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Multifunctional Nanoparticles as Biocompatible Targeted Probes for Human Cancer Diagnosis and Therapy

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Abstract

The use of nanoparticles in biological application has been rapidly advancing toward practical applications in human cancer diagnosis and therapy. Upon linking the nanoparticles with biomolecules, they can be used to locate cancerous area as well as for traceable drug delivery with high affinity and specificity. In this review, we discuss the engineering of multifunctional nanoparticle probes and their use in bioimaging and nanomedicine.

1. Introduction

Cancer is the second leading cause of death, with millions of newly diagnosed cases worldwide each year. In the United States, it is estimated that one out of four deaths is cancer-related. Since the early 1970's, the survival rate has only improved by ~10% as more powerful imaging tools have been made for early cancer detection. The yearly cancer death rate for men and women over the past few decades has not significantly decreased despite the emergence of new drugs and surgical techniques. The main reason for this dismal picture is that even with the current state of the art of cancer diagnosis, usually this disease is detected in an advanced stage, when the primary tumor has metastasized and invaded other organs, and is beyond surgical intervention. In addition, current chemo and radiation therapies have relatively poor specificity toward malignant tissues. Therefore, in order to advance cancer prevention and therapy, two important issues must be addressed, (a) fabrication of novel probes for diagnosis of this disease at an earlier, and therefore curable, stage, and (b) development of potent, targeted therapeutic methods that would selectively eliminate only diseased cells/tissues, without causing collateral damage.

A synergistic combination of nanotechnology and biotechnology provides unprecedented opportunities for addressing many of the current challenges in cancer diagnosis and therapy¹. In particular, multifunctional nanoparticles carrying multiple diagnostic probes can provide both structural and metabolic information specifically from diseased sites, thus leading to significantly improved imaging techniques for the detection of a variety of human cancers (e.g. breast, pancreatic, lung, and prostate), including improved staging for occult metastases². In addition, these nanoparticles can carry therapeutic payloads (anticancer drugs, siRNA, etc.) and efficiently deliver them to cancer sites in a target specific manner. Moreover, the unique properties of various materials at the nanoscale have given rise to novel and localized therapeutic approaches such as photothermal and magnetic hyperthermia therapies, which have already shown promising anticancer efficacy. Also, since nanoparticles are capable of carrying both diagnostic and therapeutic probes, it is possible to monitor the therapeutic action in real time. With further development of these directions, nanotechnology has the promise to revolutionize cancer diagnosis and treatment in the coming years.

In the field of nanobiotechnology today, there are broadly two types of nanoparticles used for biomedical applications, which are inorganic and polymeric, respectively². Polymeric nanoparticles are flexible and biodegradable, and therefore, can be used for controlled release of encapsulated biomolecules such as anti-cancer drugs³. However, these nanoparticles tend to be of relatively large size (200–500 nm) and thus, may not be ideal for drug delivery to solid tumors and metastatic sites. The stability of these particles on systemic administration (as opposed to topical delivery) may be compromised, and finally, these particles may lead to development of an immune response which might counteract therapy. Inorganic nanoparticles, on the other hand, can be synthesized in the nano-size range (5 – 30 nm)⁴, which not only renders them suitable for unimpeded circulation in the bloodstream and extravasation into tumor tissues, but also permit excretion through renal filtration, without the need of their biodegradation⁵. Several such materials, including silica⁶, gold nanoshells^{7,8}, and gold^{9,10} nanoparticles are known for their non-antigenicity. Many such materials have unique characteristics by virtue of which they can also be used as image contrast agents, including quantum dots (QDs)¹¹ and iron-oxide nanoparticles. However, their rigid matrix does not generally allow for encapsulating and subsequently releasing active molecules for conventional drug delivery purposes. Nonetheless, drug molecules can be linked onto the nanoparticle surface for targeted delivery purpose.

This review is focused on the latest advances from our group on the synthesis of multifunctional nanoparticles intended for application in early detection and treatment of human cancer. We describe the use of solution and vapor phase synthesis methods to produce biocompatible QDs, quantum rods (QRs), organically modified silica particles (ORMOSIL) and gold nanorods (NRs), and thorough characterization of their physical properties. In addition, bioimaging and therapeutic applications of these novel nanomaterials will be described. The QDs and QRs are particularly interesting from the point of view of their tunable optical properties, which strongly depend on the particle size, shape, composition, and surface coating^{12–14}. The benefits of using nanoparticles for biomedical applications are first briefly discussed in section 2. The basic methods of synthesizing QDs are described in section 3, since their composition and sizes directly affect their final optical properties. Section 4 describes our recent results on fabrication and functionalization of QRs. Section 5 summarizes our strategy of using various suitable additives (e.g. co-surfactants and electrolytes) to tailor the morphology of gold NRs. Section 6 focuses on using a microemulsion technique to synthesize biocompatible organically modified silica (ORMOSIL) nanoparticles and methods to functionalize them. Section 7 briefly discusses the use of ORMOSIL nanoparticles for photodynamic therapy (PDT) of cancer. The use of QDs as targeted bioconjugates for specific labeling of cancer cells is described in section 8. Section 9 focuses on using functionalized QRs for confocal, two-photon, and multiplex imaging of cancer cells. Section 10 considers the employment of QDs as targeted anticancer drug “nanocarriers” for selective destruction of cancer cells. Section 11 demonstrates the potential of functionalized QDs and QRs as early detection cancer probes for *in vivo* targeted imaging. Section 12 describes the use of gold NRs as multimodal optical contrast agents for dark-field and transmission electron microscopy imaging of cancer *in vitro*. Section 13 discusses the use of ORMOSIL nanoparticles for selective cancer labeling *in vitro*. Finally, section 14 briefly describes the *in vivo* distribution, clearance, and toxicity of these functionalized nanoparticles.

The authors wish to stress that there many more varieties of biocompatible multifunctional nanoparticles (e.g. PET-, SPECT-, CT-, and MRI-sensitive nanoprobe) reported in the literature and show promising for biomedical applications. However, due to the page limits in this feature article, the authors were forced to narrow down the scope of this feature article that are most closely related to their research. The authors sincerely apologized for not mentioning, including, or citing other nanoparticles formulation reports in this article that fit into the category of “multifunctional nanoparticles”.

2. Functionalized nanoparticles for biomedical applications

Nanoparticles provide a novel class of biocompatible contrast agents that has great potential for cancer imaging and therapy¹⁵. The use of nanoparticles for bioimaging has several advantages. First, the sensitivity of optical imaging can be significantly improved using functional nanoparticles. For example, luminescent QDs and QRs have been used as contrast agent probes for cancer imaging^{16,17}. QDs and QRs are usually crystalline cadmium selenide (CdSe)-based semiconductor nanoparticles. They are ultra-small and bright, and have a narrow luminescence emission spectrum¹⁸. Optically-doped nanoparticles such as dye-doped organically modified silica particles (ORMOSIL) represent another important class of nanomaterials for cancer detection and therapy. In dye-doped nanoparticles, each silica particle typically contains hundreds to thousands of dye molecules, which enhances the fluorescence signal. Dye-doped nanoparticles are generally smaller than cells, which make them suitable for intra-cellular application. Gold NRs have been well studied for the last few years and have been successfully used for dark field imaging of cancer cells and for photothermal therapy.

