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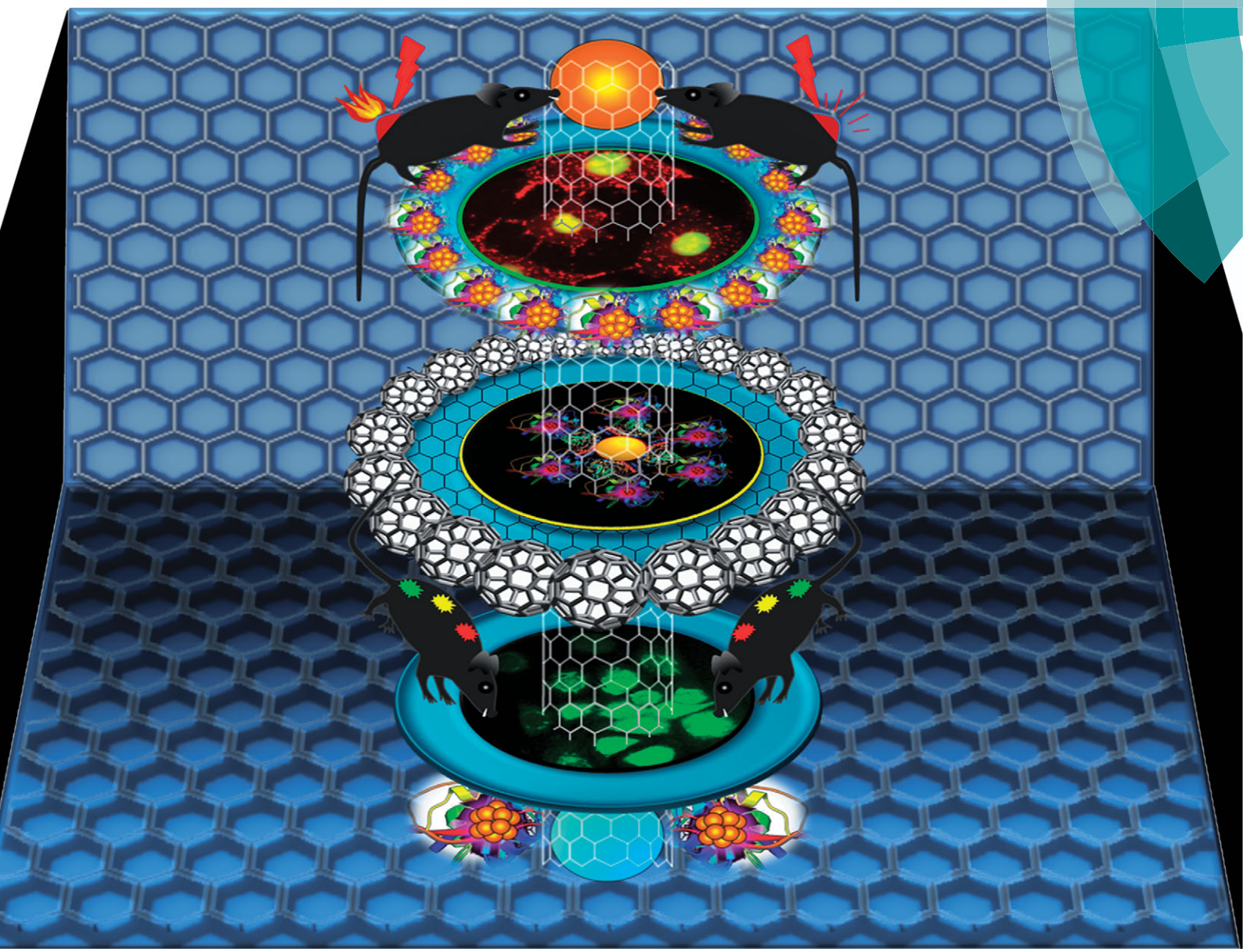

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TUTORIAL REVIEW

Vasudevanpillai Biju

Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy

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Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy

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As prepared nanomaterials of metals, semiconductors, polymers and carbon often need surface modifications such as ligand exchange, and chemical and bioconjugate reactions for various biosensor, bioanalytical, bioimaging, drug delivery and therapeutic applications. Such surface modifications help us to control the physico-chemical, toxicological and pharmacological properties of nanomaterials. Furthermore, introduction of various reactive functional groups on the surface of nanomaterials allows us to conjugate a spectrum of contrast agents, antibodies, peptides, ligands, drugs and genes, and construct multifunctional and hybrid nanomaterials for the targeted imaging and treatment of cancers. This tutorial review is intended to provide an introduction to newcomers about how chemical and bioconjugate reactions transform the surface of nanomaterials such as silica nanoparticles, gold nanoparticles, gold quantum clusters, semiconductor quantum dots, carbon nanotubes, fullerene and graphene, and accordingly formulate them for applications such as biosensing, bioimaging, drug and gene delivery, chemotherapy, photodynamic therapy and photothermal therapy. Nonetheless, controversial reports and our growing concerns about toxicity and pharmacokinetics of nanomaterials suggest the need for not only rigorous *in vivo* experiments in animal models but also novel nanomaterials for practical applications in the clinical settings. Further reading of original and review articles cited herein is necessary to buildup in-depth knowledge about the chemistry, bioconjugate chemistry and biological applications of individual nanomaterials.

Key learning points
- Large surface area and hidden space in nanomaterials
- Chemistry goes common on the surface of nanomaterials
- New horizons for drug and gene delivery using nanomaterials
- Chemistry of nanomaterials opens up corridors for nanomedicine
- Chemical tools for surfing and skiing around nanomaterials

1. Introduction

The size-dependent tunable electronic, optical, magnetic and mechanical properties of nanomaterials are fundamental to the current excitement and growing applications of nanomaterials. The most attractive nanomaterials are derived from silica, noble metals, III–VI and IV–VII semiconductors, lanthanides, metal oxides, polymers, and carbon. Among such nanomaterials, silica nanoparticles, gold nanoparticles and quantum clusters, semiconductor quantum dots, upconversion nanoparticles, polymer nanoparticles, carbon nanotubes, nanodiamonds, fullerenes, and graphene are subjects of fundamental research, device technology and biomedical technology. Combinations among fluorescence, magnetic resonance, positron emission, photothermal and photoacoustic effects, surface plasmon, and Raman and surface enhanced Raman scattering (SERS) provided by the above nanomaterials enable us to resolve various problems in biology and medical science. Furthermore, the abilities of nanomaterials to accommodate multiple functional groups, targeting biomolecules, drugs, and genes allow one to use them for not only the detection of the structures and functioning of subcellular organelles and biomolecules but also the targeted imaging and treatment of various cancers. Synthesis of colloidal nanoparticles with well-controlled size- and shape-distributions, well-defined surface chemistry, and unique optical and electronic properties is the basis of all such biological applications

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of nanomaterials. The large surface to volume ratios of nanomaterials and their surface modifications using various chemical and bioconjugate reactions have moved the interface among chemistry, materials science and biology towards biomedical science. Recent research trends at this interface show focusing effects on the *in vitro* and *in vivo* applications of nanomaterials, such as multiplexed detections, biosensing, multimodal bioimaging, gene and drug delivery, and cancer chemotherapy and phototherapy (Fig. 1). Formulation of nanomaterials into nanomedicines can cosset the common side effects such as toxicity and multidrug resistance in conventional chemotherapy. This review provides an introduction to newcomers about the chemical and bioconjugate formulations of nanomaterials for applications such as biosensing, bioimaging, drug and gene delivery and cancer therapy. Further reading of original articles in this area is highly recommended.

2. Silica nanoparticles

Silica nanoparticles can be classified into solid or non-porous and mesoporous. Owing to the straightforward and size-controlled synthesis, chemical and physical stabilities, large surface area, hydrophilic surface and well-defined surface chemistry, silica nanoparticles have become common platforms for various catalytic and chemical reactions, and bioimaging and drug and gene delivery applications. The same physico-chemical properties that make these materials ideal for the above applications also make them suitable as a protecting shell material for a spectrum of nanomaterials. Such silica shells not only render nanomaterials with hydrophilic, biocompatible and chemically active surfaces but also protect them against chemical and biochemical degradations, release of toxic ions, and activation of immune response. The Stöber process, which is the controlled hydrolysis of silyl ethers such as tetraethyl silicate into silanols using ammonia in a mixture of water and alcohol followed by the condensation of silanols into 50–2000 nm silica particles, is the basis of silica nanoparticle preparation. In this preparation, the size of silica particles can be controlled by either varying the concentrations of silyl ether and alcohol, or the normal or reverse microemulsion method, which are modifications of the Stöber process.[1] The reverse microemulsion method is the ammonia-catalyzed polymerization of silyl ethers loaded in a microemulsion. Mesoporous silica nanoparticles are synthesized by the sol–gel process, which is the *in situ* polymerization of silyl ethers stabilized in amphiphilic templates provided by surfactants such as cetyltrimethylammonium bromide (CTAB).[1] Aggregation of silica nanoparticles during the synthesis can be prevented by setting the concentration of silyl ether at a low level or by the addition of non-ionic surfactants, polymers, triethanolamine or propanetriol. Finally, the templates are removed by solvent extraction, thermal decomposition, dialysis or oxidation. The pore-size of mesoporous silica nanoparticles thus synthesized can be controlled by varying the pH of the solution and composition of the solvents, and the addition of certain swelling agents.

Fig. 1 Examples of nanomaterials and their functional groups for biological applications.

Vasudevanpillai Biju

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2.1. Surface functionalization and bioconjugation of silica nanoparticles

As-synthesized silica nanoparticles by the standard procedures discussed above own highly hydrophilic surfaces, which make

them one of the friendliest nanomaterials for biomedical applications such as drug and gene delivery, bioimaging and therapy. However, assorted surface functional groups are often necessary for the conjugation of various biomolecules, contrast agents and drugs to the surface as well as inside the pores of silica nanoparticles. Reactive functional groups such as a primary or a secondary amino, carboxyl, hydroxyl, alkyl halogen, or azide group can be introduced onto the surface of silica nanoparticles by either the co-condensation process during the preparation or by post-synthesis surface modification.[2] Although co-condensation provides uniform distribution of such functional groups, post-synthesis surface modification is preferable for maintaining the size-distribution and morphology of silica nanoparticles during the synthesis. In post-synthesis surface modifications (Fig. 2), various trialkoxysilanes are condensed with the surface silanol group. 3-Aminopropyl tri(m)ethoxy silane and 3-mercaptopropyl tri(m)ethoxy silane are extensively employed in such condensation reactions, which provide primary amine and thiol groups on the surface of silica nanoparticles.[3] The direct condensation of silane-functionalized molecules such as organic dyes, polyethylene glycol (PEG), *etc.* to the silanol group on as-synthesized silica nanoparticles can also be practiced.[3] Amino-functionalized silica nanoparticles can be conjugated with other nanoparticles, small organic molecules, proteins, peptides, nucleic acids, fluorescent dyes, *etc.* by coupling with functional moieties such as carboxylic acids, alkyl halides and NHS esters. These reactions are summarized in Fig. 2. Common coupling agents commercially available for such reactions are 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), diisopropyl carbodiimide (DIC), *N*-hydroxysuccinimide (NHS) esters, [*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate] (HBTU), (*N*-succinimidyl 3-[2-pyridyldithio]-propionate) (SPDP) and sulfo-succinimidyl-(4-*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC).[4] Here, the succinimidyl group in the hetero-bifunctional cross-linkers such as SPDP and sulfo-SMCC reacts with the amino group on silica; whereas, the pyridyldithio and maleimide moieties can be utilized for the conjugation of sulfhydryl groups in thiols and cysteine-bearing proteins. Primary amino groups on silica nanoparticles can also be coupled with aldehydes and ketones to form Schiff's bases, isothiocyanates to form thiourea derivatives, or metal complexes to form chelate bonds. Reactions of surface amino groups on silica with Wilkinson's catalyst and cisplastin are typical examples for chelate bond formation. Isothiocyanates of rhodamine and fluorescein are commercially available agents for the direct fluorescence labeling of amino-functionalized silica nanoparticles. As-synthesized silica nanoparticles are abundant in hydroxyl groups, which can be conjugated with isocyanates to

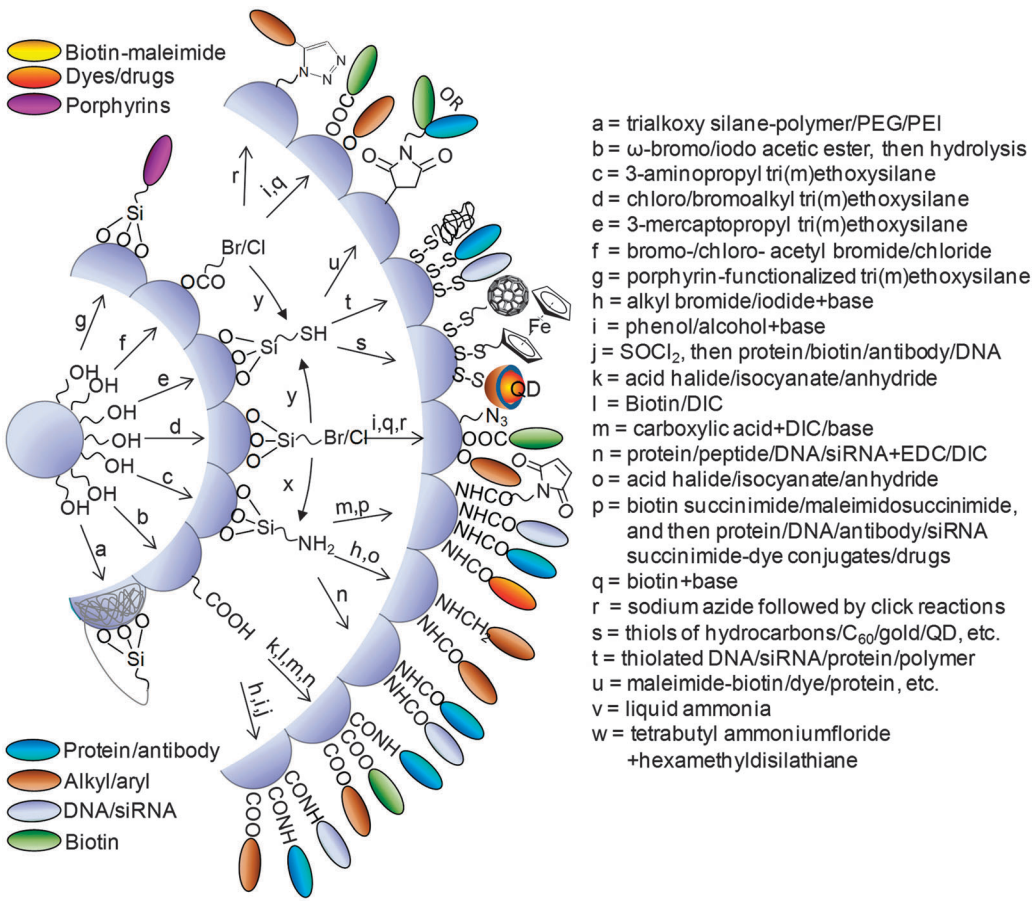

Fig. 2 Common methods for the functionalization and bioconjugation of silica nanoparticles.

form urethanes or carboxylic acids to form esters. The condensation of 3-mercaptopropyltriethoxy silane or its analogues with silanol groups on the surface of silica nanoparticles offers thiol functionalized silica nanoparticles, which can be further conjugated with gold nanoparticles, proteins, antibodies, peptides, fluorophores, polymers, *etc.* by reductive addition, disulfide coupling or maleimide reaction. Thiol functionalized molecules react with thiol groups on the surface of silica nanoparticles to form redox-active disulfide bonds, which are candidates of redox-sensitive drug delivery systems.[5] Similarly, maleimide-functionalized molecules react with thiol groups on the surface of silica nanoparticles to form stable thioether bonds. Introduction of azide groups and the subsequent click chemistry with alkyne substituted molecules is also a versatile method for the functionalization of silica nanoparticles. Other common methods for the functionalization of silica nanoparticles include polymerization by the attachment of functional groups suitable for chain transfer or free radical polymerization. Common methods for the chemical and bioconjugate reactions of silica nanoparticles are summarized in Fig. 2.

