare DNA cannot enter the cells by themselves, rather they need the aid of positively charged transfection agents. There is a need for cationic gold nanoparticles which can bind DNA and can be easily internalized by the cells (Giljohann et al., 2007). It was found that as the number of attached DNA molecules increases, there is an increment in the recruitment of proteins onto the nanoparticle surface, which also leads to enhanced cellular uptake of DNA. This was then compared to nanoparticles that are orchestrated by octaethylene glycol. Moreover, the capability of delivering DNA or RNA into the cells is not important but the potential to increase or decrease the gene expression is of the utmost importance. Mirkin et al. showed that nuclear targeting is achieved via binding DNA or RNA along with peptides such as TAT or NLS. This could enhance the perinuclear delivery of the conjugates, thus leading to a 50% increase in gene silencing as compared to antisense−gold nanoparticle conjugates only (Patel et al., 2008). Positively charged amino acid, lysine, can be used as a capping agent for gold nanoparticles and helps in the formation of dense and compact packing of negatively charged DNA on its surface. Such polycationic gold nanoparticles and DNA conjugates prove to be an efficient gene delivery system and that too in the absence of any cytotoxicity (Agasti et al., 2009). The compaction of DNA on

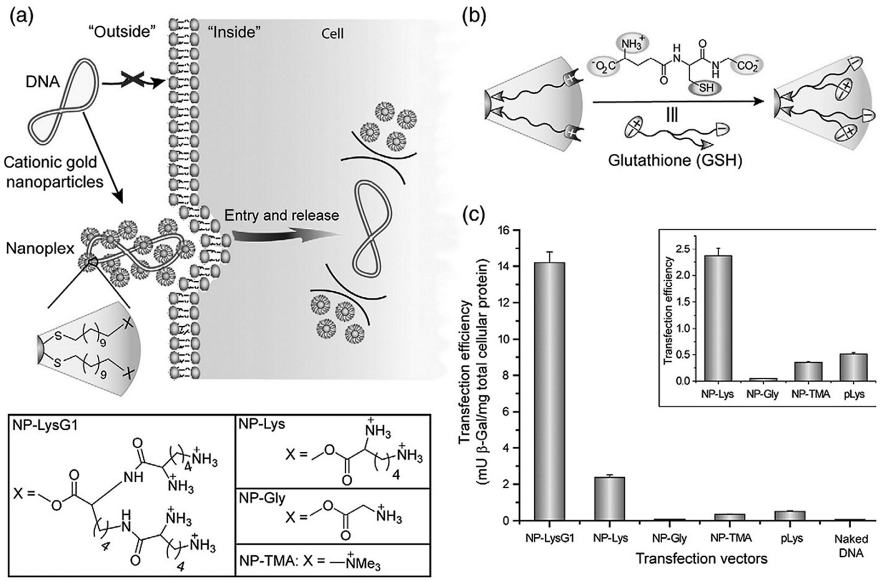

FIGURE 14.8

(a) Schematic illustration of DNA transfection using nanoplexes containing NP-LysG1 and DNA. The chemical structures of the head groups present on the surface of the NPs are given in the underneath box. (b) Schematic depiction of place-exchange between cationic ligands on NP surface and cellular glutathione (GSH), which is proved to be the mechanism of DNA release in this case. (c) Enhanced transfection using NP-LysG1 and NP-Lys relative to NP-Lys, NP-TMA, polylysin, and naked DNA.

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gold nanoparticles was enhanced via increased ammonium group density, thus helping in proficient cellular delivery which is shown by the lysine dendrons (NP-LysG1) (Figure 14.8). In comparison to polylysine, lysine dendrons acted as an efficient ligand for transfection of plasmid DNA exhibiting 28-fold higher reporter gene expression. Such conjugates released DNA, based on the intracellular concentrations of glutathione (GSH) levels (Ghosh et al., 2008b).

The most important criterion for an efficient gene delivery system is that it must escape the reticuloendothelial clearance system, once the RNA–nanoparticle conjugate enters inside the body. Moreover, the conjugate must not elicit an immune response against the gene. There is an advantage to nucleic acids that they can elicit least response of immune system. The next challenging task for such conjugates is to interrelate with the cells. It was shown that the immune response can be analyzed by measuring the levels of interferon-β inside macrophages *in vitro*, which can be modulated by the nanoparticle–DNA conjugates (Massich et al., 2009). They showed that as the surface packing of bound DNa increases, there is a decrement in

the immune response. This was compared with cationic lipids which exhibited 25-fold more IFN-β levels when tightly packed with gold nanoparticles and they maintain equal gene silencing capability. Therefore tight packing of DNA around gold nanoparticles has a great impact on cellular response. The cellular response was studied in HeLa cells and genome wide profiling of gene expression was analyzed for oligonucleotide-functionalized gold nanoparticles (ssDNA, dsDNA, and dsRNA) as well citrate-capped gold nanoparticles in terms of cell cycle progression and apoptosis induction. It was found that citrate-capped gold nanoparticles attributed to the initiation of apoptosis as compared to densely packed nanoparticles. Gene delivery applications are further needed to be performed for the comprehension of biological effects of such oligo-conjugated nanoparticles.

14.4.1.3 Delivery of drugs

Controlled release of drugs is an important criterion for designing a good drug delivery system. Other parameters, such as high surface area, tunability, and decreased physiological side-effects, are playing a critical role in deciding whether the delivery system can be used for delivering the drugs.

There are many antibiotics which have gained resistance against microrganisms and are sometimes toxic to host cells. Gold nanoparticles have come to the rescue of such drugs and have been conjugated with them for transforming resistant bacterial cells to sensitive ones. Moreover, this conjugation has also led to a reduction in the toxicity of host cells. Notably, vancomycin is the antibiotic to which *Enterococci* and Gram-negative bacteria such as *Escherichia coli* have become resistant (Figure 14.9).

Such strains have been tested after conjugating vancomycin with gold nanoparticles via thiol linkage which showed very good activity against both bacterial strains. The modus operandi of such particles is due to multivalency, since multiple drug molecules on a single nanoparticle led to the increased binding to the D-Ala-D-Ala moieties at the terminal position (Gu et al., 2003; Rao et al., 1998; Xing et al., 2003).

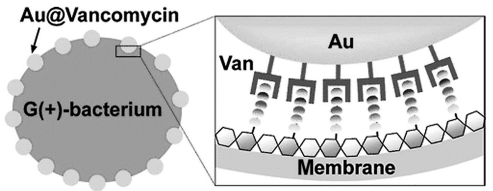

FIGURE 14.9

Illustration of a possible multivalent interaction between a Van-capped Au nanoparticle (2) and a VanA genotype VRE strain (hexagons: glycosides; ellipses represent the amino acid residues of the glycanpeptidyl precursor with different colors: L-Ala (yellow), D-Glu (orange), L-Lys (green), D-Ala (blue), and D-Lac (purple)).

The orientation of vancomycin is very critical for enhancing the binding with bacteria. Hence covalent tethering between gold nanoparticles and vancomycin is an important method to be exploited. Moreover bis(vancomycin) cystamide has been exploited to surface functionalize nanoparticles since they can absorb near-IR wavelengths of light maximally (Kell et al., 2008; Huang et al., 2007). Gold nanoparticles can also be utilized for binding a variety of bacterial types such as vancomycin-resistant *Enterococci* (VRE) and meticillin-resistant *Staphyloccus aureus* (MRSA) strains. Once such nanoparticles are bound to resistant strains, they can cause photothermal destruction with maximal efficiency. This method is even nontoxic for nonbacterial cells, which was determined by the MTT assays with human epidermoid carcinoma epithelial cells. Instead of antibiotics, phosto-sensitizers such as toluidine blue O (TBO) were also covalently attached to tiopronin-functionalized gold nanoparticles, that were highly stable (Gil-Tomas et al., 2007). There was a fourfold decrease in the minimal inhibitory concentration when irradiated with white light or laser light when TBO-functionalized gold nanopàrticles are used in comparison with free TBO. In another instance, many anticancer drugs have been utilized for their attachment with gold nanoparticles, since they tend to exhibit systemic side-effects and are nonspecific, affecting normal cells too. The chemotherapeutic drugs such as cisplatin, paclitaxel, tamoxifen, and doxorubicin are a few examples of this class. Gold nanoparticles have proved to be advantageous in the sense that after their attachment with anticancer drugs, the conjugate is less toxic with increased specificity towards cancer cells as compared to free drug. Table 14.1 summarizes gold nanoparticle–drug conjugates for bactericidal applications.