Nanoparticles can be much more photostable and biocompatible (if properly functionalized) than traditional chromophores (e.g. organic dye molecules). This forms the basis for their use for ultrasensitive and real-time monitoring of cancer progression. Photostable nanoparticles will allow non-invasive imaging of cancer tissue multiple times for monitoring tumor growth and the effect of cancer drugs during therapy¹⁹. For example, QDs and QRs are extremely photostable and exhibit low toxicity if their surface is carefully passivated. The effective surface passivation of QDs or QRs with a wide-band gap semiconductor material such as zinc sulfide (ZnS) will make them photostable and lower their toxicity. In the case of optically-doped ORMOSIL nanoparticles, chromophores remain embedded in the particle-matrix, which protects the dyes from photobleaching and prevents the dyes from interacting with the biological environment. This allows the safe use of dyes that are known to be toxic. Improved photostability of the embedded dyes is achieved because the silica particle matrix protects the dye from interaction with oxygen molecules that otherwise causes the chromophores to degrade irreversibly.

Finally, multiple functionalities can be combined in a single or multi-component nanoparticle, allowing simultaneous monitoring via multiple modalities²⁰. If a nanoparticle incorporates two or more contrast agents, it can be used for precise location and non-invasive imaging using positron emission tomography (PET), CT, and magnetic resonance imaging (MRI) simultaneously for pre-surgical assessment. During the surgical process, tumor tissue could be directly visualized in real-time, using fluorescent or luminescent nanoparticles. This mode of tumor visualization would provide direct guidance to surgeons for effective and complete tumor resection, enabling them to clearly differentiate the boundary between the tumor and normal tissues. Already, some of these nanosystems have been successfully used *in vitro* and *in vivo* for diagnostic imaging purposes, prominent examples being dye-doped silica, organically modified silica (ORMOSIL) nanoparticles, dextran coated iron-oxide nanoparticles and targeting QDs. Our group has extensively demonstrated the utility of these multifunctional nanosystems for therapy, including drug and gene delivery. Examples of such nanomaterials created and applied in our laboratory include (a) magnetic 'nanoclinics' which can selectively target to tumor tissues, followed by application of a DC magnetic field resulting in local cytotoxicity²¹; (b) ORMOSIL nanoparticles, which serve as carriers of photosensitizing anticancer drugs for photodynamic therapy (PDT)²²; and (c) ORMOSIL nanoparticles that carry genetic materials for robust non-viral gene delivery *in vivo*, with efficiency comparable to or better than engineered viruses²³. These studies strongly underline the need for further applied research in the field of inorganic-polymeric hybrid nanomaterials in order to fully exploit their diagnostic and therapeutic potential in human cancer.

3. Synthesis and Surface Functionalization of Quantum Dots (QDs)

When the diameter of a semiconductor nanocrystal becomes comparable or smaller than the Bohr exciton radius of the material from which it is made, the energy levels (conduction and valence bands in the bulk) become size dependent and increasingly made up of discrete states rather than continuous energy bands^{24–26}. Such semiconductor nanocrystals are called “Quantum Dots” (QDs)²⁷. QDs are luminescent semiconductor nanocrystals with sizes ranging from 3 to 10 nm. These contain hundreds to thousands of atoms, and may be made up of group II–VI elements (e.g. CdSe, CdTe, CdS, and ZnS), group III–V elements (e.g. InP), group IV–VI elements (e.g. PbS and PbSe)²⁸, or group IV elements alone (e.g. Si and Ge)¹⁵. Because of their unique tunable spectral and optoelectronic properties, QDs have already been extensively used for a variety of biomedical research applications over the past few years. These materials have significant advantages over traditional fluorescent probes such as organic dyes and fluorescent proteins. QDs of multiple emission colors can be simultaneously excited with a single light source, with minimal spectral overlap, providing significant advantages for multiplex imaging of molecular targets (see Figure 1). QDs have been demonstrated to remain brightly emissive even after long periods of excitation that would lead to rapid photobleaching of organic dyes²⁹. More importantly, QDs can be systematically tailored and manipulated to emit in a range of wavelengths by changing their size, shape³⁰, and composition²⁸, whereas new organic dyes must be fabricated to shift their emission towards optically detectable wavelengths in an *in vivo* system. This tunability allows the synthesis of QDs that emit in the near infrared (NIR) spectrum, which is optimal for deep tumors such as lung, pancreatic, breast, and prostate cancers. Thus, because of the size-tunable luminescence emission from the visible to infrared wavelength, broad absorption spectra, and high levels of brightness and photostability of QDs, these nanomaterials have recently attracted much attention as new generation probes for diagnostic imaging.

Hot colloidal synthesis is the most popular route to synthesize monodispersed, high quantum yield QDs. Bawendi's^{31–33} and Alivisatos's^{34–36} group are among the pioneers, who introduced the use of the hot colloidal synthesis approach to fabricate monodispersed QDs made from cadmium sulfide (CdS), cadmium selenide (CdSe), or cadmium telluride (CdTe). Following their discovery, the synthetic chemistry for CdSe QDs quickly received intensive attention and advanced, generating brightly luminescent QDs with emission wavelengths spanning from the visible to the near infrared spectral regions. As a result, CdSe has become the most popular chemical composition for QDs. Cadmium precursors such as cadmium oxide, dimethyl cadmium, cadmium carbonate, and cadmium chloride are commonly used in the QD synthesis chemistry³⁷. The selenium (or tellurium) precursor is generally prepared by dissolving selenium (or tellurium) powder in trioctylphosphine (TOP) or tributylphosphine (TBP)³⁸. When the Cd and Se precursors are mixed in the presence of surfactants at high temperatures, a short nucleation burst occurs, followed by slower particle growth. The surfactants used in the QD synthesis not only prevent their aggregation, but also yield monodispersed, functionalized, and stabilized semiconductor nanoparticles. For example, trioctylphosphine oxide, trioctylphosphine, oleic acid, myristic acid, oleylamine, and stearic acid have been commonly used as surface active agents that bind to the QDs to both limit particle growth and create a stable colloidal dispersion.

In our group, four types of QDs have most often been prepared. Their composition, sizes, surface coating, and surface charges were specifically tailored for advanced biomedical applications such as *in vitro* and *in vivo* targeted imaging. They are CdSe/CdS/ZnS core/shell/shell structured, CdTe/ZnTe core/shell structured, InP/ZnS core/shell structured, and silicon (Si) QDs. Both CdSe/CdS/ZnS core/shell/shell and InP/ZnS core/shell structured QDs are prepared by hot colloidal synthesis process, where CdSe or InP is used as core to grow a thin layer of high-band gap semiconductor material such as zinc sulfide on their surface. Growing

a thin shell of a wide band gap semiconductor on the outside of the emitting nanocrystal allows substantial improvement of their optical and colloidal stability. CdTe/ZnTe QDs are specifically prepared by using a one pot green chemistry aqueous phase synthesis approach, without the need of aggressive chemical reactants and high boiling point solvents. Most importantly, their surfaces are passivated with small functional molecules that make their overall hydrodynamic size very small, which is suitable for *in vivo* studies. Lastly, Si QDs are prepared by laser-driven pyrolysis of silane, followed by HF-HNO₃ etching and surface functionalization. In the next few sections, we briefly discuss the synthesis methods for each type of QDs as well as their physical and optical properties.