2.2. Biological applications of functionalized silica nanoparticles

Their large surface area, versatile functional groups, biocompatibility and porous nature make silica nanoparticles one of the most investigated nanomaterials for applications such as gene and drug delivery, multimodal bioimaging, and photodynamic-, photothermal- and chemo-therapies. Selected biological applications of functionalized silica nanoparticles are summarized in Fig. 3.

Gene and drug delivery. The large surface area, porosity, and flexible surface chemistry of silica nanoparticle make it an ideal host material for the loading of drugs, siRNA, pDNA and contrast agents for bioimaging in the nanomedicine settings.[5,6] Recently, functionalized silica nanoparticles have been extensively used in the conjugation of various cargo molecules such as anticancer drugs, proteins and DNA, and the subsequent site-specific controlled release of the cargo by chemical, charge-based, thermal, magnetic, biochemical or photochemical methods.[5,6] In chemical methods, at first, the amount of cargo physically loaded in silica nanoparticles is maintained with gate-keeper moieties such as nanoparticles, proteins, polymers, rotoxanes, oligosaccharides, liposomes or dendrimers. The site-specific drug delivery by these gate-keepers is controlled using redox-sensitive disulfide bonds or enzyme/pH/conformation-sensitive functional groups.[5,6] Redox-active molecules such as disulfides and tetrathiafulvalenes are excellent gate keepers, which release cargo upon reduction into thiols. A complex between polyethyleneimine and cyclodextrin-based pseudorotaxane loaded in silica nanoparticles is a typical example of pH-sensitive gates, which opens and releases cargo during the protonation of polyethyleneimine. The release of insulin from silica nanoparticles using glucose is a typical example for the release of cargo, which is based on molecular recognition.[5] Redox or pH sensitivity can also be applied without the use of gate-keepers for the release of cargos attached to silica nanoparticles. Photoresponsive derivatives of coumarins, azobenzenes and nitrobenzenes are extensively used in the photochemical delivery of cargos from silica nanoparticles by the 2+2 photocycloaddition reactions of coumarins, *cis–trans* photoisomerization of azobenzes, or photouncaging of nitrobenzene derivatives.[5] These methods work well in the loading and release of antibacterial agents (erythromycin, gentamycin, vancomycin, *etc.*), anti-inflammatory drugs (aspirin, dexamethasone, ibuprofen, *etc.*), chemotherapeutic anticancer drugs (doxorubicin, cisplatin, camptothecin, methotrexate, fluorouracil, *etc.*), and photosensitizer drugs (chlorine e6, Zn-porphyrin, Rose Bengal, *etc.*). Drug delivery systems based on silica nanoparticles are fast-developing nanomedicines with great potential for *in vivo* human use, which require rigorous analysis of the toxicity, biodistribution, clearance, and immune response of silica nanoparticles and their chemical and bioconjugates.

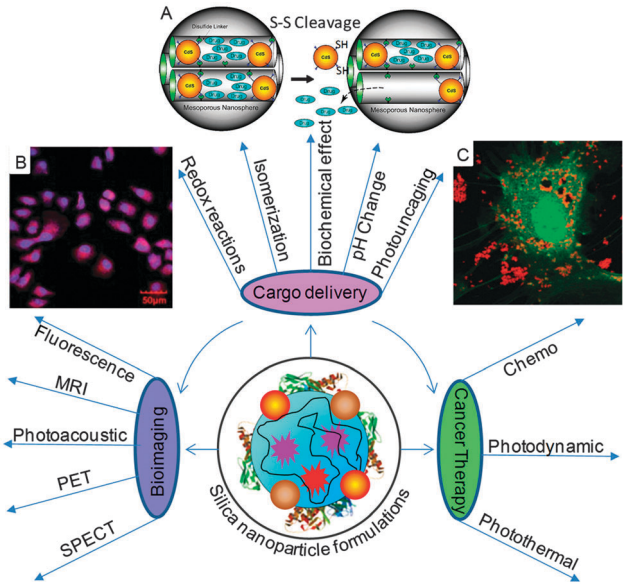

Fig. 3 Biological applications of functionalized silica nanoparticles: (A) drug delivery by the reductive removal of gate-keeper quantum dots, (B) fluorescence image of HeLa cells incubated with doxorubicin- and iron oxide-conjugated fibrous and mesoporous silica nanoparticle, and (C) fluorescence image of a rat neural glia cell in which GFP-is expressed and Texas red-loaded silica nanoparticles are delivered. Reprinted with permission from (A) C.-Y. Lai *et al.*, *J. Am. Chem. Soc.*, 2003, **125**, 4451 (copyright ACS 2003); (B) S. Gai *et al.*, *J. Mater. Chem.*, 2011, **21**, 16420 (copyright RSC 2011); and (C) D. R. Radu *et al.*, *J. Am. Chem. Soc.*, 2004, **126**, 13216 (copyright ACS 2004).

Bioimaging. Examples of bioimaging using silica nanoparticles are vast due to their ability to accommodate fluorescent/MRI/PET/SPECT contrast agents and drug/DNA molecules to their adaptable surface and pores.[7] Chemical modifications of the surface of silica nanoparticles discussed in the previous section can be conveniently chosen during the incorporation of various contrast agents. Fluorescent probes such as fluorescein isothiocyanate, rhodamine isothiocyanate, cyanine dyes, methyl violegen, alexafluor dyes, coordination complexes of ruthenium, terbium and europium, and semiconductor quantum dots can be conjugated to silica nanoparticles and efficiently delivered in different cell lines or injected *in vivo*.[8] Alternatively, fluorescence imaging modality

can be indirectly incorporated in silica nanoparticle by using it as a vector for the expression of fluorescent proteins. Carrier molecules such as transferrin, RGD peptide, epidermal growth factor, Tat-peptide, folic acid and antibodies can be recruited on the surface of such fluorescence-encoded silica nanoparticles for extracellular labeling of or intracellular delivery in selected cell lines.[7] Subsequently, the labeled cells are visualized using one or two photon fluorescence microscopy. Additional imaging modalities such as MRI, PET and SPECT can be brought together with fluorescent molecular tomography (FMT) by the conjugation or adsorption of super paramagnetic Fe_3O_4 nanoparticles, Gd complexes, Gd or Mn-based nanoparticles, ^{18}F, ^{64}Cu, ^{99m}Tc and ^{62}Cu to silica nanoparticles. Recently, the potentials of such multimodal silica nanoparticles for targeted as well as non-targeted *in vitro* and *in vivo* bioimaging are extensively investigated. Silica nanoparticle-based contrast agents conjugated with RGD peptide have been found very useful in the targeted *in vivo* imaging of $\alpha_v\beta_3$ integrin over-expressing melanoma and related lymph node metastasis in mice. Similarly, the conjugation of folic acid enables accumulation of silica nanoparticles is HeLa tumor model *in vivo*. Biodistribution, bioavailability and renal clearance of silica nanoparticle-based imaging probes can be improved by the conjugation of polymers such as PEG and PEI. Highly-charged silica nanoparticles injected *in vivo* first accumulate in the liver and eventually clear by the hepatobiliary excretion; whereas, accumulation of silica nanoparticles with hydroxyl or carboxyl functionalities, large hydrodynamic size and poor surface charge in the reticuloendothelial system continues to be a challenge in the nanomedicine field.

Therapy. The potential of silica nanoparticles functionalized or loaded with anticancer drugs, photosensitizer molecules or photothermal agents for cancer therapy have been extensively investigated in the recent past.[9] As discussed in the previous section, the main advantages of silica nanoparticles in such therapeutic interventions are their biocompatibility, and ability to accommodate high densities of therapeutic agents, ligands or antibodies against cancer markers, and fluorescence/MRI/PET contrast agents. Also, silica nanoparticles are ideal host materials for the controlled release of drugs at desired locations.[5] Because of these advantages, silica nanoparticles are extensively used for *in vitro* and *in vivo* chemotherapy, photodynamic therapy and photothermal therapy of various cancers. Treatment of breast cancer or melanoma cells/xenografts using silica nanoparticles loaded with anticancer drugs such as doxorubicin, methotrexate or camptothecin and cancer targeting molecules such as RGD peptide, folic acid, aptamers, or anti-cancer antibodies has shown remarkable cell death and suppression of tumor growth.[9] Similarly, silica nanoparticles functionalized with photosensitizer drugs such as porphyrins, phthalocyanines or methylene blue combined with one of the above targeting molecules are found to be highly efficient in the photodynamic therapy of cancers.[10] The efficiency of cancer therapy is improved by not only exploiting the large surface area for efficient drug loading but also combining photodynamic therapy with chemotherapy.[9–11]

Silica nanoparticles engineered with photothermal agents such as nanoshells, nanorods or nanoparticles of gold, or carbon nanotubes are promising candidates for photothermal therapy of cancer. Gold-shelled or gold-speckled silica nanoparticles with or without polyethylene glycol coating, cancer targeting molecules and chemotherapeutic agents have been extensively investigated in the treatment of cancers both *in vitro* and *in vivo*.[11] Furthermore, the photothermal effect of gold nanostructures loaded in silica nanoparticles is useful for the thermal release of anticancer drugs at desired locations, which is a promising approach for site-specific treatment of cancers. Combination of different imaging, drug-targeting and treatment modalities in a single silica nanoparticle is one of the most promising and emerging aspects of cancer management in the nanomedicine settings.

3. Gold nanoparticles and quantum clusters

Colloidal gold nanoparticles have been utilized as coloring agents for centuries. The brilliant color and size- and shape-dependent tunable surface plasmon of colloidal gold nanoparticles make them attractive for various applications such as solar energy harvesting, sensing, catalysis, bioimaging, drug delivery and photothermal cancer therapy. Optical and electronic properties of gold nanoparticles for such applications can be tuned by not only varying the size and shape but also their surface chemistry and aggregation state.

Michael Faraday's preparation of colloidal gold by the reduction of gold chloride using phosphorous is the milestone discovery in modern colloidal chemistry. The importance of colloidal gold was realized only in the latter half of 20th century when the preparation of monodispersed gold nanoparticles by the citrate reduction of gold ions was introduced. Although many modifications to the preparation of colloidal gold have appeared since then, the Shiffrin–Brust biphasic synthesis method introduced in 1994 has become popular due to the simple steps and the reagents involved.[12] In this method, thiol-protected gold nanoparticles are formed by the $NaBH_4$ reduction of gold ions transferred from the aqueous phase into an alkane thiol supplemented organic phase using tetraoctyl ammonium bromide. Although several methods for the preparation of hydrophobic- or hydrophilic-capped colloidal gold are recently introduced, stabilization of gold nanoparticles using thiol capping agents reported by Giersig and Mulvaney[13] and the biphasic reduction of Au^{3+} reported by Brust and Shiffrin[12] continue to be the bench-level standards in the colloidal synthesis of gold nanoparticles. Subsequent advancements in this area show that gold nanoparticles with different size and shape such as nanorods, nanocages, nanocubes, *etc.* can be prepared with excellent reproducibility by the seed-mediated growth method reported by El-Sayed and coworkers[14] and by Murphy and coworkers.[15] In the seed-mediated method, gold nanorods are prepared by the seeding of small single crystal gold nanoparticles prepared by the $NaBH_4$ reduction of $HClO_4$ in the presence of CTAB into a growth solution prepared by the mild reduction of $HClO_4$. The aspect ratio of gold nanorods prepared in this method can be well-controlled by changing the seed to gold ion ratio or the concentration of added impurities.