Table 14.1 Summary of Gold Nanoparticle–Drug Conjugates for Bactericidal Applications Showing the Drugs Which Have Been Studied, any Extra Treatment Which Is Required, the Attachment Chemistry to the Gold Surface, the Type of Bacteria Tested, and the Corresponding References

Drug/Treatment	Attachment	Tested Against	References
Vancomycin	Thiol	VRE, Gram-negative	Gu et al. (2003)
Vancomycin/ photothermal	Thiol	Gram-positive, Gram-negative, VRE, MRSA, PDRAB	Huang et al. (2007)
Ampicillin, streptomycin, kanamycin	Amine (putative)	Gram-positive, Gram-negative	Saha, et al. (2007)
Cefaclor	Amine	Gram-positive, Gram-negative	Rai et al. (2010)
Toluidine blue O/photosensizitization	Thiol	Gram-positive	Gil-Tomas et al. (2007)

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Apart from this, depending on the size of the nanoparticles the conjugate tends to passively target cancer cells via an enhanced permeability and retention effect (EPR) or target actively via antibodies or targeting molecules such as LDL or folic acid. There are a plethora of covalent as well as noncovalent methods utilized for attaching drugs on the gold nanoparticles. There are both advantages and disadvantages in both methods.

1. Covalent binding leads to stable interaction throughout the vascular travel, but needs intracellular treatment of the prodrug (Morgan et al., 2006). There are many anticancer therapeutic agents such as paclitaxel, doxorubicin, tamoxifen, cisplatin, and 5-fluorouracil that can tether gold nanoparticles via covalent linkage (Figure 14.10). These organic chemotherapeutic agents possess reactive functional groups or are modifiable in such a way that they can interact with gold nanoparticles. There are two methods by which such a type of tethering takes place.

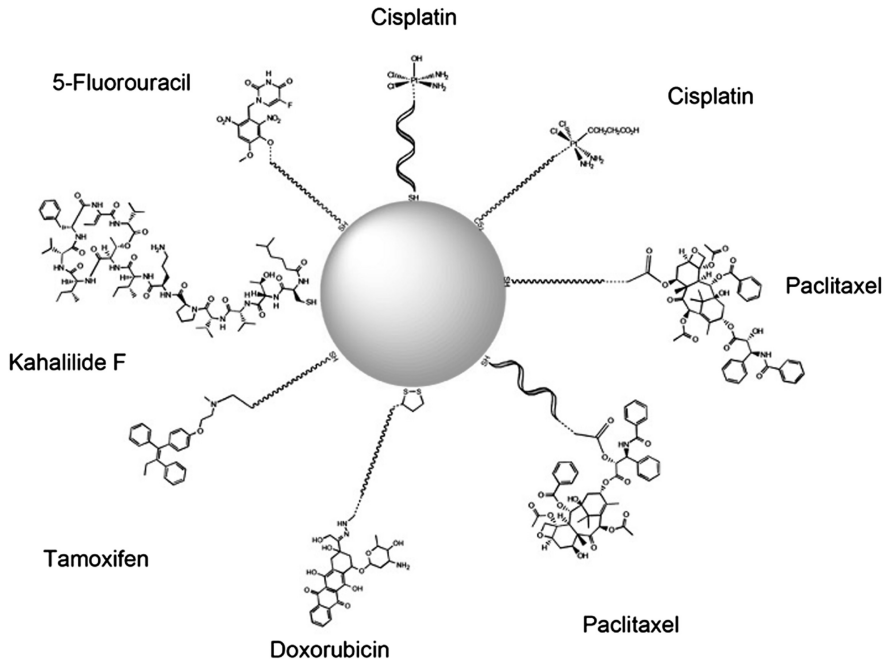

FIGURE 14.10

Overview of anticancer drugs covalently conjugated to gold nanoparticles. Dashed lines represent an omitted portion of a linker. The helical segment represents a DNA oligonucleotide. The wavy line indicates an oligo- or polyethylene glycol-containing segment.

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a. Surface orchestration of gold nanoparticles so that drugs can interact with the nanoparticles directly via covalent chemistry set on the surface.
b. Functionalization of drugs in such a way that they can easily interact with gold nanoparticles via modifications done in the organic molecule.
c. Paclitaxel, a chemotherapeutic drug possesses many side-effects affecting normal cells. Hence, this drug has been used at its C-7 position to undergo carbodiimide-based esterification with oligoethylene glycol spacer. This helps in conjugating gold nanoparticles via an oligoethylene glycol linker. These NP−paclitaxel conjugates themselves act like self-therapeutics, that is, such well-presented paclitaxel molecules by individual nanoparticle do not require any release of drugs for efficacy, they are themselves capable of acting like a drug (Gibson et al., 2007). In another instance, paclitaxel was functionalized via phosphodiester linkage at the C-2 position of the molecule, which was in turn tethered to a thiolated PEG, for binding gold nanoparticles. The phosphodiester bond was supposed to be cleaved by phosphodiesterases in cancer cells. This intracellular degradation of drugs is pertinent, as the drug must not be released in the vascular system. Moreover, free paclitaxel is a water-insoluble drug but when conjugated with gold nanoparticles, becomes water-soluble. The traditional trend to solve the insolubility problem of paclitaxel is to encapsulate inside micelles of Cremophor El solvent, which has some inherent side-effects (Hwu et al., 2009). There are other illustrations where covalently attached pro-drugs are released via enzymatic treatment such as hydrolysis done by phosphodiesterase enzyme *in vitro* (Hwu et al., 2009).
d. There are certain drugs which are highly toxic, such as platinum (Pt)-containing drugs, especially Pt (II) which is more toxic and active as compared to Pt (IV), which is less toxic and inert. Intracellular activation of prodrugs is the main requirement, which helps in the delivery of inert forms, thus decreasing the side-effects. Covalent conjugation of anticancer drugs having Pt (IV) to oligonucleotide-tethered gold nanoparticles and thus sending the prodrug synaphically into tumor cells has been illustrated. Further, such prodrugs when treated intracellularly are converted into Pt (II), thus resulting in cytotoxicity (Gibson et al., 2007). Pt (IV) prodrug was also attached covalently to gold nanorods via diamino(polyethylene glycol) functionalization. One amino end was coupled to dithiocarbamate while another amino end was coupling with carboxyl-containing platinum (IV) compound (Min et al., 2010; Tong et al., 2007). Cytotoxicity of gold nanorods conjugated with platinum prodrugs was higher as compared to free cisplatin. The reason behind higher toxicity was an increment in the intracellular levels of platinum, thus confirming that the delivery vehicle is most significant for enhanced uptake via endocytic mechanisms.
e. The covalent linkage of tamoxifen to gold nanoparticles was done through a thiolated PEG linker. The tertiary amino group was transformed into a secondary amine which was followed by alkylation with a thiolated PEG.

This tamoxifen-thiolated PEG was then simply mixed with gold nanoparticles which exhibited 2.7 times more potency than that of free tamoxifen. This was purely due to enhanced uptake rates of the conjugate and not because of the multivalency effects as discussed for antibiotic−gold nanoconjugates. This enhanced uptake was mediated by overexpressed estrogen receptors in the case of breast cancers (Dreaden et al., 2009).

f. Another 13 residue peptide chemotherapeutic drug known as Kahalilide F was conjugated to gold nanoparticles via cysteine modification of the drug. The peptide was bound covalently to gold nanoparticles, along with a multilayered coating of the peptide on the surface of the nanoparticles, thus increasing the drug loading, followed by enhanced cellular uptake. This showed higher cytotoxicity of cancer cells (Hosta et al., 2008).

g. There was a report on 5-fluorouracil which was modified through a terminal UV-photocleavable ortho-nitrobenzyl group and then conjugated with gold nanoparticles. Gold nanoparticles were precapped by pentanethiol which then interacted with zwiterrionic thiolated fluorouracil. The controlled release of drug was stimulated by 365 nm UV light, which was responsible for cytotoxic effect, immediately after the detachment of the drug from the surface of the nanoparticles (Longley et al., 2003; Agasti et al., 2009).

h. Doxorubicin, a DNA gyrase inhibitor, when attached to the thioctic acid-PEG linker, can then be exploited to tether 30-nm citrate-capped gold nanorods via a hydrazone group (Figure 14.11). This group is hydrolyzed at the reduced pH levels which are only found intracellularly inside the endosomes. Such nanoconjugates possess the ability of inhibiting the growth of drug-resistant breast cancer cells, due to enhanced uptake of particles. This is then followed by doxorubicin release inside the cancer cells due to acidic endosomes, thus circumventing the problem of drug efflux, which is a matter of concern in drug resistance.

i. Therefore, as the cellular uptake of drug increases, the cytotoxicity of cancer cells also increases likewise (Bae et al., 2003; Szakacs et al., 2006). In another instance, a hydrazone attached thiolated doxorubicin was also tethered with gold nanoparticles and analyzed in tumor cylindroids, a 3-D *in vitro* cancer model. Cationic gold nanoparticles exhibited higher cellular uptake as compared to anionic ones. Doxorubicin release was studied using fluorescence studies, but cytotoxicity was meager (Kim et al., 2010; Kasinskas and Forbes, 2006).