3.1. CdSe/CdS/ZnS QDs

In our group, CdSe/CdS/ZnS QDs are prepared by growing a CdS/ZnS-graded shell on a CdSe core. Details of the preparation procedure can be obtained from our previous reports. Figure 2 shows a representative TEM image of the QDs, showing spherical particles with average diameter about 6 nm. The dispersion of the QDs in aqueous media is achieved by adapting the method from Chan's group³⁹. Basically, the surface of the QDs is modified by capping them with a hydrophilic lysine shell. These lysine-coated QDs served as a multifunctional platform for further conjugation to biomolecules. The quantum yield of the QDs is estimated to be ~40 to 60%, which is sufficiently high for cell labeling studies. These QDs also exhibit two-photon excited fluorescence, which makes them suitable for two-photon excited cellular imaging where cellular autofluorescence can be avoided.

QD bioconjugation is the linking of targeting biomolecules (e.g. antibodies, peptides, or DNA) to the QDs surface, forming a bioconjugate, having both the inorganic and the biological materials in one system. QDs are comparable in size, or slightly larger than, many proteins. In general, there are three major routes to attach biomolecules to the QD surface. First, using the carbodiimide chemistry, carboxylated QDs are covalently conjugated to specific biomolecules through the formation of stable amide bonds. Second, disulfide bonds can be formed between surface sulfur atoms (from the ZnS surface) and peptides containing cysteine residues. Lastly, positively charged proteins can be non-covalently adsorbed onto the negatively charged QDs surface through electrostatic interactions. For specific targeting, it is highly desirable that the targeting proteins (e.g., antibodies) are properly oriented and fully functional. Thus, proper QD bioconjugation is extremely important in obtaining success in targeted *in vitro* and *in vivo* imaging.

In our approach, the carbodiimide chemistry was used to attach different targeting molecules to the lysine coated QDs. This method is simple and straightforward. The main requirement for successfully synthesizing targeted bioconjugates is optimizing the ratio of lysine coated QDs to targeting biomolecule concentration. Antibodies and proteins generally have multiple reactive sites and can cause aggregation to take place if the reactant concentrations are not optimized.

3.2. InP/ZnS QDs

InP QDs are an example of group III-V semiconductor nanocrystals⁴⁰, which are well known for structural robustness. This results from the greater degree of covalent (as opposed to ionic) bonding in these materials, relative to those made up of II-VI semiconductors (such as ZnSe, CdSe and CdTe)⁴¹. This structural robustness leads to enhanced optical stability, and most importantly, reduced toxicity as a result of reduced possibility of release of constituent heavy metal ions. We found that the optical properties of the core-shell structures strongly depend on the nature of the shell around the InP. By simply coating the InP core with a ZnS, ZnSe, or CdSe shell, the emission wavelength can be systematically tuned from ~525 to ~590 and from ~590 to ~680 nm, respectively. These features have made InP QDs a very attractive candidate

for replacing cadmium-based QDs for biological studies. Details of the preparation procedure can be obtained from our previous reports. The dissolution of QDs in the aqueous phase was achieved by a surface ligand exchange process, in which mercapto acid was used to displace the existing hydrophobic moieties on the QD surface, thereby rendering them dispersible in aqueous buffer.

3.3. CdTe/ZnTe QDs

Direct aqueous synthesis of QDs using short carbon chain thiols as stabilizing agents provides a unique alternative to the widely used hot colloidal synthesis approach⁴². The use of thiols as small surface capping molecules allows us to control the kinetics of the NCs synthesis, passivates surface dangling bonds, and provides stability, and surface functionality of the QDs. CdTe NCs capped by thioglycolic acid (TGA), 1-thioglycerol, and mercaptoethylamine, have found applications in nanotechnology. However, the use of these particles for biomedical applications is still in its infancy, due to their colloidal instability. This is because these QDs are passivated by weakly bound ligands that are highly prone to desorb from the QD surface, allowing them to aggregate.

To overcome these challenges, our group has developed a facile synthesis approach to synthesize CdTe/ZnTe core/shell structured QDs with better colloidal stability and tunable optical properties by using a one-step aqueous phase synthesis method. Our approach is able to produce core/shell QDs with quantum yield as high as ~60%, in high yield. The emission wavelength of the QDs can be easily tuned from ~500 to ~850 nm by changing the core size (see Figure 5 and Figure 6) and composition (e.g. CdSe_xTe_{1-x}) of the dots. The high yield production and quantum yield of the QDs in aqueous media will allow them to be used in bio-imaging, biosensing, and various optoelectronic applications.

The main ingredients used to synthesize water-dispersible CdTe/ZnTe core/shell QDs are cadmium perchlorate, zinc perchlorate, tellurium powder, sodium borohydride and a biocompatible thiol-stabilizing reagent (e.g. cysteine, (mercaptopropionyl)glycine, glutathione, etc). These stabilizing agents possess amino and carboxyl groups, which can be readily conjugated with biomolecules once they are anchored to the QD surface. The ingredients used in the synthesis are inexpensive and less toxic than other precursors to these materials. Different emission spectra (500 to 700 nm) can be obtained by changing the refluxing time, precursor concentrations, type of surfactants, and composition of growth solution. In addition, near-infrared emitting nanocrystals can be obtained by doping the CdTe/ZnTe dots with mercury (Hg).

3.4. Silicon QDs

Silicon QDs are expected to be less toxic than group II–VI QDs. However, they have not been widely used in biomedical applications because of the challenges inherent to making them water-dispersible and compatible with biological fluids. Fabricating colloidal and optically stable water-dispersible Si QDs remains the most important challenge in making them useful for *in vitro* and *in vivo* studies⁴³. To date, very limited studies have reported the synthesis of water-dispersible Si QDs⁴⁴. For example, water dispersible Si QDs have been produced by functionalizing the QD surface with carboxylic acids or amines. Silicon QDs are generally made dispersible in organic solvents such as chloroform and hexane, because hydrophobic moieties (e.g. styrene and octene) can be easily anchored to the nanocrystal surface. However, Si QDs show poor photoluminescence and weak colloidal stability in aqueous media. Photoluminescence degradation occurs within a few days of storage after preparation of an aqueous dispersion of Si QDs using current reported methods. Therefore, new approaches to fabricate water-dispersible Si QDs, while maintaining spectral and colloidal stability continue to be of great interest. Recently, the synthesis of water-dispersible Si QDs with blue

luminescence upon anchoring allylamine onto silicon QD surfaces was reported. The use of red-emitting Si QDs grafted with poly(acrylic acid) for fixed-cell labeling was also reported. However, so far, these nanoparticles have not maintained satisfactory colloidal and spectral stability in biological fluids.