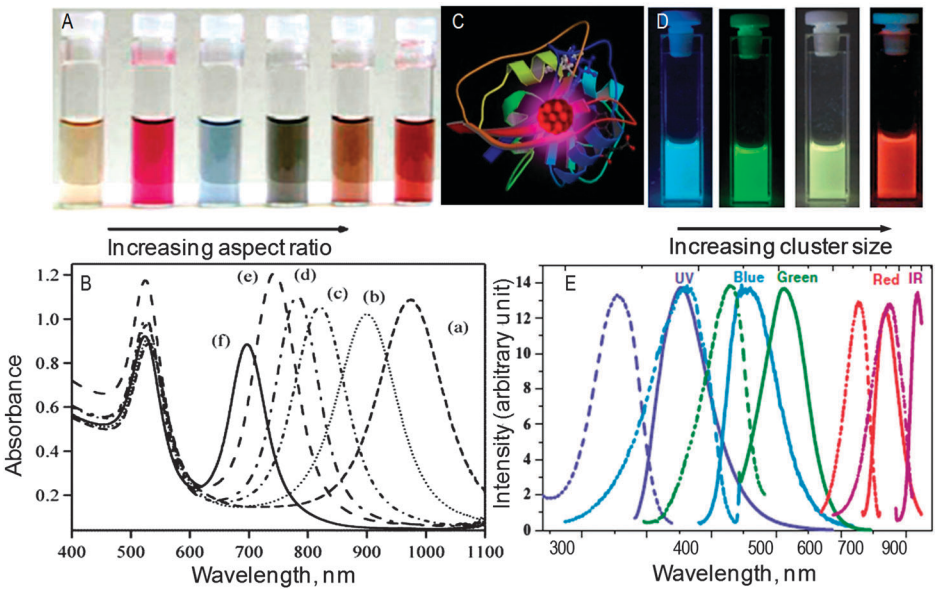

Fig. 4 (A) Photograph of an aqueous solution of 4 nm gold nanospheres (left) and progressively higher aspect ratio gold nanorods. (B) UV-Visible absorption spectra of gold nanorods with different lengths: (a) 108 ± 7 nm, (b) 89 ± 7 nm, (c) 75 ± 6 nm, (d) 73 ± 4 nm, (e) 61 ± 5 nm, and (f) 46 ± 6 nm. (C) Au_{25} quantum cluster in BSA. (D) Photographs of polymer-coated gold quantum clusters having different size under UV excitation. (E) Absorption and photoluminescence spectra of UV (Au_5), blue (Au_8), green (Au_{13}), red (Au_{23}), and near IR (Au_{31}) quantum clusters stabilized in PAMAM dendrimers. Reprinted with permissions from (A) C. J. Murphy *et al.*, *Chem. Commun.*, 2008, 544–557 (copyright RSC 2008); (B) M. Hu *et al.*, *J. Am. Chem. Soc.*, 2003, **125**, 14925–14933 (copyright ACS 2003); (D) S. Palmal *et al.*, Chem.–*Eur. J.*, 2013, **19**, 943–949 (copyright Wiley VCH 2013); and (E) J. Zheng *et al.*, *Phys. Rev. Lett.*, 2004, **93**, 077402 (copyright APS 2004).

Photographs and absorption spectra of colloidal solutions of gold nanorods are shown in Fig. 4.

Subnanometer atomic clusters of gold and silver stabilized in pockets formed by proteins or small organic ligands are other promising candidates for biological applications in the nano-gold family.[16,17] Although these molecule-like clusters were first detected in the gas phase, detailed characterization of their properties, and their biological applications have become possible only recently with the large scale preparation in the organic and aqueous phases.[18] Gold quantum clusters show broad absorption of light in the UV-Vis region and bright Vis-NIR photoluminescence (Fig. 4D and E) due to the 'molecule-like' electronic transitions and the confinement of conduction electrons within the Fermi electron wavelength. Recently, quantum clusters are prepared by the alkaline reduction of Au/Ag ions in the aqueous or organic phase. Ligands such as bovine serum albumin (BSA), glutathione or alkane thiols are ideal capping agents for the nanoclusters formed. Yet another method of preparation of quantum clusters is the core-etching using ligands, where small clusters of Au atoms are formed from larger clusters or small nanoparticles. The straightforward synthesis, broad absorption spectra, size-dependent tunable photoluminescence color (Fig. 4D), exceptional photostability, non-toxic nature and the ability to produce singlet oxygen make quantum clusters promising candidates for bioimaging and photodynamic therapy.[19]

3.1. Functionalization and bioconjugation of gold nanomaterials

As-synthesized gold nanoparticles and quantum clusters with well-controlled size, shape, and plasmon or photoluminescence properties have only a few types of surface capping ligands and functional groups. Therefore, ligand exchange reactions and chemical modifications have often become necessary for adapting these materials towards various nanotechnology and biology applications. Alkane thiols are the common surface capping ligands on as-synthesized gold nanoparticles. Murray and coworkers have introduced various ligand exchange reactions on alkane thiol protected gold nanoparticles, which are further extended to the preparation of water-dispersive gold nanoparticles with terminal functional moieties such as carboxyl acids, alkyl halides, amines, azides, maleimides, phenols, alcohols, carbohydrates, amphiphilic polymers, amino acids, nucleic acids, peptides and proteins. Common molecules involved in the ligand exchange reactions on gold nanoparticles and the corresponding surface functional groups on gold are summarized in Fig. 5. The key about ligand exchange is that one of the functional groups in the newly added ligand should be thiol, which facilitates the exchange reaction due to the high affinity of thiols for gold as first reported by Giersig and Mulvaney.[13]

Gold nanoparticles in which the ligands are exchanged with thiolated carboxylic acids can be conjugated with any amino acid, peptide, protein, antibody or another nanoparticle that carries a primary or secondary amino group using carbodiimide coupling chemistry. The same method applies to the conjugation of amine-functionalized gold nanoparticles to carboxylic acid-bearing molecules or materials. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and dicyclohexylcarbodiimide (DCC) are examples for zero-length cross-linkers that form amide bonds in such coupling reactions.[4] Commercially available *N*-hydroxysuccinimide (NHS)

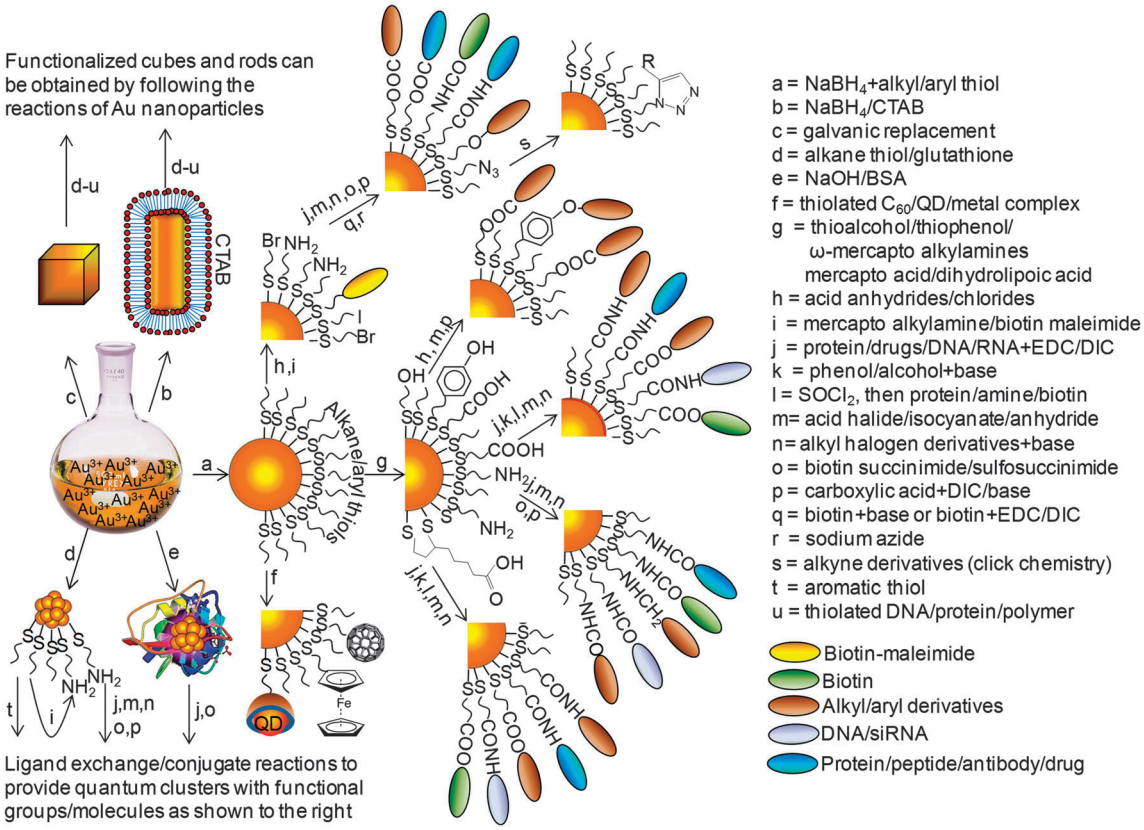

Fig. 5 Ligand exchange and bioconjugate reactions of gold nanoparticles and quantum clusters.

ester and 3-sulfo-NHS ester derivatives of organic dyes, biotin, *etc.* can be readily conjugated to primary or secondary amine-functionalized gold nanoparticles. Biotinylated gold nanoparticles prepared in this way allow one to attach avidin/streptavidin or streptavidin-functionalized molecules/nanoparticles to the surface of gold nanoparticles through the strong coupling between biotin and avidin. In parallel, amino groups in proteins/antibodies/peptides/DNAs or other nanoparticles can be biotinylated using biotin-NHS ester or biotin sulfo-NHS ester. However, amino groups necessary for the fundamental functioning of biomolecules should remain intact during such biotinylation reactions. Subsequently, the biotinylated molecule/nanoparticle can be attached to one of the free biotin binding pockets in the streptavidin-functionalized gold nanoparticle. In short, fluorescent dyes, other nanoparticles, drugs, proteins, DNAs, siRNA, peptides and antibodies can be attached to amine/carboxyl groups in ligand-exchanged gold nanoparticles by the above simple protocols. Azide click chemistry and esterification are other practical methods for the functionalization and bioconjugation of gold nanoparticles. Brominated alkane thiol-capped gold nanoparticles upon treatment with sodium azide provide azide-functionalized nanoparticles, which can be conjugated by click chemistry or photoactivation to any molecule that carries an alkyne group. Conjugation *via* esterification takes place during the reaction between alkyl halogen-functionalized nanoparticles with carboxylic acids/phenols or *vice versa* in the presence of a mild base. Yet another method for ester-based linkage is the reaction of carboxylic acid-functionalized gold nanoparticles with hydroxyl/phenol functionalized molecules or *vice versa*. Esterification allows one to conjugate carbohydrates, polymers, *etc.* to the surface of gold nanoparticles. The common surface cap of as-synthesized gold nanorods is CTAB, which limits the biological applications of gold nanorods due to the toxicity of CTAB. Thus, ligand exchange reactions using thiolated molecules such as carboxylic acids, polymers, carbohydrates and dendrimers on CTAB-functionalized gold nanorods are extensively investigated for rendering biocompatibility to nanorods. Such ligand exchange reactions can also be applied to other gold nanostructures such as nanocubes, nanocages and nanotubes.

As-synthesized gold quantum clusters (Au_8, Au_{15}, Au_{25}, *etc.*) are stabilized in ligands such as glutathione, alkane thiols or BSA. Dispersion of alkane thiol-functionalized quantum clusters in aqueous buffers can be carried out by the exchange of ligands with functional thiols such as thioglycolic acid, glutathione and 2-mercaptoethanol. However, size-reduction or etching of quantum clusters is a limitation in such reactions. Similar size-reduction is also observed for Au nanoparticles. Bioconjugation of quantum clusters protected in glutathione or BSA can be carried out by the coupling of amino groups in glutathione/BSA with carboxyl groups in other molecules by EDC or DIC coupling. Alternatively, biotinylated quantum clusters can be prepared by the reactions of amino groups in glutathione or BSA with biotin-NHS or biotin-sulfo NHS ester. Biotinylated quantum clusters can be readily decorated with streptavidin-functionalized molecules or nanoparticles.[19]

Ligand exchange and bioconjugate reactions of gold nanoparticles and quantum clusters are summarized in Fig. 5.

3.2. Biological applications of functionalized gold nanomaterials

Biosensing. Surface plasmon resonance (SPR) of gold nanomaterials such as spheres, cubes, rods and shells are extensively exploited in various biological applications ranging from bimolecular sensing to therapeutic interventions. Physicochemical properties of capping ligands and functional groups on gold play crucial roles in such applications. Ligand exchange reactions and chemical conjugations summarized in the previous section can be conveniently adapted for the recruitment of oligonucleotides, DNA hairpins, antibodies, peptides, proteins, organic dye molecules and fluorescent proteins to the surface of gold nanospheres and nanorods in the construction of biosensors. Successively, such surface modified gold nanoparticles become powerful sensors of DNA hybridization processes, protein–protein interactions, pathogens, cancer cells, antigens and various toxic materials.[20] Blue-shift in the SPR band and the associated color change due to aggregation of gold nanoparticles functionalized with oligonucleotides during the sensing of complementary DNA strands is a classical example for biosensing.[21] Such a shift in the SPR band is exploited in the sensing of human chorionic gonadotropin (β-hCG) using anti-hCG antibody-functionalized gold nanoparticles in pregnancy tests.[20] Fluorescence quenching of organic dyes during the hybridization of dye-functionalized oligonucleotides to complementary DNA strands tethered on gold nanoparticles as well as the enhancement in the light scattering intensity of nanoparticle-aggregates during such hybridization are exploited in various biosensing applications of functionalized gold.[20] Enhanced light scattering during the aggregation of gold nanoparticles *via* protein–protein interactions is also useful for the sensing of pharmacologically significant molecules such as urinary adenosine and immunoglobulins, toxic substances such as arsenic, lead, trinitrotoluene, and pathogenic bacteria and viruses.[20] Fluorescence quenching of the green fluorescent protein (GFP) assembled on the surface of gold nanoparticles and the enhancement of fluorescence during the detachment of GFP in cells is a biosensing method for the labeling and detection of cancer cells and microorganisms. Gold nanoparticles functionalized with oligonucleotides and dye molecules are also useful for Raman- or SERS-based detections of complementary DNA strands and pathogens.[20] These examples suggest that the functionalized gold nanoparticles with suitable oligonucleotides, proteins, and organic dye molecules offer versatile platforms for SPR-, scattering-, fluorescence-, Raman- or SERS-based biosensing.

Gene and drug delivery. Due to the straightforward synthesis, large surface area and flexible surface chemistry, gold nanoparticles have emerged as promising vectors for the intracellular and *in vivo* delivery of genes, drugs and contrast agents. Incorporation of polycationic molecules such as amphiphilic thiols, polyarginine, polylysine, polyaminoesters and polyethyleneimine (PEI) on the surface of gold nanoparticles not only enables us to efficiently load negatively charged DNA/siRNA molecules to the nanoparticle surface but also renders intracellular delivery and endosomal escape of the nanoparticle-vector/gene assembly.[22] The intracellular delivery efficiency of gold nanoparticles can be tuned beyond that of the well-known transfection agents such as lipofectamine 2000 by the recruitment of polyaminoesters to the surface. Furthermore, conjugation of cyclodextrins, PEG, or PEI to the gold-cargo assemblies improves the biodistribution and suppresses toxicity. The release of siRNA or pDNA conjugated directly or through other molecules to the surface of gold nanoparticles using disulfide bonds takes place spontaneously under the physiological redox conditions inside living cells.[23] Gold nanoparticles are also utilized for the intracellular or *in vivo* delivery of contrast agents, photosensitizers, and antibacterial and anticancer drugs. The loading of drugs and other molecules to gold nanoparticles can be covalent or non-covalent. Covalently-conjugated molecules can be released from the surface of gold nanoparticles by photouncaging or redox reactions; whereas, non-covalent conjugates are released as a result of pH change or by photothermal effect. Furthermore, the photothermal effect of gold nanorods and nanocubes is a promising property for the disruption of endosomes and lysosomes and the intracellular release of trapped cargos such as doxorubicin, siRNA, fluorescent dyes, and photosensitizers.