2. Noncovalent attachment of drug leads to release of active drugs directly within the cellular realm, but premature delivery is the biggest drawback. The noncovalent linkage between gold nanoparticles and prodrug is possible via glutathione linker, which is a nonenzymatic method for drug release (Figure 14.12) (Anderson, 1998; Sies, 1999; Jones et al., 2000).
The difference in the intracellular glutathione levels, that is, 1−10 mM

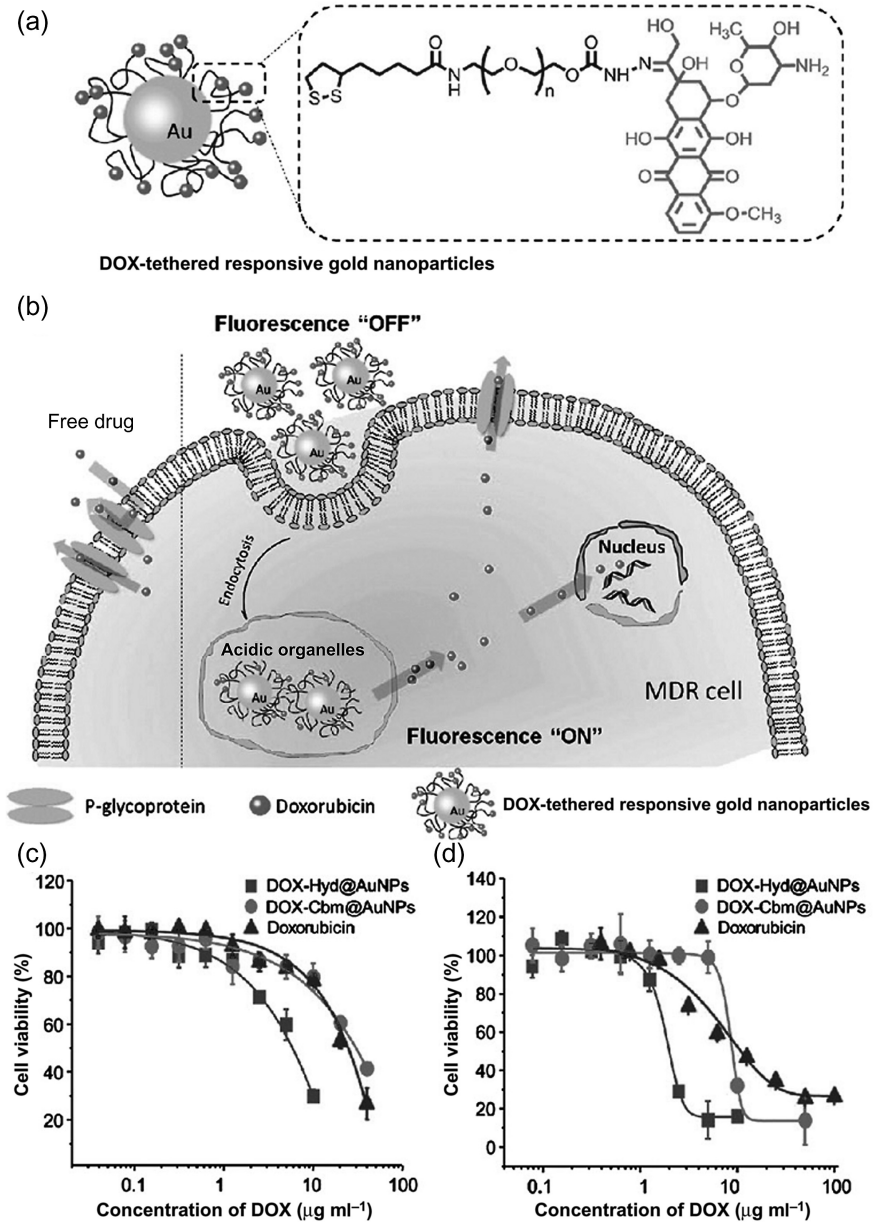

FIGURE 14.11

(a) Structure of gold nanoparticles covalently bound to doxorubicin through an acid-labile hydrazone linkage. (b) Uptake of nanoparticles and release of doxorubicin from endosomes. (c) Cell viability of drug-resistant breast cancer cells after 24 h. (d) Viability after 48 h.

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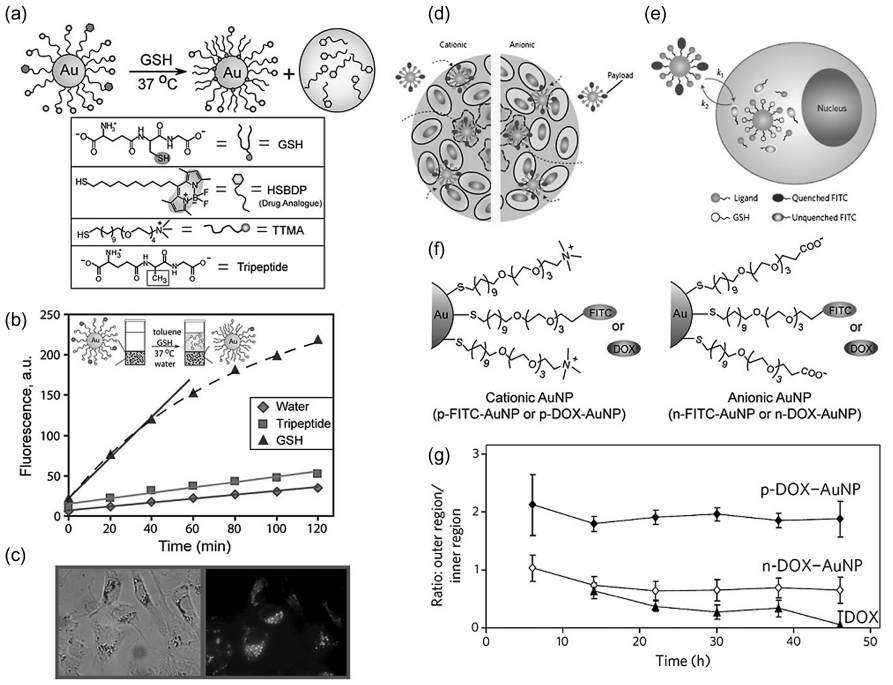

FIGURE 14.12

(a) Schematic illustration of GSH-mediated surface monolayer exchange reaction/payload release. (b) GSH-mediated release of Bodipy ligands in cuvette measured by fluorescence in toluene phase. The slopes were 2.5 (initial period), 0.33, and 0.24 for GSH, tripeptide, and water, respectively. (c) Bright field and fluorescence images of MCF-7 cells incubated with the above AuNPs displaying GSH-controlled release of the fluorophore. (d) The schematic of delivery of payloads into tumor cylindroids. Viable cells are shown by regular shape with solid boundary and necrotic cells by irregular shape with a dashed boundary. Cells containing FITC-SH are shown in green. (e) Cellular uptake and FITC-SH release by intracellular GSH. The rates of particle uptake and FITC release in extracellular regions are represented by k1 and k2, respectively. (f) FITC and doxorubicin (DOX)-loaded cationic and anionic AuNPs used in these studies. (g) The ratio of average fluorescence intensities in the outer region to those in the inner region of the cylindroids as a function of time.