We have recently prepared highly stable aqueous suspensions of Si QDs using phospholipid micelles, in which the optical properties of Si nanocrystals are maintained⁴⁵. These luminescent micelle-encapsulated Si QDs were used as targeted probes for staining cancer cells. The Si QDs were prepared by laser-driven pyrolysis of silane⁴⁶, followed by HF-HNO₃ etching⁴⁷. Styrene, octadecene, or ethyl undecylenate was used to functionalize the Si QD surfaces, thereby rendering them dispersible in chloroform (see Figure 7)^{48,49}. Phospholipid micelles were then used to enable stable dispersion of Si QDs in water, generating a hydrophilic shell with PEG groups on its surface. This paves the way for silicon QDs to be a valuable optical probe in biomedical diagnostics.

4. Synthesis and Surface Functionalization of Quantum Rods (QRs)

The effect of the shape of quantum-confined materials has recently attracted considerable attention, because certain unique behaviors are expected in the evolution from zero-dimensional (0-D) QDs to one-dimensional (1-D) QRs or quantum wires (QWs)⁵⁰⁻⁶⁶. Semiconducting nanorods are of particular interest because they may serve as probes for cancer and specific biomolecule detection⁶⁶⁻⁷⁰. Recently, our group has synthesized CdSe⁷¹, PbSe⁷², PbS⁷³, and CdS⁷⁴ QRs by a hot colloidal synthesis method (see Figure 8, Figure 9, and Figure 10). These anisotropic nanomaterials are potential candidates for biomedical research tools such as targeted probes, biomarkers, biosensors, and contrast agents⁷⁵.

CdSe-based QRs show several advantages over the corresponding QDs for use as novel functional probes for biological and medicinal applications^{65,76}. For example, QRs have larger absorption cross-section, faster radiative decay rate, and larger surface area per particle, which facilitates functionalization with multiple binding moieties⁷⁷. Color control is achievable by tuning the rod diameter, which governs the band gap energy of CdSe-based QRs. The Stokes-shift is strongly dependent on the aspect ratio (length/diameter) of the rod. Furthermore, it was reported that CdSe-based QRs emitted light that was linearly polarized along the *c*-axis of the crystallites and that the degree of polarization was dependent on the aspect ratio. This feature can be used to enhance contrast by polarization selection. Also, QRs have larger surface area per particle as compared to QDs, which can facilitate bioconjugation with targeting/diagnostic/therapeutic molecules. These unique characteristics of QRs as biological markers may provide an avenue for further improvements in sensitive imaging strategies. However, investigations on the use of QRs in bioimaging and biomedical applications began only recently, promoted by the advancement of well-controlled synthesis and surface functionalized methods. Very recently, the Alivisatos group⁷⁸ and our group have independently demonstrated live cancer cell imaging using CdSe/CdS/ZnS QRs as targeting optical probes. Both reports have illustrated the advantages of QRs as long term biocompatible imaging probes. We have shown that these QRs can be used for two-photon fluorescence imaging of cancer cells, with reduced photo-damage compared to UV-excited imaging. The Alivisatos group has shown that QRs are brighter single molecule probes than QDs.

Figure 10 shows the aqueous dispersible lysine cross-linked MUA coated CdSe/CdS/ZnS QRs. From the figure, the average length and diameter of the QRs can be estimated to be 16 and 4 nm, respectively. The PL quantum yield (QY) of the CdSe/CdS/ZnS QRs is estimated to be ~40%. The high QY is not surprising because of the expected quantum confinement of the CdS/ZnS graded shell coated rod-shaped CdSe core. Also, as seen with the QDs, these QRs exhibit two-photon excited emission.

More recently, we demonstrate the synthesis of biocompatible nanoparticles consisting of QRs encapsulated in a SiO₂ shell as an alternative route for making them water-dispersible (see Figure 11). The SiO₂ coating is used as a nanopatform to make novel hybrid nanoparticle architectures for biological applications⁷⁹. The SiO₂ coating was introduced to the QRs through a reverse microemulsion method, using Igepal CO-520 as the surfactant and cyclohexane as the “oil” solvent. The presence of the surfactant allowed the hydrophobic QRs to be encapsulated within the aqueous region of the reverse microemulsion. Though SiO₂ coated QDs have been previously reported and used for labeling cells, we are not aware of any reports of fabricating SiO₂ coated CdSe QRs.

To demonstrate that the SiO₂ coated nanoparticles could be potentially used for bioimaging, the particles were employed to label cells. We used RAW macrophage cells for the *in vitro* study. Fluorescence confocal images of live macrophage cells after 24 hours incubation with the particles are shown in Figure 12. From the confocal image, it can be seen that a large quantity of the particles were internalized and accumulated in the cytoplasm by phagocytosis. Notably, no morphological damage was caused on the macrophage cells, indicating the low toxicity of the particles. This demonstrated that the SiO₂ coated QR particles are biocompatible, stable in cytoplasm, and suitable for *in vitro* cell labeling, cell tracking, and other bioimaging applications.

5. Synthesis and Surface Functionalization of Gold Nanorods (Au NRs)

Gold NRs have received great attention for the last few years due to their unique size-dependent optical properties that have found uses in optoelectronic devices, gene delivery, bio-imaging, and biosensors^{80–85}. Gold NRs also show fundamentally interesting properties, such as strong surface enhanced Raman scattering (SERS) and fluorescence enhancement^{86,87}. Gold NRs have two distinct plasmon resonance absorption bands, a longitudinal band corresponding to electron motion along the axis of the particle, and the transverse band corresponding to motion along the short axis of the particle⁸⁸. Generally, the absorption maximum for the longitudinal band shifts to longer wavelengths with increasing aspect ratio⁸⁹.

Several approaches are available for synthesizing NRs with different sizes and aspect ratios, including electrochemical synthesis in solution, membrane templating⁹⁰, photochemical synthesis, microwave synthesis, and seed mediated growth⁹⁰. Seed-mediated methods have been developed by Murphy’s group^{91,92}, where gold seeds were prepared by chemical reduction of gold salts with a strong reducing agent (sodium borohydride)⁹³. These seeds were then transferred to a growth solution containing additional gold salts, a weak reducing agent (ascorbic acid) and cetyltrimethylammonium bromide (CTAB), which acts as a surfactant and directing agent⁹⁴. The seeds served as nucleation sites for the anisotropic growth of gold nanoparticles. Later, research groups led by El-Sayed’s group improved the seed-mediated method. They synthesized gold NRs in high yield by adding cetyltrimethylammonium bromide (CTAB)-capped seeds, and prepared a series of NRs with different aspect ratios by adding silver ions to the growth solution.