Bioimaging and phototherapy. Gold nanoparticles such as nanorods, nanocages, nanoshells, nanospheres, nanostars and nanocubes find increasing attention in the imaging and therapy of cancers *in vitro* and *in vivo*. Bioimaging modalities of gold nanomaterials include SPR, optical coherence, SERS of organic and bio molecules, and photothermal and photoacoustic responses. Other properties of gold nanomaterials that are promising for bioimaging and therapy are the straightforward synthesis, well-defined surface chemistry, non-toxic nature, and the large one- and two-photon absorption cross-sections. Ligand exchange reactions and the subsequent conjugation of one of the targeting molecules against cancer markers, such as growth hormones, RGD peptide or an anticancer antibody are prerequisites for the *in vitro* and *in vivo* labeling of cancer cells using gold nanoparticles. Conjugates of gold nanorods, nanospheres or nanocubes with folic acid, RGD peptide, EGF, anti-EGFR antibody or anti-HER2 antibody are widely investigated in such labeling. Nanospheres and nanorods of gold provide excellent contrasts in the dark-field optical and photothermal imaging of cells and tissues; whereas, nanoshells, nanospheres, nanorods and nanocages are ideal for optical coherence tomography and photoacoustic imaging of deep tissues, circulatory systems and lymph nodes.[24] Other imaging modalities offered by gold nanoparticles are X-ray contrast and stable but weak fluorescence. More recently, with the advancement in the synthesis and bioconjugation of quantum clusters, gold enters into the golden era of fluorescence-based bioimaging.[19]

Photothermal response of gold nanoparticles, in particular nanorods and nanocages, are extensively exploited in cancer photothermal therapy. The conjugation of ligands or antibodies against cancer markers, such as EGF, folic acid, anti-EGFR antibody, or anti-HER2 antibody on the surface of gold nanoparticles

enables cancer cell-specific labeling *in vitro* and *in vivo*. Subsequent photoactivation of gold nanoparticles in the labeled cells/tissues generates local heating, which results in the cell death *via* impairment of biomolecules and cell membrane.[25] Typical examples of photothermal therapy are the *in vitro* treatment of cancer cells using anti-EGFR antibody-conjugated gold nanorods and the *in vivo* non-targeted treatment of cancers in mice using PEG-conjugated gold nanorods. Other gold nanostructures exploited in photothermal therapy are nanocages, nanocubes, nanoshells and multiple-twined gold nanoparticles. Therapeutic efficiency of gold nanoparticles can be improved by the combination of photothermal therapy with photodynamic therapy and or chemotherapy. The conjugation of photosensitizer drugs such as phthalocyanines, porphyrins, toluidine blue and indocyanine green and anticancer drugs such as doxorubicin, cisplatin and camptothecin to gold nanoparticles followed by the controlled drug delivery and drug release can be practiced in such combination therapy. A typical example for phototherapy of cancer using gold nanorods is shown in Fig. 6.

4. Semiconductor quantum dots

The size-dependent tunable optical and electronic properties make semiconductor quantum dots one of the most attractive nanomaterials in the photovoltaic, optoelectronic, electro-optical and biomedical fields. Silicon-, gallium-, indium- or germanium-based group IV and VI quantum dots, or alloy quantum dots within the members of these groups or among the members of groups III and V are prevalent in our daily life in the form of semiconductor devices. On the other hand, group II–VI colloidal quantum dots such as ZnX, CdX and HgX (X = S/Se/Te), group IV–VI colloidal quantum dots such

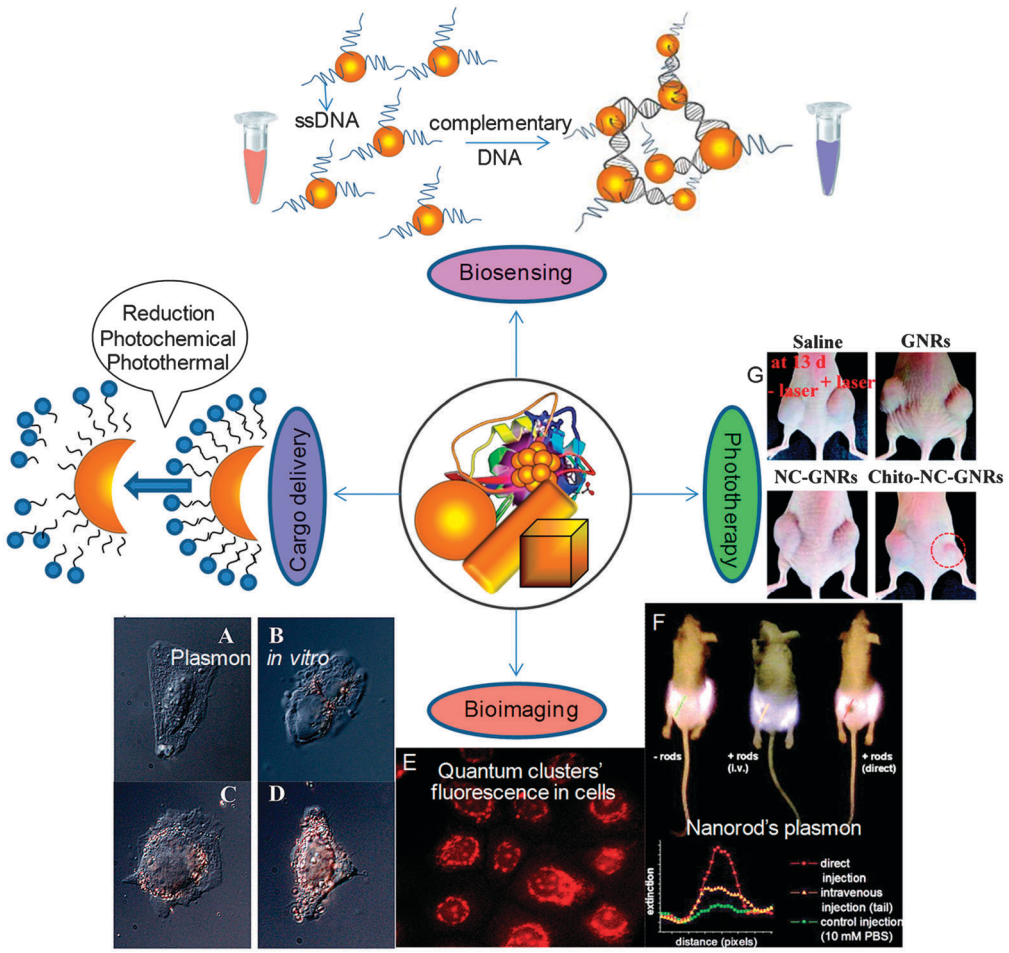

Fig. 6 Overview of various biological applications of gold nanoparticles: (A–D) plasmon images of HepG2 cells incubated with gold nanoparticle–peptide complexes, (E) fluorescence image of H1650 cells incubated with bimodal nanoparticles composed of super paramagnetic iron oxide nanoparticles, fluorescent Au_{25}–BSA complexes and EGF, (F) NIR transmission image of tumor-bearing mice treated with or without PEG-ylated gold nanorods, and the traces represent intensity line scans at tumor sites, and (G) images of tumors obtained after the injection of saline, gold nanorods, nanocarrier-gold nanorod assembly and chitosan-nanocarrier-gold nanorod assembly followed by one time irradiation with 808 nm light for 4 min. Reprinted with permissions from (A–D) A. G. Tkachenko *et al.*, *J. Am. Chem. Soc.*, 2003, **125**, 4700–4701 (copyright ACS 2003); (E) E. S. Shibu *et al.*, *Angew. Chem., Int. Ed.*, 2013, **52**, 10559–10563 (copyright Wiley VCH 2013); (F) E. B. Dickerson *et al.*, *Cancer Lett.*, 2008, **269**, 57–66 (copyright Elsevier 2008); and (G) W. I. Choi *et al.*, *ACS Nano*, 2011, **5**, 1995–2003 (copyright ACS 2011).

as PbX, and group III–V colloidal quantum dots such as InP have become prevalent in photovoltaic and light emitting devices and the imaging of biomolecules, cells and tissues. Among these colloidal quantum dots, despite the toxic cadmium content, CdSe- and CdTe-based core and core-shell quantum dots are attractive fluorescent probes for bioanalysis and bioimaging, owing to their size-dependent tunable absorption and emission of light in the UV-Vis-NIR regions, broad absorption and narrow emission bands, large molar extinction coefficients, exceptional photostability, high photoluminescence quantum efficiencies, large surface to volume ratios and flexible surface chemistry.

Recently, colloidal synthesis of high quality quantum dots has become highly reproducible, for which the seminal work by Bawendi and coworkers on the high-temperature synthesis of CdX quantum dots from dimethyl cadmium and trioctylphosphine chalcogenides or hexamethyldisilathiane is the base.[26] The surfaces of as-synthesized CdX quantum dots are passivated with trioctylphosphine and trioctylphosphine oxide, which makes these materials highly hydrophobic and incompatible in the aqueous phase. The concept of biocompatible quantum dots comes from the aqueous phase synthesis of CdX quantum dots from $CdClO_4$ by Weller and coworkers.[27] The initial challenges associated with poor quantum efficiency and broad size-distribution of quantum dots were resolved with the introduction of greener methods of quantum dots synthesis from $Cd(CO_3)_2$, CdO and $Cd(acetate)_2$ by Peng and Peng.[28] Whilst shells from any higher band-gap semiconductor materials provide physical-, chemical- and photo-stabilities to the core quantum dots, shells such as silica, ZnS and CdS offer platforms for ligand exchange and bioconjugate reactions that are necessary for various biological applications of quantum dots.

4.1. Functionalization and bioconjugation of quantum dots

The best quality quantum dots are synthesized in the organic phase and are capped with highly hydrophobic aliphatic ligands such as alkyl phosphines, alkyl phosphine oxides, aliphatic amines and aliphatic carboxylic acids. Therefore, ligand exchange reactions and surface modifications have become necessary for biological applications of quantum dots. Although ligand exchange reactions of as-synthesized core-only quantum dots using thiols facilitate aqueous dispersion and bioconjugation, major challenges such as chemical decomposition, photo-etching, toxicity due to the leaching out of cadmium ions, and unstable photoluminescence needed further attention. Shelf life of quantum dots in the organic or aqueous phase can be considerably increased by the protection of the core with inorganic shells such as silica, CdS and ZnS. Silica shells can be conveniently prepared on core or core–shell quantum dots first by the tethering of a monolayer of 3-mercaptopropyl tri(m)ethoxysilane followed by the growth of silica shells on the silane monolayer by the Stöber synthesis, which is discussed in Section 2. ZnS shells can be prepared on core quantum dots by the pyrolysis of diethyl zinc and hexamethyldisilathiane (HMDST) as reported by Bawendi and coworkers.[29] A similar procedure can be applied in the preparation of CdS shell from dimethylcadmium and hexamethyldisilathiane.

The most important aspects to be considered during the surface modification and bioconjugation of quantum dots are that the fundamental properties of quantum dots remain unaffected, uniform dispersion is maintained, and the conjugated molecules or functional groups become available for biological applications. Electrostatic and hydrophobic binding of molecules to the surface was one of the earlier attempts for the functionalization of quantum dots. On the other hand, stable covalent chemistry can be utilized more efficiently for the replacement of hydrophobic ligands on core and core–shell quantum dots using thiogycolic acid (TGA), dihydrolipoic acid (DHLA) or mercaptosilanes. Such bifunctional ligands can be further exploited in the conjugation of hydrophilic dendrimers, amphiphilic block copolymers, carbohydrates, micelles, peptides, proteins, antibodies, DNA, siRNA, photosensitizers and anti-cancer drugs.[30] Chemical and bioconjugate modifications of silica-shelled quantum dots should follow the reactions of silica nanoparticles described in Section 2.1. Ligand exchange reactions of core-only, or ZnS- or CdS-shelled quantum dots render surface functional groups such as carboxylic acids, amines and hydroxyl groups, which can be efficiently utilized for the conjugation of biomolecules or nanoparticles as with gold and silica nanoparticles. Steps involved in the preparation, surface modification and bioconjugation of quantum dots are summarized in Fig. 7, among which, the simplest protocol for the covalent conjugation of quantum dots with biomolecules is EDC coupling. For example, the carboxylic acid group in thioglycolic acid or dihydrolipoic acid functionalized quantum dots can be conjugated with amino groups in biomolecules such as peptides, proteins, antibodies or DNA by EDC coupling. Here, the possible complexity associated with cross-linking among multiple carboxyl and amino groups present in most biomolecules should be carefully considered and minimized. Amine terminated quantum dots can be biotinylated by the reaction with biotin NHS or biotin-sulfo NHS ester. Both streptavidin- and biotin-functionalized quantum dots, which are readily available with Life Technologies can be used in various bioconjugate reactions. Common bifunctional cross-linking reagents such as EDC, DIC, SPDP, sulfo-SMCC and maleimido succinimide can be used in the conjugation of amino, carboxyl and sulfhydryl functionalized quantum dots with any desired biomolecule. Details of some cross-linking reactions are provided in Sections 2.1 and 3.1.