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concentration, as compared to the extracellular thiol levels, that is, 2 μM glutathione and 8 μM cysteine, creates a concentration gradient and hence numerous disulfide-based drug carriers are dependent on such mechanisms (Jones et al., 1998; Bajaj et al., 2008; Khemtong et al., 2009). But the most exigent task is the disulfide exchange with cysteine of serum proteins. Gold nanoparticles forming an interfacial association with the thiols create a steric

shield, thus proving to be stable against this exchange. To demonstrate that GSH is involved in cellular delivery and release of a hydrophobic dye (Bodipy) inside the cells, it was demonstrated that the cationic gold nanoparticles easily cross the membranous barrier. The release of dye was corroborated from the fluorescence-quenching property of gold nanoparticles. GSH-mediated release of the dye can be studied by monitoring the regeneration of fluorescence when the dye is released from gold nanoparticles in cuvette or in human liver cells (HepG2). GSH-mediated release of the payload was further studied by using glutathione monoester, which is used as an external stimulus for releasing the thiol-linked dye. It is worth mentioning that the release of dye or drug conjugated to AuNPs via biogenic thiols is possible due to the cationic surface charge on NPs, which is more sensitive than anionic analogs (Hong et al., 2006; Sapsford et al., 2006; Chompoosor et al., 2008).

14.4.2 CARBON NANOTUBES

There are different types of novel drug-delivery systems, which are of fundamental importance for improvement of pharmacological properties of many therapeutic substances. Carbon nanomaterials are one such proficient armamentarium for transportation and translocation of therapeutic macromolecules such as bioactive peptides, proteins, nucleic acids, or small organic molecules such as drugs. These nanocontainers are exploited as delivery systems to translocate cargos inside organs and cellular monarchy. Carbon materials such as carbon nanotubes (single-walled carbon nanotubes and multiwalled carbon nanotubes) possess high surface-to-volume ratio, long chain of carbon having space for functionalization, lower toxicity, and a plethora of drug/gene tethering properties.

Carbon nanotubes are produced from the rolling of graphene sheets and hence develop unparalleled physical, chemical, and biomedical properties. They possesss diameters of 1−2 nm and length ranging from 50 nm to 1 cm. Single-walled carbon nanotubes (SWNTs) are flexible one-dimensional tubes which bend to allow multiple binding sites of a functionalized nanotube to one cell. These SWNTs exhibit multivalence effect and enhanced binding affinities of nanotubes which binds with their targeting ligands. All the atoms are mostly exposed on the surface of carbon nanotubes, hence they possess extremely high surface area (theoretically 1300 m^2/g), thus causing efficient loading of multiple molecules along the length of the nanotube. Moreover, aromatic molecules can be easily bound to the polyaromatic surface of nanotubes which is caused due to $\pi-\pi$ stacking of those molecules. SWNTs are semiconducting in nature, with small band gaps of the order of 1 eV, thus exhibiting photoluminescence in the NIR range. Thus they have their applications in photothermal therapy and photoacoustic imaging. This NIR emission of SWNT is responsible for the absorption by the biological tissue. SWNTs possess Raman sugnatures for Raman detection and

imaging due to their large scattering cross-sections. Hence their intrinsic characteristics make them useful for therapeutics and imaging.

MWNTs possess large diameters as compared to SWNTs since they possess multiple layers of graphene sheets. Since the size of MWNTs are more, their application in the delivery of biomolecules, such as plasmid DNA into cells, is something which can be exploited.

All the above applications of SWNTs and MWNTs are possible only when they are properly functionalized, since nonfunctionalization leads to their toxicity towards cells and organisms. Moreover, nonfunctionalized CNTs are water-insoluble and cannot be utilized for any biomedical applications. Surface functionalization is pertinent since they tend to make CNTs water-soluble, less toxic, and efficient in biodistribution and biocompatibility. Surface functionalization is of two types: covalent and noncovalent (Liu et al., 2009).

1. *Covalent functionalization of CNTs* involves mostly oxidation, which is carried out by oxidizing agents such as nitric acid (Figure 14.13) (Niyogi et al., 2002; Rosca et al., 2005). In this process, carboxyl groups are produced at the end as well as at the defects of sidewalls of CNTs. There are reports where sp3 carbon atoms are found on the surface of SWNTs after the oxidation process and then forms covalent linkage with amino acids (Zeng et al., 2008). Such oxidized CNTs are soluble in water, but they tend to aggregate in high ionic strength due to charge screening effects. This makes it difficult to be exploited in high salt concentrations of biological suspensions. Hence, to protect from such high salt content, such oxidized CNTs must be covalently functionalized by polyethylene glycol. This conjugate can then be utilized both for *in vivo* as well as *in vitro* applications.

   Cycloaddition reaction is another covalent functionalizion method of CNTs occurring on the aromatic sidewalls and not on the ends or defects of nanotubes. This is possible by the photochemical reaction between azides and CNTs or via Bingel reaction (Lee et al., 2005; Moghaddam et al., 2004; Coleman et al., 2003). There are recent reports on 1,3-dipolar cycloaddition reaction on CNTs (Georgakilas et al., 2002; Tagmatarchis and Prato, 2004). Condensation of α-amino acids and an aldehyde leads to the formation of an azomethine-ylide on the graphitic surface. This leads to the formation of a pyrrolidine ring which couples to the CNT sidewall. Such amino-terminated PEG can be exploited for further interaction with drugs or genes (Pantarotto et al., 2004a; Pastorin, et al., 2006).
2. *Noncovalent functionalization* of CNTs is possible by their interaction with amphiphilic surfactants or polymers. The ultrasonication treatment involved during noncovalent binding of such molecules does not disrupt the π-network of CNTs. Hence such water-soluble CNTs can be utilized for biomedical purposes. Pyrene derivatives have been functionalized noncovalently on the surface of CNTs due to $\pi-\pi$ stacking of such aromatic molecules on polyaromatic CNTs (Chen et al., 2001; Wu et al., 2008). There are reports on protein immobilization (Chen et al., 2001) and glycodendrimer conjugation

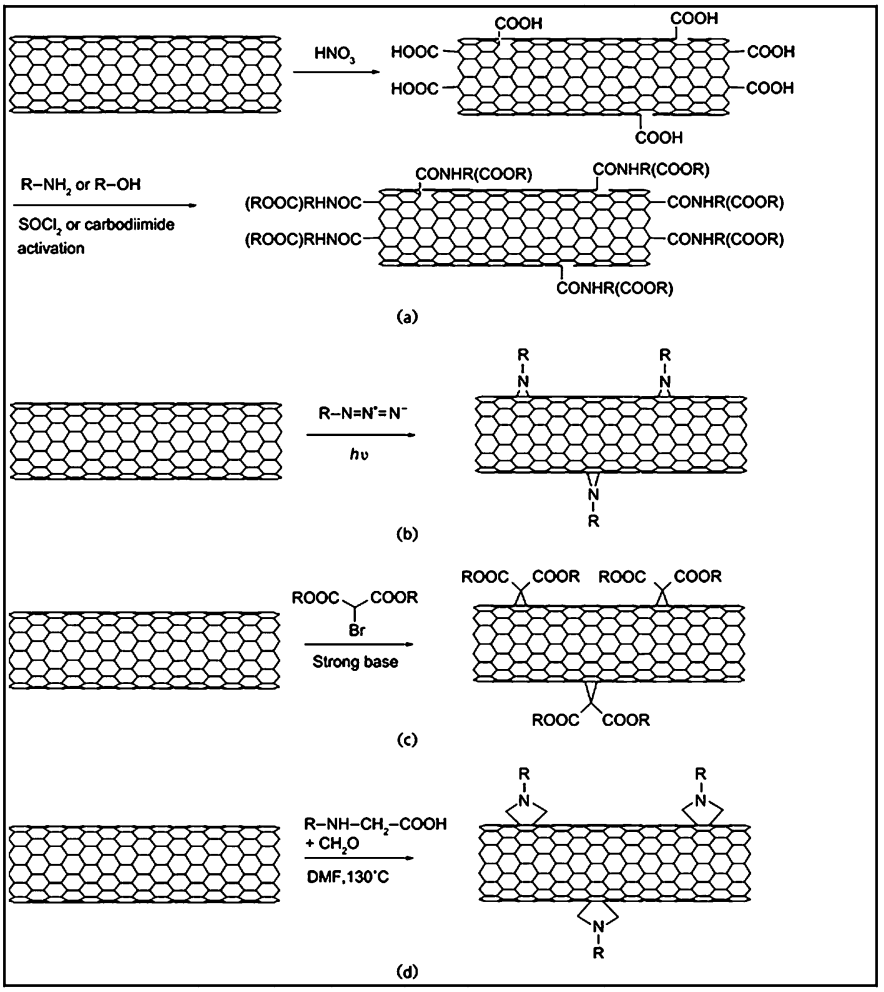

FIGURE 14.13

Schemes of covalent functionalization of carbon nanotubes: (a) CNTs are oxidized and then conjugated with hydrophilic polymers (e.g., PEG) or other functional moieties; (b) photoinduced addition of azide compounds with CNTs; (c) Bingel reaction on CNTs; (d) 1,3-dipolar cylcoaddition on CNTs. For biological applications, "R" in the figure is normally a hydrophilic domain which renders CNTs water-soluble. Further conjugation of bioactive molecules can be applied based on such functionalizations.