In our approach, CTAB was used as the primary surfactant where the growth of rod shaped gold nanoparticles take place. Silver nitrate was used to promote growth of NRs. Non-ionic surfactants such as Tween and Triton and electrolytes such as sodium chloride and potassium chloride have been used as additives to tailor the shape, size, and aspect ratio of the gold NRs. It was discovered that with increasing concentrations of these additives in the growth solution, the aspect ratio of the NRs increases to a critical limit, after which it decreases again. Furthermore, upon carefully controlling the content of Triton X-100 or Tween 20 in the growth solution, these nonionic surfactants aided in fine-tuning the shape of gold NRs (e.g. to yield rectangular and “dogbone” shapes). Figure 13 shows a TEM image of typical monodispersed

gold NRs sample prepared with sodium chloride in the growth solution. The growth pattern of the NRs fits into the soft template model based on the micellar structures expected to form from the mixtures of CTAB and non-ionic surfactants. Figure 14 shows the visible absorption spectra of gold NRs with three different aspect ratios.

Gold NRs possess optical scattering properties that are very different from spherical gold particles. To demonstrate the dependence of optical scattering properties on nanorod aspect ratio, dark-field scattering images from gold NRs of different aspect ratio are shown in Figure 15. As expected, the longer rods preferentially scatter lower-energy orange/red light whereas shorter rods scatter blue-green and yellow light. Also, because both plasmon bands are in the visible for the short rods but only the transverse one is in the visible for the long rods, the short rods scatter more visible light and appear brighter than the longer ones (Figure 15). Our group has exploited this unique feature for *in vitro* optical scattering imaging and this subject will be further elaborated in section 13.

As mentioned earlier, CTAB is commonly used for the synthesis of gold NRs, at a concentration as high as 0.1 M. Other groups and we have observed adverse effects (cytotoxicity) on cells from CTAB at concentration as low as 10^{-5} M. This result strongly suggests that for Au NRs to be useful in biological applications, one has to eliminate or at least minimize the concentration of CTAB molecules to the level that is harmless to animal or human. To overcome these challenges, CTAB-coated NRs can be “wrapped” with multilayers of polyelectrolytes, to generate a charged polymer shell around Au NRs and mask the CTAB layer. This polymeric multilayering is essential in order to generate positively charged Au NRs, while masking the CTAB layer. These positively charged and coated NRs can then be linked to biomolecules with net negative charge, producing a neutral or slightly anionic complex bioconjugates for imaging cancer cells⁹⁵.

6. Fabrication of Functionalized Organically Modified Silica (ORMOSIL) Nanoparticles

Organically modified silica (ORMOSIL) nanoparticles are generally synthesized by condensation-polymerization of their organosilane precursor molecules, where one or more of the alkoxy ‘arms’ of the tetra-alkoxysilane precursor has been replaced by a hydrocarbon group. When this synthetic step is carried out in the confined dimensions of a microemulsion (either oil-in-water or water-in-oil), highly monodispersed and ultrafine (diameter less than 50 nm) nanoparticles are precipitated, which can be purified as an aqueous suspension through simple steps such as centrifugation or dialysis. Moreover, a number of bioactive molecules, either hydrophobic or hydrophilic, can be encapsulated within these nanoparticles. Since these particles are mesoporous, they can serve as sustained release vehicles for encapsulated biomolecules. In addition, the rich chemistry of silica can be exploited in order to synthesize particles presenting a variety of active groups (carboxyl, thiol, or amino) on the surface, which can be further modified to incorporate imaging/diagnostic probes, genetic materials, and biorecognition molecules, such as tumor-specific monoclonal antibodies. Thus, ORMOSIL can serve as an ideal nanopatform for the assembly of ‘multimodal’ nanoparticles for targeted diagnostics and therapeutics.

A number of precursor organo-alkoxysilanes can be used for the synthesis of these nanoparticles, either alone or in combination. These precursors can be roughly divided into two subgroups, (a) those which make up the core formulation such as octyl-triethoxysilane (OTES), vinyl-triethoxysilane (VTES) and phenyl-trimethoxysilane (PTMS), and (b) those which provide surface functional groups such as aminopropyltriethoxysilane (APTES) and mercaptopropyltrimethoxysilane (MPTMS). An ideal ORMOSIL nanoparticle containing a suitable functional group on its surface is usually formed from a combination of these two

types of precursors. These functional groups can be used either for the conjugation of various biorecognition molecules for target specific delivery, or reversible attachment of genetic materials such as DNA and RNA for gene delivery. Various surfactant molecules, such as Aerosol OT (AOT), Tween-80, Triton-X-100, pluronics, etc., in combination with the co-surfactant 1-Butanol, can be used to form the template micelles for the synthesis of ORMOSIL nanoparticles. The size of the nanoparticles can be tuned from ~10 to 100 nm diameter by varying the amounts of individual components such as surfactant, co-surfactant, precursor organo-alkoxysilane, etc.. In summary, ORMOSIL nanoparticles with desired sizes and surface properties can be easily synthesized from a variety of commonly available chemicals, thus allowing their “customized synthesis” for particular applications.

7. Functionalized ORMOSIL nanoparticles for Imaging Cancer Cells

Since ORMOSIL nanoparticles are optically transparent, they assume the optical properties of the various fluorophores that can be encapsulated/conjugated within them. Therefore, they can be used as a probe for targeted optical imaging. Our group has demonstrated the synthesis of amino-terminated ORMOSIL nanoparticles conjugated with the fluorophore rhodamine-B, using a combination of three precursors, VTES, APTES and rhodamine-silane (where the fluorophore is covalently linked to the precursor APTES prior to nanoparticle synthesis). Furthermore, we have shown that the surface amino groups can be used for bioconjugation with the pancreatic-cancer-specific monoclonal antibodies such as anti-claudin-4 and anti-mesothelin, which allowed for targeted delivery to pancreatic cancer (Panc-1) cells *in vitro*. Figure 16 presents confocal microscopic images of Panc-1 cells, clearly demonstrating a superior cellular uptake of the bioconjugated, as opposed to non-bioconjugated, nanoparticles.

In addition to conjugation with small molecule fluorophores, ORMOSIL nanoparticles can encapsulate other smaller nanoparticles, allowing the fabrication of a ‘multimodal’ diagnostic nanoplatfrom. Our group has demonstrated such a nanoplatfrom by co-encapsulating within them, two separate nanoparticles, namely QDs and iron-oxide nanoparticles⁶. The resulting nanosystem had both optical and magnetic properties. Using two-photon confocal bioimaging we have shown that they can be targeted to cancer cells *in vitro* using magnetic-field guidance. This targeting to cancer cells was further enhanced significantly by conjugating the tumor targeting molecule transferrin (Tf) on the surface of ORMOSIL, where a synergistic interaction between magnetic-guidance and transferrin-receptor mediated biotargeting was observed. Such experiments provide a foundation for the development of a broad class of ORMOSIL based multimodal nanoprobess that are expected to play a critical role in the next generation of diagnostic probes.