4.2. Biological applications of quantum dots

Among the unique properties of quantum dots, broad absorption and narrow emission bands, large one- and two-photon absorption cross-sections, bright and stable photoluminescence and large surface area are attractive over organic fluorophores for the *in vitro* and *in vivo* detection/imaging of biomolecules, cells and tissues. Since 1998, bioconjugated quantum dots have become regular parts of biology for sensing, gene and drug delivery, cell and biomolecular imaging, and photodynamic therapy experiments. Selected biological applications

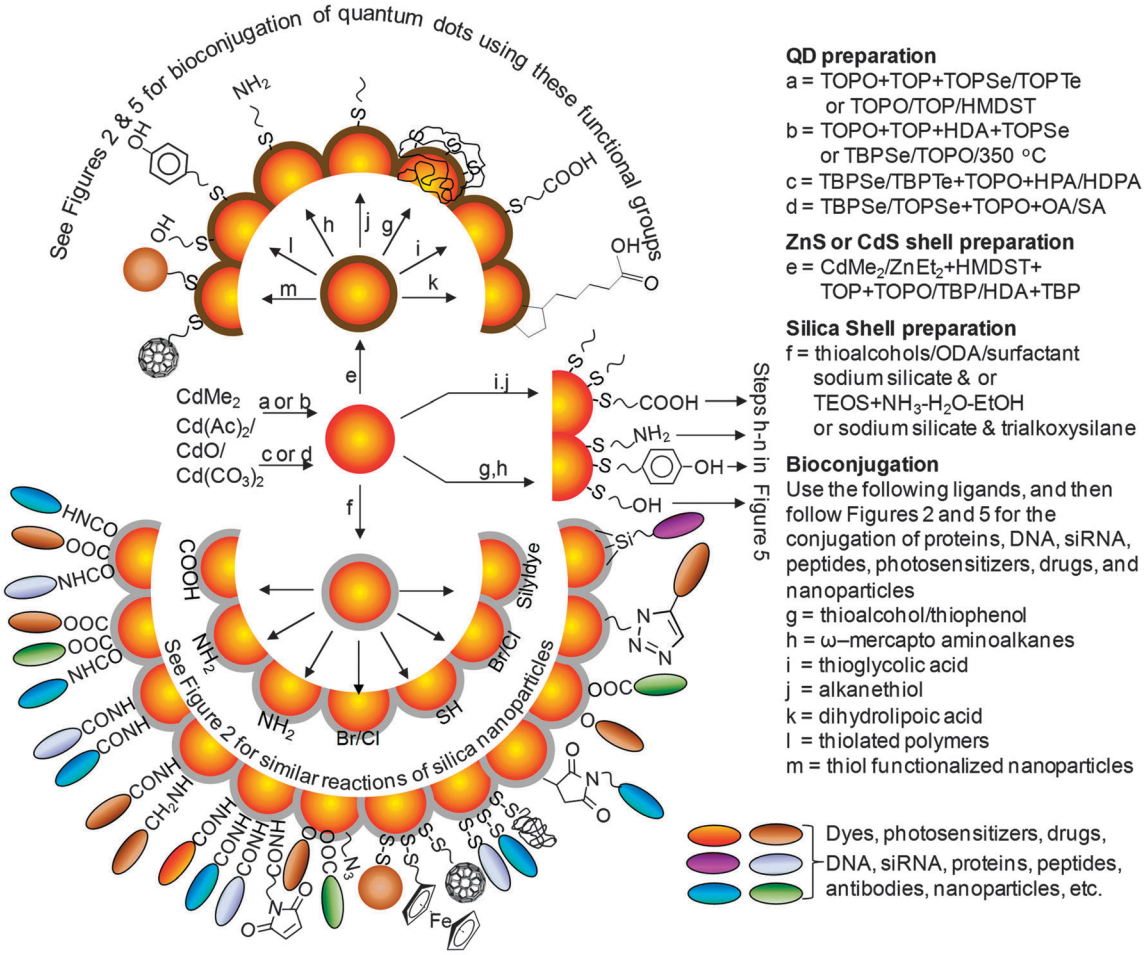

Fig. 7 Surface modification and bioconjugation of quantum dots. TOP: trioctyl phosphine, TOTO: trioctyl phosphine oxide, HMDST: hexamethyldisilathiane, HPA: hexylphosphoric acid, HDPA: hexadecylphosphoric acid, HDA: hexadecyl amine, TBP: tributyl phosphine, OA: oleic acid, SA: stearic acid, TEOS: tetraethylorthosilane, ODA: octadecyl amine.

of quantum dot are shown in Fig. 8. Additional applications can be found in ref. 30 and 31.

Biosensing. Quantum dots are extensively exploited in the construction of fluorescence- and FRET-based biosensors for the ultrasensitive detection of biomolecules such as nucleic acids, sugars, enzymes, antigens and antibodies.[31] One of the most interesting properties of quantum dots for biosensing is their narrow emission band, which allows for multiplexed detections of DNA strands or gene fragments in DNA finger printing, and antibodies or toxins in pathogen detections. The detection of glucose on quantum dot-based higher order structures and TNT using functionalized quantum dots are interesting biosensor applications. Quantum dots assembled with organic dyes such as fluorescein serve as FRET or fluorescence intensity-based ratiometric pH sensors. Quantum dots conjugated with various anticancer antibodies find applications such as the detection of cancer biomarkers in immunomicrochannels or immunochromatographs. Quantum dots conjugated with oligonucleic acids are powerful platforms for the FRET-based detection of dye-labeled complementary strands, which is analogous to the surface plasmon-based detection of oligonucleotides using gold nanoparticles. Nevertheless, the distance between the dye and quantum dot is one of the key factors in the sensitivity of such detections. Therefore, preparation of FRET-based biosensors composed of quantum dots with large hydrodynamic diameter needs careful consideration of donor-to-acceptor distance.

Gene and drug delivery. Functionalized quantum dots are extensively exploited as either carriers of genes and drugs or reporters of gene/drug delivery processes. Incubation of a quantum dot-siRNA complex in which siRNA is adsorbed onto quantum dots functionalized with multiple amines efficiently transport and deliver siRNA in the cytosol by the proton sponge effect offered by amines. Intracellular delivery of siRNA, sense or antisense RNA, or DNA conjugated to quantum dots through disulfide linkers can be efficiently released under the reducing environment in the cytosol. Conjugation of quantum dot-drug/gene composites with cationic peptides such as Tat, or incorporation of quantum dots inside or on the surface of drug/gene-loaded liposomes are attractive approaches for the monitoring of drug/gene delivery processes. Amine- or carboxyl-functionalized quantum dots can be conjugated with carboxyl- or amine-functionalized liposome *via* EDC/DIC coupling as

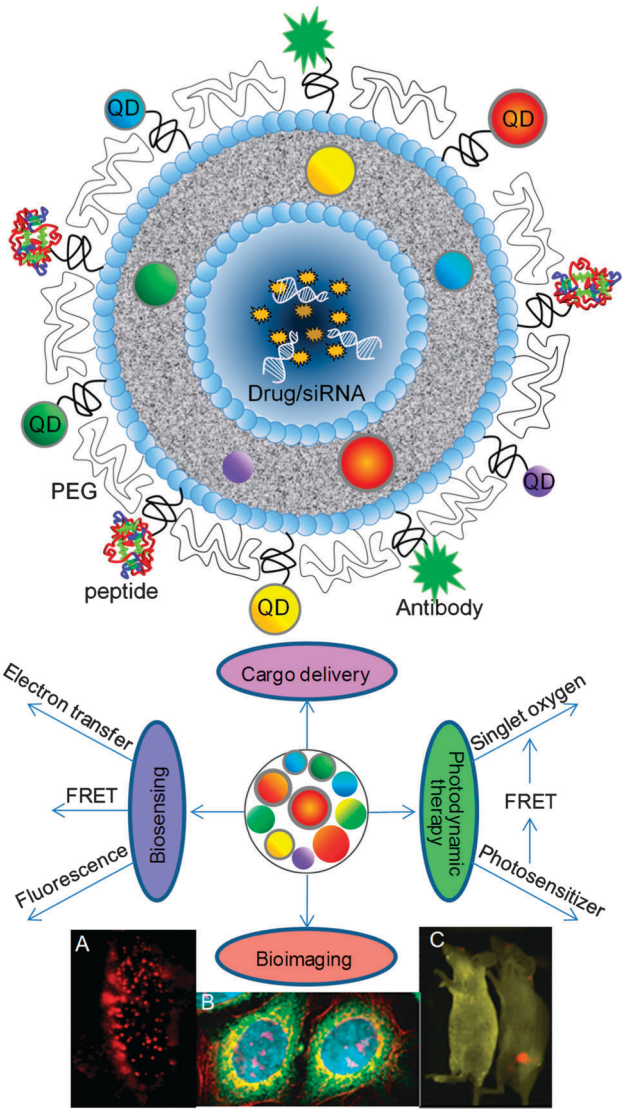

Fig. 8 Biological applications of semiconductor quantum dots: (A) fluorescence image EGFR single-molecules in a A431 cell labeled using quantum dot-EGF conjugates, (B) pseudo-colored image showing five-color labeling of fixed human epithelial cells using quantum dot-bioconjugates, and (C) prostate tumor in a mouse labeled using quantum dot-PSMA antibody conjugate. Reprinted with permission from (A) N. Kawashima *et al.*, *Chem.–Eur. J.*, 2010, **16**, 1186–1192 (copyright Wiley-VCH 2010); (B) I. L. Medintz *et al.*, *Nat. Mater.*, 2005, **4**, 435–446 (copyright McMillan publishers 2005); and (C) X. Gao *et al.*, *Nat. Biotechnol.*, 2004, **22**, 969–976 (copyright McMillan Publishers 2004).

discussed in the previous section. Further, incorporation of genes or drugs in the hydrophilic cavity of liposome, and cell-targeting moieties such as transferrin, EGF, folic acid, RGD peptide, aptamers, anti-EGFR antibody, anti-HER2 antibody or an antibody against prostate specific membrane antigen (PSMA) on the surface of liposomes facilitate intracellular transport and delivery of drugs and genes, which can be monitored by following the fluorescence of quantum dots. Here, the selection of biomolecules for conjugation to the surface of liposome or quantum dot depends on the biomarker in the target cell. Drug delivery can also be monitored by following FRET from quantum dots to other fluorophores or drugs such as doxorubicin. Other quantum dot-based systems for the intracellular delivery of drugs and genes are composed of imine conjugates, which facilitate proton sponge-mediated endosomal escape and cargo release. Intracellular delivery of quantum dot-bioconjugates and their endosomal escape are summarized in ref. 30.

Bioimaging. Cell imaging is the main biological application of quantum dots. Cell biological applications of quantum dots can be classified into nonspecific or cell-specific, which depend on the property of molecules recruited to the surface of quantum dots. Nonspecific labeling can be applied to any cell type.[30] Quantum dots conjugated with or encompassed in small molecules, cationic lipids, liposomes, cell penetrating peptides, polymers, proteins or carbohydrates non-specifically attach to the cell membrane by the electrostatic or hydrophobic interactions, which is followed by macropinocytosis. Quantum dot capped with small organic molecules such as TGA, DHLA or mercaptopropionic acid directly interacts with cells and enters into the cytosol. Quantum dots conjugated with peptides such as nucleus localization signal (NLS) or mitochondrial localization signal (MLS) facilitate the targeted intracellular labeling and imaging of nucleus or mitochondria. Although cytotoxic, liposomes and surfactants efficiently transport quantum dots into the cytosol. Lipofectamine 2000 is one of the most promising candidates in this category, which is a common vector for the intracellular delivery of drugs, genes and various contrast agents. Lipids/liposomes fuse with the cell membrane and deliver the contents into the cytosol. Thus, multilamellar vesicles are employed in the delivery of cargoes into the cytosol and nucleus of cells. Nonspecific intracellular transport of quantum dots can be carried out using various other molecules such cationic or amine-rich co-polymers, and carbohydrates such as chitosan, glucose and cationic polysaccharides.

Whilst nonspecific extracellular and intracellular labeling is useful for the detection and imaging of any cell type, biomarker-specific targeted cell labeling is often necessary for practical applications such as the detection and imaging of cancer cells and tumor milieu, and gene and drug delivery. Targeted labeling of cells using quantum dots can be carried out by the conjugation of growth hormones such as EGF, VEGF and transferrin, and antibodies such as anti-EGFR antibody and anti-HER2 antibody. Since antibodies are expensive and scarce, alternative agents such as folic acid, hyaluronic acid and RGD peptide are widely exploited in such targeted cell labeling. Here, targeted labeling of cells takes place by the binding of quantum dots conjugated with EGF to EGF receptor, D-galactose to asialoglycoprotein receptor, transferrin to transferrin receptor, folic acid to folate receptor, aptamers to PSMA, neuroligin-1b(Nrx1b) to neurolignin-1 (Nlg1), hyaluronic acid to CD44, RGD peptide to $\alpha_v\beta_3$ integrin, neurotrophins to nerve growth factor receptors, anti-mouse Fab fragments to glycine receptors, and mannose to glycoproteins.[30] Certain other biomarkers recruited to the surface of quantum dots for cell labeling are ligands or antibodies against P-glycoprotein, cell adhesion molecules, Her2 receptors, prostate stem cell antigen, vascular

endothelial receptor, E-cadherin, insulin-like growth factor receptor, integrin receptors, gamma-aminobutyric acid (GABA) receptor, target of rapamycin (mTOR), estrogen receptor, progesterone receptor, claudin 4, α-fetoprotein, α-CD3, wheat germ agglutinin receptor, anti-α-tubulin antibody, and anti-nuclear antigen.[30] If the direct labeling of a biomarker using a quantum dot-primary antibody conjugate is not practical, labeling can be carried out in multi-steps such as labeling using biotinylated primary antibody followed by treatment with streptavidin- or secondary antibody-conjugated quantum dots. FRET and bioluminescence resonance energy transfer (BRET) are also valuable processes for the imaging of biomolecular functioning in cells.[31] In BRET, luciferase enzyme conjugated to quantum dot offers energy transfer mediated bioimaging without the use of any external light source.

Some of the quantum dot conjugates discussed above for *in vitro* imaging are also useful for *in vivo* imaging of tumor vasculature, metastasis, and cell migration.[30] Although non-targeted quantum dots with surface molecules such as polymers, sugars, gelatin, carboxylic acids, Tat and polyarginine are exploited in the imaging of lymph nodes, blood vessels and tumors, conjugates of quantum dots with RGD peptide or anti-PSMA antibody show efficient and selective localization in tumor milieus. Another example for the targeted *in vivo* applications of quantum dots is the labeling and imaging of leukocytes using quantum dot-D-lactose conjugate. Also, quantum dots conjugated with RGD peptide show enhanced binding to $\alpha_v\beta_3$ integrin over-expressed under angiogenesis and metastasis of tumors. Autofluorescence of cells and tissues and poor penetration of excitation light and emitted fluorescence are challenges associated with the *in vivo* fluorescence imaging using quantum dots, which can be lifted by using NIR fluorescent quantum dots, two-photon excitation and BRET. Furthermore, multimodal imaging probes based on quantum dots for *in vivo* applications can be prepared by the conjugation of quantum dots with contrast agents for SPECT, PET and MRI.