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(Wu et al., 2008), as well as ssDNA binding on such pyrene functionalized CNTs (Kam et al., 2005b; Zheng et al., 2003; Tu and Zheng, 2008). However, there is a report on DNA functionalized SWNTs which are unstable in biological environments against nucleases (Moon et al., 2008). Fluorescein

(FITC) terminated PEG chains are capable of solubilizing SWNTs, hence they can be utilized for biological imaging due to their visible fluorescence. Aromatic molecules, such as porphyrin moieties, can also be exploited for noncovalent functionalization (Moon et al., 2008). There are different types of amphiphiles which can be used for suspending CNTs in water, with hydrophobic domains interacting with nanotube surface via van der Waals forces and hydrophobic effects, while the polar heads for solubilization with water. Amphiphiles such as Tween-20 and a Pluronic triblock polymer are exploited for nonspecific binding of proteins on their surface when CNTs are used for biosensor applications. But Pluronic tri-block coating is not that stable *in vivo* since serum proteins are attracted towards such Pluronic-coated SWNTs and replace the surfactant making CNTs highly unstable. There are other surfactants such as sodium dodecyl sulfate and Triton X-100 which can be used for suspension of CNTs in water (Wang et al., 2004). CNTs are found to be more stable when conjugated with phospholipids (PL) and PEG, since phospholipids are a part of cell membrane, hence they can be used for biological systems, while PEG imparts a hydrophilic coating on the surface of CNTs. Hence, such PL-PEG functionalized CNTs are highly water-soluble and biocompatible and can be exploited for a variety of biomedical applications such as imaging and drug (Liu et al., 2007a,b,c; Kam et al., 2005b; Welsher et al., 2008) (Figure 14.14).

Both covalent as well as noncovalent functionalization of CNTs prove to be successful in decreasing the toxicity of CNTs and can easily enter the cells. The mechanism of cellular entry is either receptor-mediated endocytosis or passive diffusion. CNTs are considered to be efficient shuttle vectors for transportation of biomacromolecules such as DNA/RNA or small molecules such as drugs. Once the conjugates deliver their cargoes, CNTs exit from the cells via exocytosis.

14.4.2.1 Delivery of siRNA/DNA

Carbon nanotubes after undergoing modification with positive charges attain the ability to interact with DNA plasmids for the transfection of genes (Pantarotto et al., 2004b; Liu et al., 2005; Singh et al., 2005; Gao et al., 2006). Both amine-terminated SWNTs and 1,3-dipolar cycloaddition-linked MWNTs have been used to tether DNA plasmids for enhancing the efficiency for transfection of DNA plasmids (Pantarotto et al., 2004b; Singh et al., 2005). In another research, oxidation of MWNTs is followed by amino orchestration and then binding of DNA plasmid and then later transfection. This is helped in successful expression of green fluorescence protein (GFP) in the cells. The transfection efficiency was less than the commercially available agents, such as lipofectamine 2000, but MWNTs showed less toxicity (Gao et al., 2006). In another work, polyethylenimine-grafted MWNTs were exploited for DNA attachment and its delivery (Liu et al., 2005). Small interfering RNAs (siRNA) are considered to silence specific expression of

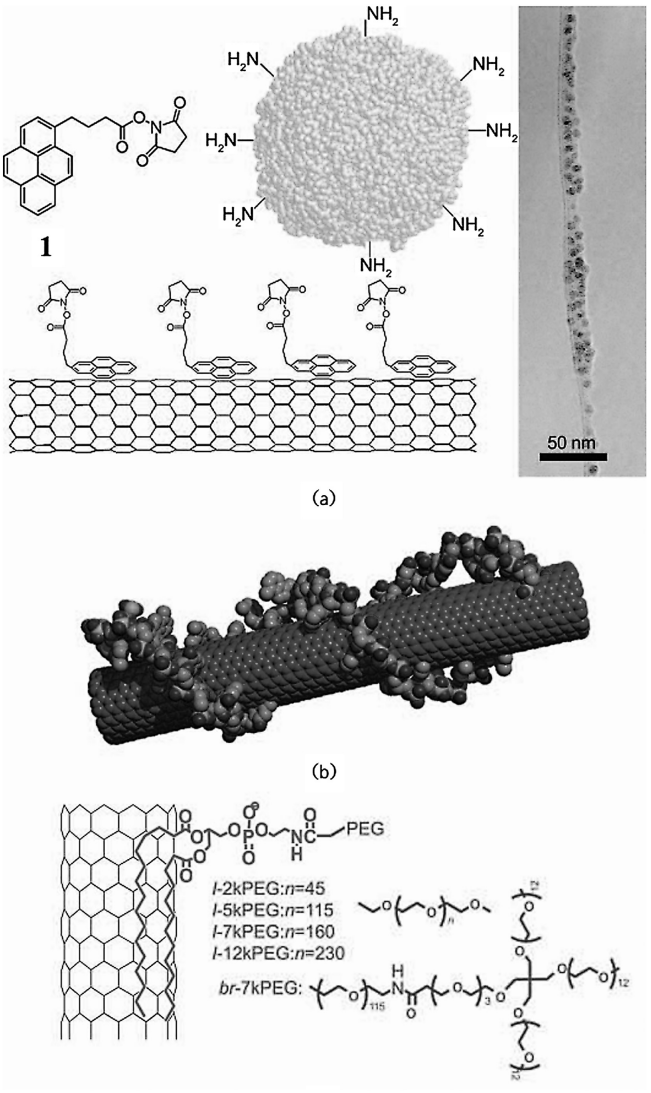

FIGURE 14.14

Schemes of noncovalent functionalization of carbon nanotubes. (a) Proteins are anchored on the SWNT surface via pyrene $\pi-\pi$ stacked on a nanotube surface. Right: A transmission electron microscope (TEM) image of an SWNT conjugated with proteins. (b) An SWNT coated by a single-stranded DNA via $\pi-\pi$ stacking. (c) An SWNT functionalized with PEGylated phospholipids. Both linear PEG (*l*-PEG) or branched PEG (*br*-PEG) can be used in this method.

(a) (Adapted with permission from Chen et al. (2001) © 2001 American Chemical Society); (b) (Adapted with permission from Kam et al. (2005b) © 2005 the National Academy of Sciences); (c) (Adapted with permission from Liu et al. (2008b) © 2008 the National Academy of Sciences).

genes via RNA interference (Mello and Conte, 2004). Viral vectors for siRNA delivery have shown some safety concerns, hence there is a need to develop non-viral vectors for their delivery. siRNA, after conjugating with SWNTs via cleavable disulfide linkage, was successfully delivered inside the cells and caused gene silencing (Kam et al., 2005a) (functionalization of carbon nanotubes via cleavable disulfide bonds for efficient intracellular delivery of siRNA and potent gene silencing). Such SWNT-based siRNA delivery helped in transfection into human T cells and primary cells, which are considered too difficult for transfection (Liu et al., 2007c).

Surface functionalization also played an important role for SWNT-siRNA uptake by cells such as long PEG (5.4 kDa), coating as a linker was not that useful as compared to shorter PEG (2 kDa) that exposed enough hydrophobic surface, showed more cellular uptake, and favors siRNA delivery into cells. Short PEG retains some hydrophobicity as the whole nanotube wall is not completely covered, thus helping in binding cells due to the hydrophobic interactions with the cellular membrane domain. Cell binding is a pivotal step towards cellular entry via endocytosis, which can be attributed to the water solubility and biocompatibility of the anoconjugates (Zhang et al., 2006) (Figure 14.15).