8. ORMOSIL nanoparticles for photodynamic therapy (PDT) of cancer

Photodynamic therapy (PDT) of cancer is based on the concept that light-sensitive species or photosensitizer (PS) molecules can be preferentially localized in tumor tissues upon systemic administration. When such photosensitizers are irradiated with an appropriate wavelength of visible or near infrared (NIR) light, the excited molecules can transfer their energy to molecular oxygen in the surroundings, resulting in the formation of reactive oxygen species (ROS) such as singlet oxygen, which are responsible for their therapeutic action. Therefore, under appropriate conditions, photodynamic therapy offers the advantage of an effective and selective method of destroying diseased tissues without damaging adjacent healthy ones.

Our group has demonstrated that PS molecules can be either encapsulated within or conjugated to ORMOSIL nanoparticles, thus rendering a stable aqueous formulation of the hydrophobic PS molecules. Although in this approach the drug is not released from the nanoparticles, it was found that the immobilized drug still retained its anticancer activity following light irradiation.

This is because the pores of the nanoparticles allow free diffusion of molecular and singlet oxygen in and out of the ORMOSIL matrix. The drug-doped nanoparticles were found to be actively taken up by cancer cells *in vitro*, and subsequent light irradiation of these treated cells resulted in significant destruction of the cells through PDT action. These data represent one of the first examples where a 'non-release' nanoparticulate drug delivery formulation has resulted in anti-cancer therapeutic action. This is considered to be a significant improvement over the existing micellar/liposomal based PDT drug delivery system, where the drug can prematurely release at off-target sites leading to reduced efficiency.

The PDT efficiency of a PS/ORMOSIL nanoparticulate system can be further enhanced by co-incorporation of other molecules in the same system that would synergistically overcome some of the drawbacks of conventional PDT. One such drawback is limited tissue penetration of visible light where most PS molecules absorb, thus hampering the treatment of deep-sitting tumors using this modality. Our group has demonstrated that PS molecules within the ORMOSIL nanoparticles can be excited using wavelengths in the near infrared (NIR) region that have much higher tissue penetration than visible wavelengths. This was achieved by co-incorporating a two-photon absorbing dye molecule in the nanosystem, which absorbs two-photons of the NIR light and transfers its up-converted emission energy in the visible range to the nearby PS molecules, through a process called fluorescence resonance energy transfer (FRET). As a result, the PS molecules robustly generated singlet oxygen following two-photon irradiation, and exhibited significant cytotoxic effects on cancer cells that were treated with these nanoparticles.

The singlet oxygen generation efficiency of a PS molecule can be significantly enhanced by the 'heavy-atom' effect. Here, when either a heavy atom is substituted chemically with a lighter-atom within the PS molecule, or a heavy-atom containing molecule is present in the vicinity of a PS molecule, following light irradiation the excited PS molecule will have increased propensity to cross-over to its reactive triplet state, which in turn enhances its energy transfer to molecular oxygen, leading to increased production of singlet oxygen. We have shown that when an iodinated ORMOSIL precursor was used to synthesize nanoparticles encapsulating a PS molecule, the singlet oxygen generation efficiency of the nanosystem was almost twice that generated when a non-iodinated ORMOSIL precursor was used. This heavy atom effect has also been successfully translated to *in vitro* PDT studies, where the iodinated PS/ORMOSIL nanoparticles have resulted in higher cytotoxicity than their non-iodinated counterparts. The above studies, coupled with the systemic non-toxicity of ORMOSIL, demonstrate the immense potential that an ORMOSIL-based multimodal nanoplatform has towards a successful clinical translation of nanoparticulate PDT.

9. Quantum Dots for Targeted Bioimaging of Cancer Cells

QDs are the best developed of nanoparticulate agents for cellular labeling and have attracted great attention in the biotechnology community⁹⁶. Many reports illustrate that QD labeling allows long term visualization of cells under continuous illumination as well as multiplex imaging, highlighting their unique advantages relative to traditional organic dyes. In our group, functionalized CdSe/CdS/ZnS, InP/ZnS, CdTe/ZnTe, and Si QDs were used for *in vitro* imaging and each was shown to have its own advantages and drawbacks. Figure 17 and Figure 18 show confocal images of MiaPaCa pancreatic cancer cells stained with CdSe/CdS/ZnS and CdTe/ZnTe QD-Tf bioconjugates, respectively. From the confocal images, the Tf-mediated targeting can be observed by the robust optical signal from the cells treated with Tf-conjugated QDs, as opposed to a complete absence of signal from the cells treated with the lysine coated QDs. Similar staining was also observed for L3.6PL and CoLo-357 pancreatic cancer cell lines⁹⁷. In addition, we have observed similar cellular staining using two-photon excited confocal imaging. Though cadmium QDs can be safely made and protected by surface

modification, there is still some concern, especially from clinicians, health scientists, and biologists, that these QDs will slowly decompose releasing Cd^{2+} and Se^{2-} ions, due to the ionic character of the II-VI semiconductor bonding. In view of this, we have formulated functionalized indium phosphide (InP) QDs, which provide a more stable alternative to CdSe QDs in specific biological applications that are highly sensitive toward the presence of cadmium ions. Similar cellular staining was observed for pancreatic cancer cells treated with the anti-mesothelin-InP/ZnS QD bioconjugates. Cellular uptake of the QD-bioconjugates can be observed from the robust optical signal from the Panc-1 and MiaPaCa cells, while no signal is observed in the case of cells treated with non-bioconjugated QDs, which again demonstrates the specific nature of the antibody-mediated targeting on the pancreatic cancer cells. Though the alloyed InP/ZnS QDs presented here do not contain cadmium, they do have other heavy metals. Zinc and phosphorus are usually found in minerals and vitamins, which are beneficial to humans in small quantities. However, indium is a heavy metal whose toxicity is a potential concern. For *in vivo* studies using InP/ZnS QDs, ~150 pmol of injected QDs contains 22 μg of indium⁹⁸. An average American ingests on the order ~10 μg of indium daily from food and water. Therefore, despite being composed of potentially toxic materials, the dosage may be small enough that overall toxicity is low. In addition to heavy metal based QDs, we have developed a procedure for preparing biocompatible Si QDs (non-heavy metal based) using PEGylated phospholipids and have used them for imaging of live cancer cells. This provides a simple, facile, and straightforward method for the production of colloiddally and optically stable water-dispersible Si QDs, with no observable *in vitro* toxicity. Nevertheless, there are other challenges, such as improving the Si QDs quantum yield and achieving higher nanoparticle yields and narrower emission spectrum before Si QDs can be broadly used for *in vivo* experiments.