Therapy. Quantum dots alone or in combination with classical photosensitizers such as porphyrins, phthalocyanines, organic dyes, inorganic complexes, *etc.* are investigated in photodynamic therapy *in vitro* and *in vivo*. Photosensitized production of singlet oxygen and other reactive oxygen species (ROS) by the transfer of excitation energy or electron to oxygen is the fundamental process in photodynamic therapy.[32] Although quantum dots are exceptionally photostable, their poor efficiency (<5%) to activate singlet oxygen is the major limitation in photodynamic therapy. The efficiency of singlet oxygen production is improved up to 70% by the conjugation of standard photosensitizers such as chlorine e6 or Rose Bengal to quantum dots. In these cases, photoactivation of quantum dots results in the efficient transfer of energy to the attached sensitizer, which undergoes efficient intersystem crossing and transfers energy to oxygen. Singlet oxygen sensitizers can be conjugated to the surface of quantum dots using amines, carboxylic acids or peptides by following the steps summarized in Fig. 7. Further, the conjugation of cancer specific antibodies discussed in the above section allows one to localize the quantum dot-sensitizer complex in cancer cells or tumor milieu. Nevertheless, cytotoxicity of cadmium-based quantum dots continues to be a major challenge in the advancement of quantum dot-based *in vivo* imaging and photodynamic therapy. Also, renal clearance of quantum dots applied *in vivo* is decided by multiple factors such as the surface charge, surface functional groups, and the size. Rigorous *in vivo* studies about the toxicology and pharmacokinetics of quantum dots are under way in many laboratories worldwide, results of which are necessary to assess the clinical prospective of quantum dots.

5. Carbon nanomaterials

Carbon nanomaterials such as carbon nanotubes (CNTs), fullerenes, graphenes, carbon nanoparticles, nanodiamond, carbon nanohorns and carbon nanocaps show unique mechanical, electronic, optical and magnetic properties that emerge from the π-electron cloud and their unique structures. The most attractive properties of these materials for biological applications are the broad absorption of light in the UV-Vis-NIR region, NIR photoluminescence, unique Raman signals, exceptional photothermal response, photosensitized production of singlet oxygen, and large surface area for the covalent and non-covalent conjugation of contrast agents and drugs/DNA/RNA. Thus, carbon nanomaterials infiltrate into various biological applications such as bioimaging, biosensing, gene and drug delivery, and photothermal and photodynamic therapy of cancers and certain infectious diseases. However, as-prepared carbon nanomaterials are highly hydrophobic and not biocompatible, which often demands covalent or non-covalent conjugation of hydrophilic molecules to the surface. This part of the review is mostly focused on the surface functionalization and selected biological applications of CNTs, Buckminster fullerene (C_{60}) and graphene. Since many chemical reactions are common to these carbon nanomaterials, their functionalization and bioconjugation are summarized in Fig. 9 by taking CNT as an example.

5.1. Carbon nanotubes

CNTs are 1–100 nm diameter rolled up graphene structures in quasi-1 dimensional tubular carbon network forms with single or multiple walls (SWCNT or MWCNT). Although CNTs were detected 50 years ago, they became prevalent in optoelectronic, photovoltaic and nanobio research subjects with the seminal publication about the characteristics of CNTs by Sumio Ijima.[33] CNTs were first prepared by the direct current evaporation of carbon rods in a high pressure (100 Torr) argon atmosphere. Modification of this preparation by setting the pressure at >500 Torr is adapted for the large scale production of CNTs. Although samples prepared by these methods contain CNTs with a varying number of walls and thickness of carbon coating, high-temperature reactions of as-prepared samples with a mixture of oxygen and carbon dioxide render uniform size CNTs *via* oxidative removal of outer layers. More recently, a seed-mediated chemical vapor deposition (CVD) method by flowing a mixture of an inert gas, hydrocarbon and alcohol through a

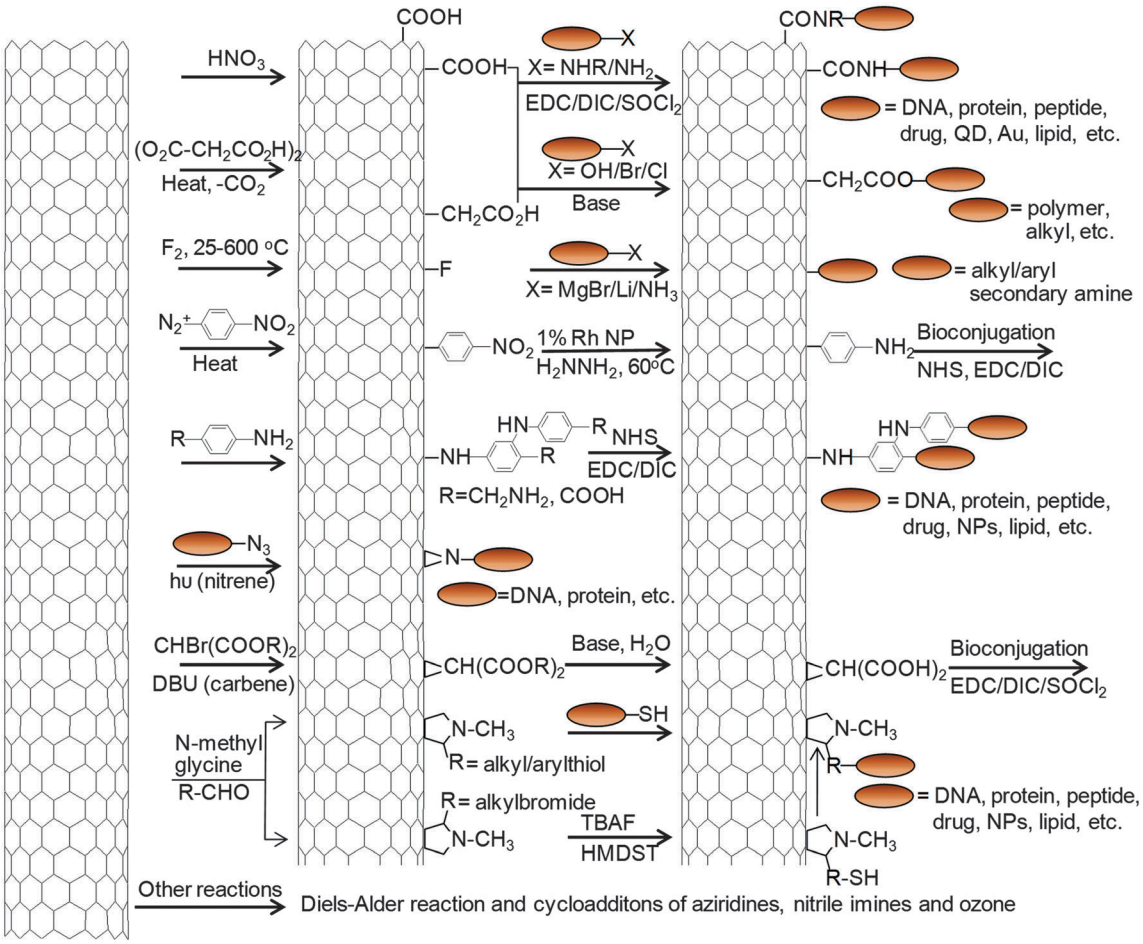

Fig. 9 Chemical and bioconjugate reactions of CNTs. These reactions work well for the functionalization and bioconjugation of fullerenes and graphene as well.

catalyst (Fe, Co or Ni) deposited on substrates such as alumia has been extensively utilized in the patterned and controlled growth of SWCNT and MWCNT.[34] Metal and carbonaceous impurities in the product are removed by oxidative and acid treatments. Further, metallic and semiconductor CNTs formed in these preparations need to be separated for various device applications of CNTs. Different methods for the production of CNTs are summarized in ref. 34. Recently, CNTs have become popular in nanomedicine and bioimaging areas owing to their large molar extinction coefficient, broad absorption of light in the UV-Vis-NIR regions, NIR photoluminescence, excellent photothermal response, large surface area and characteristic Raman bands such as radial breathing mode (RBM), graphene (G) mode, defect (D) mode, and defect induced overtone (G′ or D′) modes. Photoluminescence of CNTs is contributed by c_1–v_1 carrier recombination which follows activation of exciton by S_{22} transition and phonon-assisted intra-band relaxations. However, the major pathway of carrier recombination is non-radiative, which results in the exceptional photothermal effect, but poor quantum efficiency (<0.01) of photoluminescence. Although the electronic and optical properties of CNTs are affected to various extents by chemical reactions, the introduction of hydrophilic molecules and reactive functional groups is often necessary for applications such as biosensing, bioimaging, drug and gene delivery, and phototherapy.

5.1.1. Functionalization and bioconjugation of CNTs. As-prepared CNTs by the classical methods mentioned above exist in the aggregate or bundle form due to the high hydrophobicity. Because separation and rearrangement of individual nanotubes are tedious, CNTs for various device and biosensor applications are grown site-specifically. Nevertheless, uniform suspensions of CNTs in aqueous buffers are required for bioimaging, drug delivery and photothermal therapy. The simplest method for the dispersion of CNTs in the aqueous phase is the controlled sonication of an aqueous suspension of CNTs and amphiphilic molecules or surfactants. However, covalent functionalization is the most effective method for the stable dispersion of CNTs in the organic or aqueous phase. Furthermore, covalent functionalization and bioconjugation of CNT are essential for not only minimizing the *in vivo* toxicity but also the effective biodistribution and excretion of CNT. The chemistry of CNT can be classified into covalent functionalization such as halogenation, hydrogenation, oxidation, ozonolysis, cycloaddition, free radical/electrophilic/nucleophilic addition,

and non-covalent functionalization such as hydrophobic and π-stacking interactions. Among these functionalization reactions, cycloaddition and oxidation are widely investigated. Nonetheless, defects generated during such chemical reactions on the side-wall adversely affect the electrical, electronic and optical properties of CNT. Carboxyl groups on the ends of CNT, which are produced by oxidation, can be used for bioconjugate reactions without considerably affecting the optical and electronic properties of CNT. The carboxyl group can be reacted with alcohols, phenols, alkyl halides, anhydrides, amines, *etc.*, as shown in Fig. 9, for the direct or step-wise conjugation of desired biomolecules. Also, CNT derivatives soluble in the organic or aqueous phase and having various functional groups can be prepared by the [2+1] addition reactions of *in situ* generated carbenes or nitrenes. Acyclic adducts on CNTs are mostly formed by the addition of free radicals generated *in situ* by the oxidation of amines, reduction of aryldiazonium salts, or decarboxylation of acid anhydrides or azylperoxides. 1,3-Dipolar cycloaddition reactions of azomethine ylides generated by the thermal decomposition of aziridines or nitrile imines or by the condensation of an α-amino acid with an aldehyde introduce pyrrolidine rings on the side-wall of CNT. Yet another method for the side-wall functionalization of CNT is the Diels–Alder [4+2] cycloaddition of 1,3-dienes. Reactive functional groups such as carboxyl, amino, hydroxyl, and alkyl halogens can be introduced on CNTs by suitably selecting the reagents in such addition reactions (Fig. 9). Details about chemical reactions of CNTs can be found in ref. 35. Further, the reactive functional groups introduced in CNTs can be used for the conjugation of organic dyes, siRNA, DNA, anticancer drugs, peptides, proteins, antibodies, nanoparticles and radionuclides, for which the reagents and reactions are given in Fig. 2, 5 and 9.

5.1.2. Biological applications of functionalized CNTs. Biological applications of CNTs can be classified into electrical/SERS/Raman-based biosensing, drug and gene delivery, photoluminescence/Raman/photoacoustic/photothermal bioimaging, and photothermal therapy of cancer. Chemical modifications of CNTs allow one to recruit various functional groups, fluorescence/positron emission/MRI probes, drugs, and biomolecules such as DNA, siRNA, proteins, peptides and antibodies to the surface of CNTs. Cutting edge and emerging biological applications of functionalized CNTs are discussed below.

Biosensing. Changes in the electrical conductivity, photoluminescence spectrum and SERS and Raman bands of CNTs provide label-free methods for biosensing, which have been widely exploited in the recent past. For example, electrochemical detection of molecular markers or immunosensors such as PSMA, carcinoembryonic antigen and interleukin 6 or 8 using bioconjugated CNTs show great potential for the early detection of cancers.[36] Another example for sensing is the use of polymer–CNT hybrid systems for the detection of volatile organic chemicals in the breath of lung cancer patients. Further, the modulations of the Schottky barrier and electrical conductivity of CNT-based field-effect transistors during the binding of biomolecules such as oligonucleotides and antibodies to native or bioconjugated CNTs offer nanomolar to femtomolar sensitivity. Similarly, a decrease or increase in the intensity, as well as spectral shifts associated with the intrinsic NIR photoluminescence of CNTs during the recognition of proteins, DNA, and glucose have been exploited in the construction of CNT-based biosensors.[36] Nevertheless, the sensitivity of detection is adversely affected in many cases by the low photoluminescence quantum efficiency, which is because of the non-radiative carrier recombination and the non-emissive nature of metallic CNTs. On the other hand, hypsochromic shift in the NIR photoluminescence band of oligonucleotide-conjugated CNTs during the recognition of complementary DNA strands is a highly sensitive method for the detection of specific DNA sequences, which is comparable with the sensitivity offered by the bathochromic shift in the SPR band of gold nanoparticles during DNA sensing. Due to the unique Raman scattering features of CNTs, evaluations of the Raman and SERS signal intensities during the binding of proteins, antibodies, DNA, antigens, *etc.* directly to CNTs or to the counterpart biomolecules covalently or non-covalently attached to CNT are powerful methods for microarray-based biosensing with sensitivity in the femtomolar regime.