14.4.2.2 Delivery of drugs

Carbon nanotubes after surface orchestration with 1,3-dipolar cycloaddition can then be used for conjugating with fluorescent dyes and drugs such as anticancer, antibacterial, and antifungal drugs (Feazell et al., 2007; Wu et al., 2005; Pastorin et al., 2006). In another instance, phospholipid pegylated SWNTs are exploited for attaching a platinum complex (IV), a prodrug that can be endocytosed by cancer cells. After receptor-mediated endocytosis, the complex can then enter inside endosomes, where reduced pH stimulates reduction of Platinum (IV) to Platinum (II). This causes cytotoxicity of cancer cells. In a similar fashion, paclitaxel was also tethered to branched PEG-coated SWNTs through a cleavable ester bond and was tested for their cytotoxicity both *in vitro* and *in vivo* (Liu et al., 2008a). Doxorubicin was also loaded onto PEGylated SWNTs with an extremely high loading rate due to the extremely high surface area of nanotubes (Figure 14.15). The binding of DOX on nanotubes is a pH-dependent mechanism and the release takes place inside endosomes or lysosmes or the milieu of tumor. The supramolecular approach has unlocked many avenues for drug loading on CNTs (Liu et al., 2007b).

There are a plethora of synaphic ligands which have been used for targeting cancer cells both *in vitro* and *in vivo*, such as folic acid (Dhar et al., 2008), peptides (Liu et al., 2007a), and antibodies (Welsher et al., 2008; McDevitt et al., 2007). Folic acid has been used to conjugate with Pt(IV), a prodrug compound, which further interacts with SWNTs. This complex showed enhanced cytotoxicity towards folate receptor (FR)-positive cells, as compared to FR-negative cells due to synaphic delivery (Dhar et al., 2008), Moreover, for targeted delivery of DOX, the drug has been attached onto the surface of nanotubes

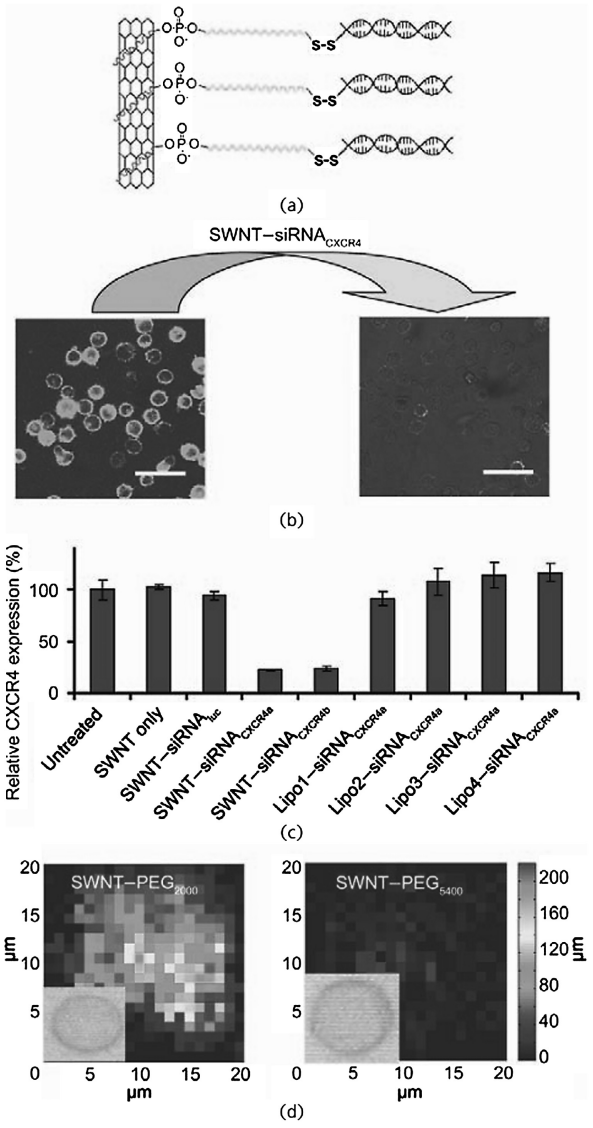

FIGURE 14.15

siRNA delivery by carbon nanotubes: (a) a scheme of SWNT siRNA conjugation via disulfide linkage; (b) confocal images of untreated cells (left) and SWNT siRNACXCR4-treated cells (right) after PE-anti CXCR4 staining. Scale bars: 40 μm. (c) CXCR4 expression levels on CEM cells 3 days after various treatments, including four types of liposomes (Lipo1−4) and luciferase (Luc) siRNA control; (d) G-mode Raman intensity maps of single CEM cells after incubation for 1 day in SWNTs functionalized by PL-PEG 2000 (left) and PL-PEG 5400 (right) chains respectively. Inset: optical microscope images of CEM cells.

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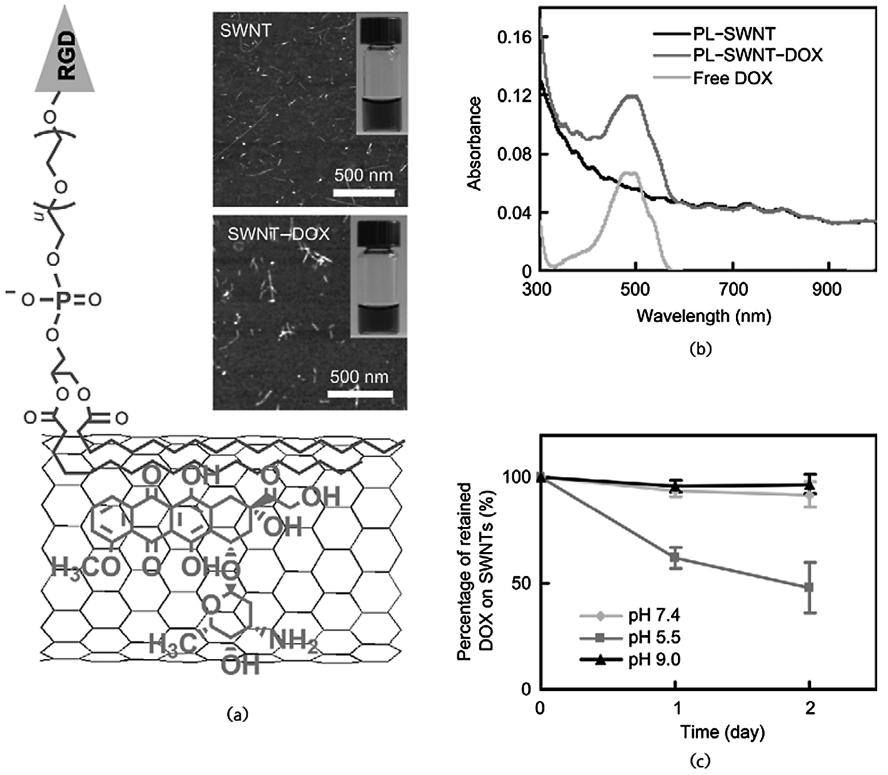

FIGURE 14.16

Supramolecular chemistry of functionalized SWNTs for efficient drug loading and delivery. (a) Schematic of doxorubicin (DOX) π-stacking onto a nanotube prefunctionalized by PL-PEG. Targeting ligands such as RGD peptide can be conjugated on the PEG termini for targeted drug delivery. Inset: AFM images of SWNT before (top) and after (bottom) DOX loading. The height of SWNTs increased after DOX loading. (b) UV vis NIR absorbance spectra of solutions of free doxorubicin (green), plain SWNTs (black), and DOX loaded SWNTs. (c) Release curves of DOX from SWNTs at different pH. DOX loaded on SWNTs is stable at basic and neutral pH with very slow release but exhibits faster release in acidic environments.

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through π−π stacking and moreover the targeting is possible via Arg-Gly-Asp (RGD) peptide (Figure 14.16) (Liu et al., 2007b). These drugs not only bind on the surface but also get encapsulated inside the hollow structure of the carbon nanotubes. A large number of molecules are encapsulated, such as fullerene balls (Kataura et al., 2001), metal ions (Jeong et al., 2003), metallocenes (Li et al., 2005), and even DNA (Kaneko et al., 2007). But drug release is rarely reported by such an encapsulation method done by CNTs.