10. Quantum Rods for Confocal, Two-Photon, and Multiplex Imaging of Cancer Cells

Following the above rationale mentioned in section 4, we have synthesized and characterized CdSe/CdS/ZnS QRs using the synthesis strategy described there. These QRs were bioconjugated with pancreatic cancer specific antibodies (anti Claudin-4 and anti-mesothelin) using carbodiimide chemistry^{99–101}. *In vitro* confocal microscopic studies revealed that these bioconjugated QRs were robustly taken up by a variety of pancreatic cancer cells (Panc-1, MiaPaCa, and CoLo357), as shown in Figure 19. More importantly, we have shown that these QRs can be used for two-photon fluorescence imaging of cancer cells with reduced photo-damage compared to UV excited imaging. In addition to targeted *in vitro* delivery, we have also demonstrated the co-imaging of two different QRs emitting at spectrally separable wavelengths¹⁰². This multiplex bioimaging will enable simultaneous detection of two or more optical probes within the same experiment. To demonstrate this, CdSe/CdS/ZnS QRs with two different aspect ratios (3.5, orange-emitting, and 4, red-emitting) were prepared, and the orange-emitting and the red-emitting QRs were conjugated with transferrin (Tf) and folic acid (FA), respectively. *In vitro*, we have treated KB cells, which are known to overexpress the corresponding receptors for both transferrin (TfR) and folic acid (FAR), with both of these QR bioconjugates. Both of these QRs (orange and red) were internalized within the same cell, with some areas of emission overlap. This result clearly suggests that it is possible to study the simultaneous uptake of multiple QR-bioconjugates by using *in vitro* confocal microscopy.

11. Quantum Dots as Targeted Theranostic Probes for Traceable Selective Destruction of Cancer Cells

In addition to cell labeling, we have also fabricated drug-antibody-QD bioconjugates that can specifically target and deliver anticancer drug to cancer cells and selectively destroy targeted

tumor cells based on the specific receptor-mediated uptake. The localization of the bioconjugates can be observed by fluorescence imaging using the quantum dots. Figure 20 shows *in vitro* cytotoxicity effects of the specifically engineered doxorubicin-anti-claudin-4-QD bioconjugate on pancreatic cancer (Panc-1), HeLa and KB cells. The antibody used here is known to be overexpressed in both primary and metastatic pancreatic cancer, but not in HeLa or KB cells. As shown in Figure 20, the multifunctional nanoprobe caused marked cytotoxicity following treatment of the pancreatic cancer cells for 24 hours, even at low dosages. In comparison, for the KB and HeLa cells treated with the same probe, the relative viability was always high for 48 hours post treatment. The cell viability is as high as 90–100% for the maximum dosage. This data not only confirms the specific cytotoxicity of the probe towards the pancreatic cancer cells, thereby justifying our strategy of the using an antibody for targeting, but also demonstrates the low uptake of the probe on the HeLa and KB cells and highlights its potential for selective destruction of pancreatic cancer *in vitro* and *in vivo*.

12. Functionalized Quantum Dots and Rods for *in vivo* fluorescence imaging

A new area of research for QDs is *in vivo* imaging application^{103–106}. To date, there is very little literature reporting the use of QDs as *in vivo* biomarkers^{107,108}. For example, Akerman et al. demonstrated the use of QD bioconjugates for targeting tumors in tumor-bearing mice¹⁰⁹. Later, Gao et al. reported the use of multifunctional nanoparticle probes based on QDs for cancer targeting and imaging in live animals¹¹⁰. Kim et al. demonstrated that QDs allow a major cancer surgery, sentinel lymph node mapping, to be performed in large animals under complete image guidance¹¹¹. Rao et al. showed the use of bioluminescence resonance energy transfer to excite QDs for *in vivo* imaging without the use of a laser excitation source.

More recently, QDs that emit red to near-infrared (IR) wavelength (600–900 nm) have been of great interest because this is an important spectral region for biological imaging and detection^{111,112}. In practice, *in vivo* bioimaging using visible light is hindered by native biological fluorescence ("autofluorescence"), light absorption, and scattering by biological tissue constituents, such as amino acids, hemoglobin, and water. NIR fluorescence imaging can address these challenges because biological autofluorescence and absorbance are both minimized in the NIR range. Traditionally, organic fluorophores have been used for near-IR-luminescence optical imaging. Cell and tissue imaging have been performed using these NIR fluorophores. However, these near-IR-emitting organic fluorophores not only tend to photobleach, but also have relatively narrow absorption profiles and broad emission spectra. These problems limit the use of current NIR organic dyes for ultrasensitive and multiplexed biological imaging in cells, tissues, and whole animals. Thus, there is a great need for development of a series of biocompatible NIR QDs for imaging purposes. The use of NIR QDs in the biomedical field has been reported but has not gone beyond proof-of-concept demonstration. This is mostly due to difficulties in producing high quality CdTeSe QDs that maintain their useful properties in various biological fluids. Our group has recently fabricated NIR CdTeSe alloy QDs for bioimaging using a one-pot solution phase synthesis approach. Phospholipid micelles were then used to provide stable dispersion of the NIR QDs in water, generating a hydrophilic shell with PEG groups on their surface. To demonstrate the proof of concept, phospholipid-PEG encapsulated QDs were sub-Q injected into the brain of a mouse. The mouse was then imaged using the Maestro imaging system¹⁰⁸. The *in vivo* fluorescence image is shown in Figure 21.

In addition to QDs, we have also employed functional QRs for *in vivo* imaging. The exceptionally high QY of QRs and their colloidal stability enabled us to use them as targeted probes to detect cancer. For *in vivo* studies, we have used pancreatic cancer specific antibody (anti-claudin 4) conjugated CdSe/CdS/ZnS QRs as targeted optical probes for locating tumor sites in the mouse. The mice were used for *in vivo* targeting experiments two weeks after the

tumor cell inoculation. Tumor signals can be detected by injecting antibody conjugated QRs into the tail vein of a tumor-bearing mouse (see Figure 22). The mice were imaged in the Maestro (CRI) *in vivo* imaging system. The captured images at various wavelength scans were loaded into the vendor software and analyzed. Spectral unmixing using the mice as the autofluorescence signal created the pseudo-color images of the background autofluorescence (red) and QD signals (green). Studies are in progress in our group and others to drastically reduce the uptake of QRs in the liver and spleen.

13. Gold Nanorods as Multimodal Optical Contrast Agent for Dark Field and Transmission Electron Microscopy Imaging of Cancer Cells

Recently, our group has used gold NRs with a multilayer polyelectrolyte coating as localized surface plasmon enhanced scattering probes with targeting for both dark-field imaging and transmission electron microscopy¹¹³. The antibody-conjugated gold NRs were used to label cancer cells^{114–116}. Panc-1 cancer cells were the target cell line. These are known to overexpress mesothelin and claudin-4 receptors. Receptor-mediated cellular uptake of the anti-mesothelin-conjugated NRs is demonstrated by the orange/red dark field images (see Figure 23), as compared to minimal uptake in the case of unconjugated NRs. The orange/red scattering associated with the NRs originates from their strong longitudinal surface plasmon oscillation, which has a frequency in the NIR region. In addition to dark field imaging, gold NR bioconjugates were also used for transmission electron microscopy imaging. To achieve this, electron microscopy images were collected for Panc-1 cancer cells labeled with gold NR bioconjugates and unconjugated NRs separately. From Figure 24, it is clear that the NR bioconjugates are selectively taken up by the Panc-1 cells compared to the unconjugated NRs, which show no uptake. The endosomal localization of the NRs within the cells also suggests receptor-mediated endocytotic uptake. This is a promising demonstration of developing localized surface plasmon enhanced scattering probes for biological applications. Most importantly, they are highly biocompatible in comparison to cadmium-based QDs, especially for early *in vivo* cancer detection (e.g. breast, lung, and prostate cancer).