Cargo delivery. The large surface area, availability of multiple functional groups and the hydrophobic nature of CNTs are useful parameters for the efficient loading and delivery of fluorophores, drugs, proteins, DNA, and siRNA.[37] Although there are several examples of cargo delivery using non-covalent conjugates of DNA, fluorescent dyes and anticancer drugs such as doxorubicin with CNTs, non-covalent derivative of CNTs when applied *in vivo* often induce various toxicological and pharmacological complications such as immune response, oxidative stress, hyperkeratosis, fibrillation and mesothelioma. Capping agents such as surfactants or amphiphilic polymers non-covalently attached to CNTs can be easily removed in the blood, interstitial fluid or extracellular or intracellular matrix, which results in the trapping of uncapped CNTs in the reticuloendothelial system (RES) or in the plasma membrane and subsequently pose a major challenge in clinical applications of CNT-based drug delivery systems. On the other hand, the clearance of CNT administered *in vivo* can be considerably improved by increasing the number of functional groups on CNT. Also, functionalized CNTs are essentially non-toxic. In other words, functionalization of CNT allows one to not only prepare stable conjugates of dyes, drugs and DNA/siRNA but also suppress the toxicity of CNT. The covalent or non-covalent conjugation of cell-targeting molecules such as RGD peptide, folic acid, EGF, aptamers, or a spectrum of antibodies against extracellular antigens to CNTs provides bioconjugates that facilitate targeted intracellular transport of cargos. Conjugation of targeting molecules and cargos to CNTs can be carried out by following the reactions shown in Fig. 9. Further, conjugation of polymers such as PEG to CNTs is necessary for both the prolonged circulation in the blood stream and minimizing entrapment in the RES, which are common requirements for other nanomaterials as well. Intracellular cargo release from CNTs is the next step necessary for inducing the desired changes such as gene expression using plasmid DNA/siRNA, chemotherapy using anticancer drugs

such as doxorubicin, paclitaxel, methotrexate, camptothecin and cisplatin, and phototherapy by the NIR photoactivation or photosensitization. Redox-sensitive disulfide bonds, photo-uncaging linkers, and esters are extensively utilized in the chemical and photochemical release of cargos. Basic aspects about such cargo release are discussed in Section 2. Also, photothermal effect induced by CNT is an important parameter for not only the release of cargos from the surface of CNT but also endosome/lysosome disruption and the endo-lysosomal escape of CNT and cargo, which is similar to the photothermal drug delivery system based on gold nanorods. This trend in the drug and gene delivery using CNTs as the cargo carrier suggests that the clinical applications of CNT-based drug/gene delivery systems cannot be far away.

Bioimaging. Fundamental properties of CNTs for bioimaging are the broad absorption band extending in the UV-Vis-NIR region, NIR photoluminescence, excellent photoacoustic response, and unique Raman/SERS bands.[38] For bioimaging, CNTs can be delivered in living cells or administered *in vivo* with or without bioconjugation. Although passive uptake of pristine CNTs wrapped in surfactants, amphiphilic polymers or liposomes takes place in living cells or tumors, toxicity and immune response due to the partial trapping of CNTs in the cell membrane or RES is a limitation. CNTs can be conjugated with various targeting molecules such as RGD peptide, folic acid, Herceptin, transferrin, EGF, aptamers and various antibodies, or incorporated in immunoliposomes. Targeted delivery and biodistribution of such bioconjugated CNTs are efficient in living cells by the simple incubation or in tumors by the intravenous or intratumoral injection. Coating of CNTs with polymers such as PEG renders excellent biodistribution and efficient *in vivo* tumor targeting. The broad absorption band of CNT allows one to excite them using a red or NIR light source, but without stimulating autofluorescence of cells or tissues. Despite the poor photoluminescence quantum efficiency of CNT, the efficient penetration of both the excitation light and the emitted NIR photoluminescence (1 to 1.8 micron) through tissues allows one to attain excellent signal-to-noise ratios. The photoluminescence quantum efficiency of CNT varies with surface coating and the content of metallic CNT. Coating of CNT with sodium cholate or PEGylated phospholipids provides higher photoluminescence quantum efficiency than for those stabilized in simple surfactants. Resonance coupling with gold nanoparticles followed by the plasmon-assisted energy transfer is another method for improving the photoluminescence intensity of CNTs.

Raman imaging is one of the most promising and powerful bioimaging modality provided by CNT-based probes.[38] Among the various vibration modes of CNTs, the Graphite-mode (G-band) in the 1500–1600 cm^{-1} range is the most powerful fingerprint Raman band for bioimaging. NIR excitation for Raman imaging allows one to minimize autofluorescence of biological specimen and photobleaching of CNTs.[38] The photoacoustic response of CNT is another promising modality for bioimaging. The underlying principle of photoacoustic imaging is that the optical excitation energy converted into ultrasonic waves by a biological specimen is detected using ultrasonic transducers. However, the differentiation of a disease condition such as a tumor from normal tissues by photoacoustic imaging is difficult without the use of any contrast agent. CNTs offer excellent photo-to-acoustic conversion efficiency and photothermal-acoustic response, which make these materials one of the most promising contrast agents in photoacoustic imaging of tumors. Thus, CNTs conjugated with tumor targeting molecules such as RGD peptide and anticancer antibodies emerge into promising materials for biomedical photoacoustic imaging.[39] Additional bioimaging modalities can be incorporated to CNT by the conjugation of radionuclides, fluorescent dyes, other nanoparticles, or inorganic complexes as contrast agents of MRI, CT, PET, SPECT, *etc.* Selected bioimaging applications of CNT are summarized in Fig. 10.

Therapy. With the introduction and expansion of the surface chemistry of CNT and the successive applications of functionalized CNT towards cancer imaging and drug and gene delivery, CNT has become popular in the nanomedicine settings for the treatment of cancers and microbial or fungal infections. Therapeutic potentials of CNT can be classified into two main categories: therapy *via* oxidative stress and photothermal effect induced by CNT,[40] and therapy *via* drug and gene delivery using CNT as a cargo delivery system. Yet another classification of CNT-based cancer therapy is chemotherapy, gene therapy and phototherapy. As discussed in the previous section, a spectrum of anticancer drugs such as doxorubicin, methotrexate, camptothecin, paclitaxel and cisplatin can be conjugated covalently or non-covalently to CNT with excellent drug to CNT ratios. These conjugates can be selectively delivered in cancer cells or tumor milieu using various cancer targeting molecules discussed in the previous section. Subsequent release of the drugs from CNT by chemical, physical, photochemical, or photothermal activation has shown remarkable therapeutic effects on tumor models derived from gliomas, breast cancers, hepatoma, and cancers of the kidneys and pancreas. In gene therapy, CNTs are used for the targeted as well as controlled release of siRNA/DNA, which is discussed in the previous section. Efficient intracellular delivery of siRNA using CNT has been found useful in the suppression of tumor growth *via* the activation of necrosis or the inhibition of hypoxia inducible factor-1 α, which is instrumental in the regulation of growth hormones such as insulin-like growth factor-2 and transforming growth factor-α. Similarly, CNT can be used as a vector in regenerative medicine. Phototherapy using CNTs can be classified into photodynamic therapy, photo-acoustic therapy and photothermal therapy. In photodynamic therapy, CNT or a CNT-photosensitizer conjugate is first delivered in cancer cells or tumor milieus, or applied to areas affected by bacteria or fungi. The subsequent photosensitized production of singlet oxygen and other ROS by either CNT or the photosensitizing drug delivered using CNT results in the damage of vital biomolecules and the destruction of cancer cells, bacteria or fungi. Photo-acoustic and photothermal therapies are the most promising therapeutic modalities offered by CNT.[40] Photoactivation of CNTs using NIR (700–1100 nm) light or radio frequency results in the considerable local heating and

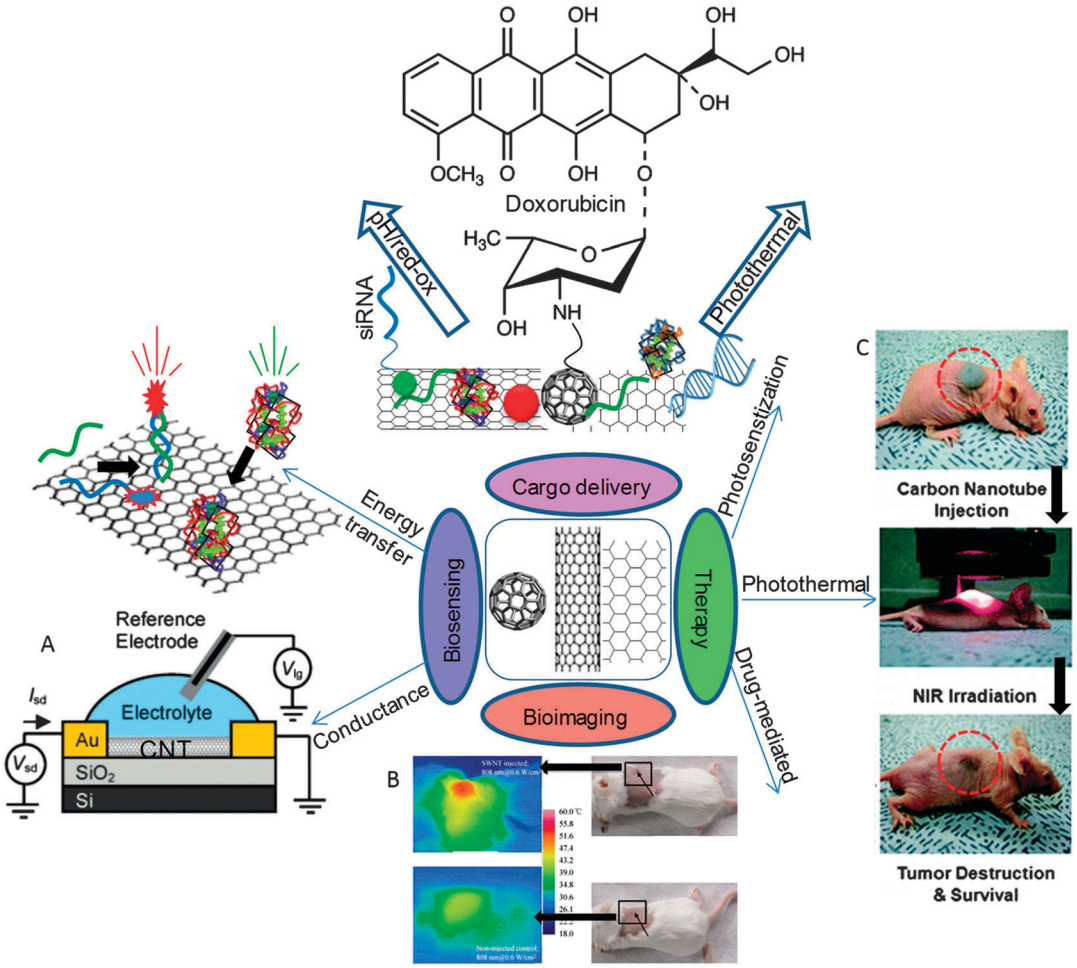

Fig. 10 Selected biological applications of functionalized CNT, fullerene and graphene: (a) scheme of a CNT-based electrochemical sensor, (b) thermal images of mice collected during photothermal treatment with (top) and without (bottom) injection of CNTs, (c) photographs of a tumor-bearing mouse intratumorally injected with PEG–SWCNT complex and recorded before, under and after NIR photothermal treatment. Reprinted with permissions from (A) I. Heller *et al.*, *Nano Lett.*, 2008, **8**, 591–595 (copyright ACS 2008); (B) J. T. Robinson *et al.*, *Nano Res.*, 2010, **3**, 779–973 (copyright Springer 2010); and (C) K. Y. Moon *et al.*, *ACS Nano*, 2009, **3**, 3707–3713 (copyright ACS 2009).

photothermal ablation of cancer cells and solid tumors. Although non-targeted CNTs conjugated with PEG show excellent biodistribution and passive localization in tumors, targeted labeling of cancer cells or tumor milieu using CNTs functionalized with antibodies or ligands/peptides against cancer markers can provide better results. Selected therapeutic applications of CNT are shown in Fig. 10. In short, the exceptionally high ability of CNT to load anticancer drugs, photosensitizers and genes, and its enormous efficiency to produce a photothermal effect place CNT in the front line in cancer nanomedicine and regenerative nanomedicine.

5.2. Buckminster fullerene (C_{60})

Fullerenes exist in various magic number forms from small ones such as C_{20} to giant icosahedral C_{540} in both natural and man-made sources. Among these, the soccer-ball-shaped or truncated icosahedron Buckminster fullerene (C_{60}) is the most abundant form. Although C_{60} was first detected in a soot sample prepared by the laser vaporization of graphite, its macroscopic scale synthesis has become possible with the introduction of the Krätschmer–Huffman method, where a vacuum arc discharge using graphite rods produces carbon soot enriched with C_{60}.[41] Although many other methods such as low pressure helium sputtering and electron beam evaporation of graphite targets were introduced over the above two classical examples, combustion of a laminar flow of benzene and oxygen is the current commercial method for the production of C_{60} and C_{70}.[42] Hydrocarbon impurities present in samples prepared by the above methods are removed by extraction with diethyl ether. Successively, fullerenes are separated by column chromatography or HPLC. With such large-scale production of fullerenes and successive optimization and characterization of their physical and optical properties, applications of C_{60} in the engineering and biomedical fields became matured. The broad absorption of light in the UV-Vis region, photothermal effect, the ability to accommodate multiple electrons and endohedral metal atoms, long-living triplet state, and singlet oxygen production are the interesting properties of C_{60}. Diagnostic and therapeutic

applications of functionalized fullerenes are the most exciting ones in the biomedical field.

5.2.1. Functionalization of C_{60}. The first step in the functionalization of C_{60} is its dissolution in an organic solvent. Fullerene is soluble at a few to several mg mL^{-1} in solvents such as chloronathpthalenes, chlorobenzenes, CS_2, xylenes, toluene, and benzene. However, it is essentially insoluble in common laboratory solvents such as hexane, chloroform, alcohols, ethyl acetate and acetone, which is improved upon functionalization. Covalent conjugation of molecules to C_{60} can be carried out by various free-radical reactions, cyclopropanation, or cycloaddition reactions such as [1+2], [2+2], [3+2] and [4+2] reactions. Furthermore, organometallic chemistry and coordination chemistry are explored in the conjugation of transition metal-based molecules to C_{60}. Among these reactions, cyclopropanation and cycloadditions of carbenes, nitrenes, aziridines, azomethine ylides, and 1,3-dienes are widely investigated.[43] The chemical reactions of C_{60} follow essentially the same steps and mechanisms of that of CNT. Thus, a separate scheme for the functionalization of C60 is not provided. Functional groups such as carboxylic acids, amines and carbohydrates are valuable for the bioconjugation and biological applications of C_{60}. The functional groups derived from cycloaddition reactions not only improve the solubility of C_{60} but also enable the conjugation of photosensitive and biologically active molecules such as ferrocenes, porphyrins, phthalocyanines, carotenes, cyclodextrins, dendrimers, peptides, proteins, DNA, and so on to C_{60}. Bioconjugation of C_{60} is mostly carried out by the reactions of amine-, carboxylic acid- or thiol-functionalized C_{60} using succinimides, carbodiimides, maleimides or thiols. Nevertheless, as the number of functional groups or cycloadduct increases, the basic properties of C_{60} due to its truncated icosahedral structure and π-electron cloud largely deviate from the original ones.