14.4.3 SUPERPARAMAGNETIC NANOPARTICLES

Magnetic materials at nanoscale possess various biomedical applications due to their unique physical properties at the cellular and molecular levels of the biological interface. They are an efficient theranostic agent since they are considered to be good for therapeutic purposes, as well as for magnetic resonance (MR) contrast imaging (Corot et al., 2006; Pedro et al., 2003). They have been exploited for the diagnosis and treatment of cancer (Ferrari, 2005), cardiovascular diseases (Wickline et al., 2007), and neurological diseases (Corot et al., 2004). The size, shape, surface charge, surface chemistries, and composition can be tailored for such nanoparticles so that their magnetic properties are improved and hence can be used proficiently for theranostic purpose, both *in vivo* as well as *in vitro* (Tartaj, 2003; Gupta and Gupta, 2005).

As a contrast agent such magnetic nanoparticles have been commercially used since they enhance the proton relaxation of specfic tissues, serving as MR contrast imaging agents. The commercially available materials are Lumiren® and Gastromark® for bowel contrast imaging, Endorem® and Ferridex IV® for liver/spleen imaging, etc. (Wang et al., 2001; Bonnemain, 1998). As a treatment agent, these nanoparticles have been utilized as a magnetic drug-targeting agent (Senyei et al., 1978; Neuberger et al., 2005) as well as an active targeting agent (Torchilin, 2006; Zhang et al., 2002; Veiseh et al., 2005). Other nanoparticles possess the defect of lack of targeting strategies, hence, when they are biodistributed systemically after conjugating with drugs, they are intrinsically inefficient to reach towards their target. But magnetic nanoparticles can be targeted towards cancer cells due to the external magnetic field. This reduces the deleterious side-effects and drug dosage due to synaphic delivery of drugs.

Magnetic nanomaterials possess the ability to penetrate the magnetic field through human tissue and manipulate in such a way that they can be exploited for medicinal purposes (Mourino, 1991). The material's magnetic property is symbolized by its magnetic susceptibility (χ), that is the ratio of induced magnetization (M) to the applied magnetic field (H). At a nanoscale of the order of tens of nanometers, ferri- or ferromagnetic materials possess a large single magnetic domain having a large magnetic moment. In the presence of an external magnetic field, this single magnetic domain having a magnetic moment is aligned parallel to the applied magnetic field (H). But at high temperatures (blocking temperature T_B) (37 °C) and in the absence of an external magnetic field, thermal fluctuations can stimulate free rotation of the particles, thus leading to a loss in net magnetization. Hence this superparamagnetic property, which renders nanoparticles without any remnant magnetization after the removal of external fields, helps them to maintain their colloidal stability and prevents any agglomeration with the vascular proteins. Moreover, these superparamagnetic particles possess individual magnetic domains and their coupling interactions leads to higher magnetic susceptibilities.

Superparamagnetism is a favorable property but the reduction of size also comes with a disadvantage, that as the size of the particle decreases, the

surface-to-volume ratio increases leading to manifestions of surface effects, that is, spin canting, noncollinear spins, that affect the overall magnetic properties (Lu et al., 2007).

Recently, there have been reports in which magnetization of iron oxide nanoparticles (Fe_3O_4, magnetite, and Fe_2O_3, maghemite) have been increased by doping them with ions such as cobalt, manganese, zinc, nickel, and iron itself. $MnFe_2O_4$ is nontoxic *in vitro* and has higher magnetic susceptibility than iron oxide nanoparticles. Both cobalt and nickel ferrites, though they possess *in vivo* toxicities, possess unique MR imaging properties. Furthermore, iron oxide nanoparticles are also coated with Fe, where iron oxide acts as a core and Fe as shell so that the magnetization value increases from 30 to 50 emu/g for plain iron oxide nanoparticles to Fe/Fe_3O_4 core–shell nanoparticles having 102 emu/g. In another instance, Pt, Au, and Ag have been used for coating the surface of iron oxide nanoparticles. FePt has been used to bind DNA and protein on its surface (Gao et al., 2007a,b), while Au/Fe_3O_4 core–shell nanoparticles have been utilized to improve the biocompatibility of iron oxide nanoparticles and bind anticancer drugs for delivery inside cancer cells (Presa et al., 2007) (Figure 14.17).

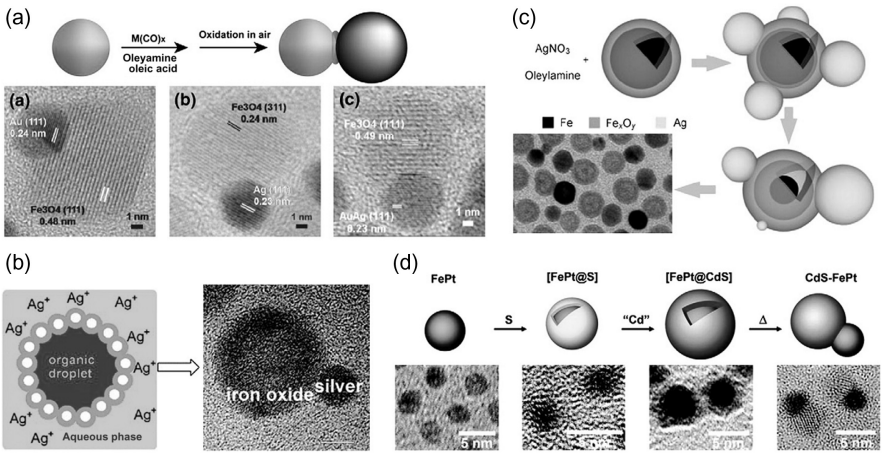

FIGURE 14.17

(a) Schematic illustration of the growth of metal-oxide dumbbell MNPs on pre-made noble metal NPs and high-resolution transmission electron microscope (HRTEM) images of (a) Au–Fe_3O_4, (b) Ag–Fe_3O_4, and (c) Au,Ag–Fe_3O_4 MNPs. (b) Schematic illustration of the growth of Ag-hollow Fe_3O_4 dumbbell MNPs in aqueous phase. (c) Schematic illustration of the growth of Ag-hollow Fe_3O_4 dumbbell MNPs in organic phase. (d) Schematic illustration of the growth of FePt–CdS dumbbell MNPs.

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14.4.3.1 Surface functionalization

Magnetic nanoparticles need to be functionalized by surface ligands such as polymeric coatings, metallic coats such as silica or gold, liposomes, and micelles, since individually they are toxic and tend to be easily recognized and cleared out by the reticuloendothelial system before reaching their target. The effectiveness of magnetic nanoparticles is increased by improving their steathiness by surface functionalization, thus increasing their retention time in blood circulation, so that there is maximum probability of magnetic nanoparticles reaching their target (Chouly et al., 1996; Gref et al., 2000; Moghimi et al., 2001).

14.4.3.2 Delivery of siRNA/DNA

The delivery of genes is unfavorable due to inefficiencies in their transfection, short half-life in blood, being nonspecific, as well as deprived diffusion through cell membranes (Brigger et al., 2002; Juliano et al., 1999). This can be overcome by conjugating magnetic nanoparticles with antisense oligodioxynucleotides (ODNs), which is also known as magnetofection. This has been successfully optimized for *in vitro* applications and now is being tested for *in vivo* applications (Scherer and Anton, 2002; Krotz and de Wit, 2003; Dobson, 2006a,b).

A dendrimer-tethered MNP has been used to transport antisense survivin ODNs to breast and liver cancer cells (Pan et al., 2007). Researchers have demonstrated that positively charged polyamidoamine-coated MNPs, when complexed with ODNs, can downregulate the survivin gene, as well as protein, in 15 min. This consequently led to the inhibition of cellular growth.

Magnetic nanoparticles after surface functionalization act as an important vehicle for delivery of small interfering RNA (siRNA) (Schillinger et al., 2005). There are a plethora of cationic polymers which are coated onto the surfaces of magnetic nanoparticles, along with polyethyleneimine-coated MNPs which have been utilized for the delivery of siRNA. There are reports on NIFR-labeled MNPs, which are covalently bound with siRNA, that can silent GFP production in a GFP-expressed xenograft in a tumor mouse model. The report confirms the *in vivo* tumor uptake of such MNP conjugates due to EPR effect, which can be used for MR imaging purposes. The future endeavor is to exploit such conjugates for therapeutic purposes (Schillinger et al., 2005; Mykhaylyk et al., 2007; Medarova et al., 2007).

14.4.3.3 Delivery of drugs

Chemotherapeutic agents possess the most disadvantageous effects of nonspecificity and augmented side-effects to healthy tissues. This can be circumvented by utilizing magnetic nanoparticles due to magnetic targeting and site-specific delivery of therapeutic cargoes (Pankhurst et al., 2003; Dobson, 2006a,b). To make it simple, the process involves attachment of an anticancer drug on to prefunctionalized MNPs. This conjugate on intravenous injection was followed by application of an external magnetic field gradient to target the nanoparticles on the

pathological site. Due to a hyperthermal effect, the drugs are released onto the desired site. But the execution of such a simple process has been complicated by physicochemical parameters such as field strength and geometry, hemodynamic shear force, depth of the tumor tissue, which play a pivotal role in drug delivery (Dobson, 2006a,b; Neuberger et al., 2005).

Magnetic nanoparticles attached to epirubicin have reached early clinical trials which have been targeted to tumors. Patients tolerate such conjugates, but the most challenging task is the occurence of embolization of blood vessels, due to limited field penetration into tissues, such conjugates cannot be used *in vivo*. There is no proper control of drug diffusion after release and toxicity of magnetic nanoparticles. For the comprehension of the effects of magnetic nanoparticles there is a creation of a mathematical model, which takes care of the hydrodynamics of the blood vessels, particle volumes, magnetic field strength, etc. This model proves that magnetic drug targeting could be useful only on the targets which are close to the surface of the body (Lubbe et al., 1996, 2001; Grief and Richardson, 2005). This study inspired attachment of mitoxantrone on the surface of starch-coated USPIO in VX2 squamous cell carcinomas on the hindlimbs of New Zealand White rabbits. It was demonstrated that such conjugates could completely eradicate tumors after 35 days of treatment (Alexiou et al., 2000; Mornet et al., 2004).

In other instances, traditional drugs such as etoposide, doxorubicin, methotrexate, and cisplatin have been either attached to magnetic nanoparticles or encapsulated inside polymer functionalized MNPs for treatment of malignant prostate and breast tumors (Kohler et al., 2005, 2006; Schulze et al., 2005; Jain et al., 2005). Moreover, there are reports in which poly(ethyl-2-cyanoacrylate) (PECA)-coated magnetic nanoparticles are attached to two different types of drugs, that is, hydrophobic cisplatin and hydrophilic gemcitabine. This helps in controlled release of cisplatin due to its hydrophobicity and rapid release of gemcitabine due to its hydrophilicity (Yang et al., 2006).

14.4.4 MESOPOROUS SILICA NANOPARTICLES

MSNs have certain advantages to be used as drug-delivery vehicles, such as tunable size and shape of the particles, high pore volume and surface area for high drug loading, flexiblity, and possibilities of surface engineering for synaphic delivery and controlled release. Surface engineering helps in preventing unregulated and unwanted biological interactions, thus facilitating bioavailability and evading immunosurveillance mechanisms, thus consequently leading to efficient pharmacokinetic release profiles, and enhanced therapeutics. Silica-based nanoparticles for diagnosis, known as C-dots (Cornell dots), were FDA-approved for the first stage of human clinical trials (Rosenholm et al., 2010a,b; Rosenholm et al., 2011). MSNs also exhibit toxicity depending on the administrative routes.

For example, nonfunctionalized particles, when administered subcutaneously, exhibit less toxicity in comparison to particles delivered intraperitoneally or systemically. Apart from morphological characteristics such as size and shape, other factors such as biological system recognition, coronation (opsonization of proteins on the surface), communication of toxicity between the cells, cell-specific reactions, and biodegradation have their greatest impact on the therapeutic efficacy of drugs (Hudson et al., 2008).

14.4.4.1 Delivery of siRNA/DNA

Naked DNA, siRNA, or mRNA cannot easily undergo cellular uptake since their bioavailability and half-life are much less when *in vivo*. Moreover their negative charge makes them difficult moieties for cellular internalization. Their biological stability is also poor. Further, even though they are internalized by cells, nucleases degrade them, making their nuclear journey almost impossible. Hence nonviral gene delivery, which involves mesoporous silica nanoparticles is the next doorstep, which material scientists would like to stand upon. This is because electroporation, magnetofection, and sonoporation, all possess some or other limitations. But MSNs are beneficial to act as vehicles for gene delivery, due to their narrow pore size distrbution which helps in releasing the plasmid at the point of delivery. Furthermore, plasmid DNA can be maximally adsorbed after surface functionalization of MSNs having cationic charge on the surface. It was found that siRNA and DNA constructs were adsorbed on the surface of MSNs only when a layer of polyethyleneimine (PEI), a known gene transfection agent, was coated on its surface (Xia et al., 2009; Meng et al., 2010). But this will not prevent the nucleases present in the plasma from cleaving the nucleic acids on the surface of MSNs. There are two strategies that can be utilized for enhanced adsorption of DNA on its surface and preventing degradation from plasma nucleases. One is optimization of the loading conditions and the other is using MSNs of larger mesopore dimensions. In another report it was demonstrated that mesopores of small-pore MSNs can be used to adsorb short salmon DNA (20−250 bp) in the presence of chaotropic salt conditions (guanidine hydrochloride, 2M, pH 5) and the core was made up of SPIONs (Li et al., 2011a,b). The chaotropic condition was favorable for maximum loading capacity and screening the electrostatic repulsions. There was no desorption of DNA at lower temperatures such as 20 °C, but as the temperature was increased to 37 °C, the physiological temperature inside the biological system, there was complete desorption of plasmid DNA within 1 h. Even siRNA was also tried under the same conditions, but there was negligible adsorption of siRNA. But when adsorption was performed in more dehydrating environments (66.7% ethanol), there was loading of 13.5 mg siRNA/g of MSN. Almost 27.5 mg siRNA/g was loaded at an equilibrium in the pores of MSNs. This was followed by adsorption of 25 kDa PEI for capping the pores. This helped in protecting the siRNA from plasma degradation

as well as degradation inside the A549 cells after internalization, which was confirmed by fluorescent labeling of siRNA. Such MSNs were capable of knocking-down the target genes (Li et al., 2011a,b).

14.4.4.2 Delivery of drugs

There are anticancer drugs which are poorly soluble, highly unstable and in which the cellular uptake is also poor. Moreover, the therapeutic window is also very narrow, due to many side-effects of the drugs (Rosenholm et al., 2011). There is a need for such a delivery system which can have the capacity of a very high payload of drugs and especially hydrophobic drugs. There are previous reports in which DOX when conjugated with silica nanoparticles was used for the treatment of malignant mesothelioma. In another instance, it was demonstrated that PEG-PEI-coated MSNs when conjugated with DOX, exhibited enhanced cellular uptake, thus leading to tumor regression after intravenous administration. Thus this complex showed less systemic, hepatic, and renal toxicity. The particles aggregated at the tumor sites, which is purely due to the EPR effect (Lee et al., 2010; Hillegass et al., 2011). Moreover, active targeting also enhances the tumor-targeting capacity, which was further analyzed by studying the targeted delivery of camptothecin (Lu et al., 2010).

Docetaxel also shows detrimental side-effects due to systemic toxicity of the drug. It was then shown that PEGylated silica nanorattles can be used for the encapsulation of docetaxel and was then subcutaneously tested on a mouse model of liver cancer. Hence MSNs are potential candidates for enhanced bioavailability, efficacy, and reduced side-effects of drugs (Li et al., 2010).

14.5 CONCLUSIONS

A large number of proof-of-concept studies on gene as well as drug delivery using inorganic nanoflotillas have been done both *in vitro* and *in vivo*. The primary focus was delivery to the brain and tumor environment. This has fueled hope amongst medical scientists for improved therapeutics and synaphic delivery for circumventing the side-effects induced by drugs and genes. The selection of an optimized delivery system using such inorganic nanoparticles with surface orchestrations based on targeted delivery of drugs and genes consequently leads to a battle won for clinical trials. The nanoparticulate system was not only compared with the free drug or free siRNA/DNA, but also to the conventional drug delivery systems that constitute desired drugs or genes for navigation of reaserch within the pharmaceutical drift. Parametric optimization of the delivery system per se, for the comprehension of size and orchestrations, needs to be paralleled with incorporation of novel targets, novel molecular markers, and novel therapeutic agents for the achievement of high efficiency in the treatment of diseases.

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