5.2.2. Biological applications of C_{60} and functionalized C_{60}. Biological applications of C_{60} derivatives are primarily on radioimaging, gene/drug delivery, oxidative stress reduction and cancer therapy. Due to their efficient distribution in the blood and localization in organs, functionalized fullerenes are widely exploited in angiography and organ-specific imaging.[44] Furthermore, biocompatibility and the non-toxic nature of C_{60} functionalized with multiple carboxylic and hydroxyl groups, dendrimers, proteins, amphiphilic polymers and carbohydrates are added advantages for biological applications. Multiple iodine functionalized C_{60} is a promising X-ray contrast agent, which shows prolonged retention in the blood and excellent *in vivo* compatibility when compared with other clinically available X-ray contrast agents such as Iopamidol and Iohexal.[44] Endohedral metallo-fullerenes with Gd, ^{166}Ho or ^{99}Tc, or C_{60} with multiple fluorine atoms are promising contrast agents for MRI, single photon emission computed tomography (SPECT), and radio tracing.[44] One of the advantages of metallo-fullerenes over inorganic nanoparticles and coordination complexes of metal ions is that the toxicity due to the release of metal atoms/ions can be reduced in the former case due to the stable caging effect provided by C_{60}. Conjugates or complexes of fullerene with polyamines, amphiphilic molecules, peptides, or lipids/liposomes are exploited in the construction of gene and drug delivery systems. Also, direct conjugates or complexes between DNA and fullerene can be used in the construction of transfection complexes. One of the early excitements about biological applications of C_{60} is the growth control of human immuno virus (HIV I), where C_{60} fits well within the hydrophobic cavity of HIV I protease and inhibits its enzymatic activity.[45] The radical scavenging property and the deactivation of both reactive oxygen species and glutamate by C_{60} polyols and polycarboxylic acids are useful for the reduction of oxidative stress, deactivation of transforming growth factor, and the control of apoptosis and neurodegenerative diseases. In contrast, photoactivation of C_{60} yields singlet oxygen in high efficiency due to the efficient intersystem crossing. Polyols of fullerene are also ideal candidates for the control of osteoporosis, where the affinity of C_{60} for hydroxyapetite is exploited to target bone tissues.[44] Antimicrobial activity of fullerene derivatives is another interesting biological application, where the toxic effects to bacteria are brought about by the insertion of fullerene into the cell membrane. In spite of these diverse biological applications, C_{60} also shows certain adverse effects in biological systems, which include oxidative stress, inhibition of neurotransmitters and enzymes, DNA damage, immune response, and the production of anti-fullerene antibodies. Although biological applications of C_{60}, such as drug and gene delivery and bioimaging are considerably limited by its small size (<0.8 nm), the broad absorption of visible light and the efficient production of singlet oxygen are promising parameters for photodynamic therapy.

5.3. Graphene

Like CNTs and fullerenes, graphene is an allotrope of carbon in the sp^2-hybridized state. It differs from CNTs and fullerenes in its 1 atom layer 2D structure. Graphene, which is the basic building structure of graphite, is first isolated by the exfoliation of graphite using an adhesive tape.[46] Interactions of the π-electron cloud in the 2D structure of graphene with the period potential of its crystal lattice produce quasiparticles, which can be defined with the Dirac Hamiltonian. With the finding of Dirac like fermions, graphene has become popular in the physics, nanomaterials and nanotechnology areas. Despite the high mobility of charge carriers and extremely low resistivity, the conductivity of graphene is low, which can be improved by doping with impurities. The zero-band gap of graphene allows absorption of light in the UV-Vis-NIR region, which under selected conditions can be tuned further to the microwave and terahertz regions. More recently, epitaxial growth of graphene by the high temperature reduction of silicon carbide under low pressure has become popular for the production of graphene with anomalous quantum Hall effect. Epitaxial growth on iridium, chemical vapor deposition on nickel or copper from carbon sources such as methane, and the reduction of graphite oxide using hydrazine are other routine methods for the large scale production of graphene.

5.3.1. Functionalization of graphene. Poor dispersion in liquids is one of the challenges associated with the functionalization

of graphene prepared by the exfoliation method. However, ultrasonication in solvents such as pyridine, *N*,*N*-dimethyl formamide, benzylamine, and perfluorinated hydrocarbons provides reasonable dispersion. Graphene samples prepared by the oxidative methods or by the reduction of graphene oxide carry carbonyl and carboxyl functional groups on both faces and around the edges. Carbonyl groups can be coupled with primary amine-containing molecules to form Schiff's bases. Carboxylic acids can be conjugated with proteins or other amine-containing molecules by EDC or DIC coupling to form amides or with alcohols, anhydrides or alkyl halides to form esters. Also, carboxylic acid-functionalized graphene reacts with thionyl chloride to form highly reactive acid chloride, which readily condenses with primary or secondary amine-containing molecules. Carboxylic groups present in graphene and graphene oxide often become the point of conjugation of DNA, proteins, polymers, and drugs. Like with CNTs and C_{60}, non-covalent functionalization of graphene and graphene oxide is practiced with polymers, aromatic hydrocarbons, DNA, *etc.* through hydrophobic interactions or π-stacking. Due to the structural similarities, most chemical and bioconjugate reactions discussed in the cases of CNT can be applied for the covalent functionalization of graphene as well. Thus, a separate scheme for the chemical functionalizations and bioconjugate reactions of graphene is not provided (Fig. 9).

5.3.2. Biological applications of functionalized graphene. Although major applications of graphene are in the photovoltaic, optical and electronic fields, recent studies show their potentials for biological applications such as biosensing, drug and gene delivery, bioimaging and phototherapy (Fig. 10). Fundamental properties of graphene underlying such applications are the large surface area, electrical conductivity, characteristic Raman and SERS bands, and the ability to act as an excellent acceptor of energy.

Biosensing. Changes in the electrical conductivity of graphene due to its charge-transfer interactions with adsorbents such as gases, glucose, metal atoms and ions, organophospates, H_2O_2, hydroxylated hydrocarbons, antibodies, DNA, proteins and enzymes are extensively exploited in biosensing.[47] For example, glucose sensing, which is clinically relevant in the screening of diabetic conditions, can be carried out by the measurement of the electrical conductivity of graphene during the binding of glucose or glucose oxidase. Oligonucleotides and nucleic acids can be electrochemically detected using graphene by the monitoring of the oxidation potential of graphene during the electrostatic binding to graphene or binding to complementary DNA strands tethered to graphene. Similarly, graphene adsorbed or conjugated with antibodies are excellent electrochemical immunosensors. In addition to the electrical conductivity and electrochemical behavior, the G and D Raman bands of graphene, which are highly sensitive to molecular adsorption, can be followed for the sensing of organic dyes, DNA, and proteins.[47] Further, the broad absorption band of graphene makes it an ideal acceptor of energy in Förster-type donor–acceptor systems, in which quenching of the fluorescence of dye molecules attached to DNA or other biomolecules by graphene is extensively investigated for the sensing of ssDNA, analysis of the melting of DNA duplexes, detection of single-base mismatches, *etc.* Nonetheless, the selective excitation of energy donors in such donor-acceptor pairs becomes often challenging due to the broad absorption band of graphene.

Gene and drug delivery. The large surface area, two dimensional π-stacked structure and CNT-like surface chemistry of graphene and graphene oxide allow one to chemically conjugate or physically adsorb a large amount of cargos such as contrast agents, siRNA, pDNA, anticancer drugs, and antibodies. Like CNT, one of the main advantages of graphene for cargo delivery is to increase the local concentration of drugs/genes/contrast agents and improve the efficiency of therapy and imaging. The structural similarity of graphene to CNT allows one to adopt the well-defined chemistry and bioconjugate chemistry of CNT discussed in the previous section (Fig. 9) during the conjugation of cargos and targeting molecules to graphene. Moreover, the introduction of reactive functional groups such as amino, carboxyl, hydroxyl, alkyne, azide, alkyl halides and thiol, which is shown in Fig. 2, 5 and 9, allows one to conjugate cargos through photo-, redox- or pH-sensitive linkers. Also, cargos can be loaded onto graphene by the direct π-stacking in the cases of aromatic drugs and fluorophores or the indirect π-stacking mediated by aromatic hydrocarbons such as pyrene linked to the cargo. Next, the drug, gene or contrast agent carried by graphene can be targeted to cancer cells or tumor milieu by the conjugation of molecules such as RGD peptide, growth hormones, ligands and anticancer antibodies to graphene. Subsequently, the site-specific delivery or release of cargos can be carried out using the photothermal effect provided by graphene, differences in the pH conditions, and the chemical reduction or photouncaging of cargo-to-graphene bonds. Such, photo-, redox- or pH-sensitive delivery of pDNA and siRNA to cells is accomplished using conjugates of graphene and polymers such as PEG, polyethylene imine or chitosan. Recently, the *in vitro* and *in vivo* treatments of cancer cells and tumors have become very efficient with the use of graphene and polymer-graphene nanocomposites conjugated or adsorbed with cancer targeting molecules such as anti-CD20 or folic acid and anticancer drugs such as doxorubicin or camptothecin.[48] Besides, the non-toxic nature and efficient clearance of graphene administrated *in vivo* suggest that bioengineered graphene can be efficient nanomedicine for cancer therapy.

Bioimaging and therapy. Just like in the case of CNTs, the fundamental properties of graphene valuable for bioimaging are the visible and NIR photoluminescence, characteristic Raman bands, and photo-acoustic and photothermal responses.[49] In particular, the NIR photoluminescence of graphene is a promising parameter for minimizing the autofluorescence during *in vitro* and *in vivo* bioimaging. Furthermore, multimodal imaging probes can be prepared by the conjugation of fluorescent moieties such as organic dyes, gold quantum clusters, semiconductor quantum dots, radionuclides such as

^{66}Ga, ^{99m}Tc, ^{125}I and ^{64}Cu, and magnetic materials such as Gd(III) complexes and super paramagnetic iron oxide nanoparticles to graphene or its derivatives.[50] Coating or conjugation of PEG to graphene helps us to improve the *in vivo* distribution of graphene-based imaging probes. As discussed in the previous section, targeted imaging of cancer cells and tumor milieu using graphene can be accomplished by the conjugation or adsorption of antibodies, peptides or ligands against cancer markers. The large photothermal effect of graphene has been recently investigated in the photothermal therapy of cancers and Alzheimer's disease.[48,49] Interestingly, *in vivo* photothermal treatment of tumors in mice treated with graphene oxide–Fe_3O_4–PEG composite under illuminations with a NIR laser shows a considerable decrease in the size of the tumor.[50] Furthermore, the unique 2D structure of graphene allows one to combine photothermal therapy with chemotherapy and photodynamic therapy on a single platform by the covalent or non-covalent conjugation and delivery of anticancer drugs such as doxorubicin and camptothesin and photosensitizers such as chlorine e6, perylenequinonoid and Zn-phthalocyanine. Excellent therapeutic effects are observed in such graphene-based combination therapy. In short, the large surface area, flexible surface chemistry and unique photoluminescence, Raman and photothermal properties of graphene show its great potentials for drug delivery bioimaging and cancer therapy, which can be improved by the construction of multimodal and multifunctional graphene arsenals for image-guided NIR phototherapy and chemotherapy of cancers.

6. Summary and outlook

Surface functionalized nanoparticles are extensively used as nanoscaffolds for various biological applications ranging from bioanalysis and bioimaging to diagnosis and therapy. This review article mainly focuses on the chemical and bioconjugate reactions on the surfaces of nanomaterials such as silica nanoparticles, gold nanoparticles and quantum clusters, semiconductor quantum dots, carbon nanotubes, fullerene and graphene. The chemical and bioconjugate reactions discussed in this article formulate these nanomaterials for various biological applications such as biosensing, bioimaging, drug and gene delivery, and therapy. The large surface to volume ratio is the common property among these nanomaterials, which is useful for the conjugation of various sensors, targeting molecules, contrast agents, drugs and genes at high local concentrations. Furthermore, the fundamental optical properties of these nanomaterials show certain common features that are valuable for multiplexed biosensing and bioimaging, and phototherapy. For example, the shape- and size-dependent tunable plasmon of gold nanorods is comparable with the size-dependent tunable photoluminescence of semiconductor quantum dots and gold quantum clusters. Other common properties of nanomaterials are the photothermal responses of silica nanoparticles, gold nanorods, carbon nanotubes, fullerene and graphene, visible to NIR photoluminescence of gold quantum clusters, semiconductor quantum dots, carbon nanotubes and graphene, photoacoustic response of silica nanoparticles, gold nanorods, carbon nanotubes and graphene, and the photosensitized singlet oxygen production by gold quantum clusters, semiconductor quantum dots and C_{60}. Furthermore, many functional groups and chemical and bioconjugate reactions are common to the formulation of these nanomaterials into biosensors, imaging probes, drug and gene delivery systems, and nanomedicine. Although multimodal and multifunctional nanoscaffolds constructed by the conjugation of various targeting molecules, drugs, genes and contrast agents to nanomaterials are extensively investigated in the imaging and treatment of cancer cells and tumors, controversial reports about toxicity and pharmacokinetics hamper the clinical applications of nanomaterial formulations. Therefore, a number of issues such as biocompatibility, toxicity, *in vivo* and *in vitro* targeting efficiency, bioavailability, renal and hepatobileary clearance, and long term pharmacological and toxicological issues of functionalized nanomaterials are under rigorous investigation. The current scenario of research in the formulation and testing of bioengineered nanomaterials as sensors, contrast agents, drugs, and drug and gene delivery systems suggest that the complete transformation of conventional medicine into nanomedicine cannot be too far away.

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