14. *In vivo* distribution, clearance, and toxicity of nanoparticles

To use the fabricated nanoparticles described above for clinical applications, there are many challenges that must be addressed¹¹⁷. Many studies have demonstrated the use of QDs^{118, 119}, QRs⁷⁸, gold nanoparticles¹²⁰, gold NRs¹¹⁵, carbon nanotubes and gold nanoshells⁷ for small animal *in vivo* imaging and cancer detection. However, there are some important issues that urgently need to be addressed before use of these materials can advance toward clinical applications¹²¹. For example, what is the *in vivo* distribution of the nanoparticles? How, if at all, will these nanoparticles clear from the body? Do the surface coating, shape, and size of the nanoparticles affect the overall distribution and clearance of the particles? How long does it take for the nanoparticles to clear from the body? What is the lethal dosage (LD₅₀) of these different types of nanoparticles, and what are the modes of toxicity? What is the minimum dosage required for imaging applications? All these questions remain to be fully addressed. For example, the reported QD doses in mice range from several nmol to over one hundred nmol of particles. This concentration range is too wide for clinicians or health scientists to use in detection and labeling. It was reported that a higher QD concentration is required for detection of a tumor when the mechanism is passive uptake rather than active targeting. So far, images of functional nanoparticles crossing the blood stream and accumulating in organs have been shown, but there have been very few reports of quantitative analysis of QD biodistribution. Quantitative studies will provide understanding of nonspecific binding *in vivo*, the specific uptake at diseased sites, and the degree of metabolism and clearance of QDs. This information can assist in the identification of the optimal concentration for enhanced signal detection with

minimal collateral effects¹²². All these factors need to be carefully considered in order to advance biomedical nanoparticles toward clinical applications.

It has been reported that nanoparticles are removed by the reticuloendothelial system (RES), including the liver, spleen and lymphatic system. The RES is a protective mechanism that removes foreign particles or substances from the body. If the nanoparticles are rapidly removed by the RES, they will not be able to target tumor sites, and as a result, their utility as an early cancer detection probe will be hindered. Thus, it is extremely important for us to solve this major hurdle in order to further advance the nanoparticles use for clinical applications. It is widely known in the field of drug delivery that the introduction of an outer layer of polyethyleneglycol (PEG) on nanoparticles endows the nanoparticles with 'stealth', or RES evading properties. PEGylation increases the circulation time of nanoparticles in blood, thereby enhancing their ability to accumulate in target organs such as solid tumors by passive diffusion, taking aid of the enhanced permeability and retentivity (EPR) effect. Therefore, surface PEGylation is a powerful strategy for evasion of the RES and enhancing the circulation time of the nanoparticles in the blood, irrespective of the size of the nanoparticle core.

Discussion of nanoparticle toxicity can be very confusing and difficult due to the diversity of nanoparticles being used. To make this review easier for the readers to understand, we want to stress that not all nanoparticles are alike. Each nanoparticle type possesses its own unique physicochemical properties, which in turn give rise to their potential toxicity. To date, there are many inconsistencies in the reports regarding the toxicity of nanoparticles, which can be attributed to several factors¹²³. A variety of nanoparticle concentrations have been used in published *in vivo* and *in vitro* experiments. Most importantly, nanoparticles of widely varying physical and chemical properties have been used in these preliminary studies of their toxicity. So far, systematic studies required for complete toxicologic assessment (e.g., dosage, duration, routes of exposure, and mechanisms of action) are very limited because these studies are complicated and required long term *in vivo* evaluation. Moreover, a large amount of collective statistical data is needed to draw meaningful conclusions for each type of nanoparticle formulation, before they can be safely applied in clinical trials. In this review, we will only cover and comment on the toxicity of QDs and ORMOSIL nanoparticles as these nanomaterials have been most widely used to date.

QDs represent the most frequent used functional nanoparticles for *in vitro* and *in vivo* biomedical applications. Cadmium and selenium are two of the most widely used constituent elements in QD metalloid complexes. These elements are known to cause acute and chronic toxicities in vertebrates at high dosage. For instance, Cd ion, a potential carcinogen, with a biologic half-life of 15 – 20 years, can cross the blood–brain barrier and placenta, and is systemically distributed to all bodily tissues, with liver and kidney being target organs of toxicity¹²⁴. However, the toxicity effects to biological system might significantly change when the Cd and Se elements are in nanoparticulate form instead of ions¹²¹. For example, it was demonstrated that cytotoxicity is not a major concern in using QDs for long-term *in vitro* studies¹²⁵. It was reported that the coating chemistry of QDs could prevent cytotoxicity in cell culture¹²⁶. For *in vivo* studies, the toxicity effects will be different. Recently, it was reported that upon intravenous administration of cadmium-based QDs to small animals, the cadmium concentration in the liver and kidneys increased over the course of a month. The cadmium signals in the kidneys reached 10% of the injected dose as compared to 40% in the liver. The presence of QDs in both the liver and kidneys was observed using fluorescence microscopy. To date, it is still unknown whether cadmium is in the form of nanocrystals or ions in the targeted organs. It was speculated that the redistribution of cadmium over time may result from degradation of QDs *in vivo*, since the natural accumulation sites of Cd ions are the liver and kidneys. Many investigations are still needed to evaluate the cytotoxicity of cadmium based QDs engineered for biomedical applications.

The *in vivo* biodistribution, clearance and potential toxicity of non-biodegradable ORMOSIL nanoparticles is another area of increasing interest as these nanoparticles have so far shown considerable promise in diagnostics and therapeutics. Since there are very few studies available in the literature on the toxicity of ORMOSIL particles, at present we can only speculate and extrapolate from the data obtained in our *in vivo* studies using these nanoparticles.

Histopathological and behavioral data obtained in our lab, following direct, stereotaxic injection of these nanoparticles complexed with plasmid DNA in the rat and mouse brain indicate no sign of chronic or acute toxicity. In addition, our observation of the ORMOSIL-DNA treated brain cells being able to robustly express the transfected gene *in vivo* indicated that the cells maintained their normal functioning. Also, our preliminary *in vivo* imaging studies have demonstrated that mice receiving systemic injection of these nanoparticles showed no signs of ill health such as infection, weight loss, lack of grooming, abnormal posture and hypoactivity. The accumulation of these nanoparticles has been mainly observed in the liver and spleen, with some indication (via fluorescence) of excretion through the fecal route. These observations strongly suggest that ORMOSIL nanoparticles do not impart any toxic and hazardous effect *in vivo* at the experimental dosages studied. However, further detailed investigations are obviously necessary before confirming the benign nature of ORMOSIL nanoparticles for *in vivo* experimentation and their eventual clinical translation.

Overall, we suggest that there are several guidelines, which must be met for the nanoparticles to be useful and safely applied in the *in vivo* biomedical field. The criteria are as follows:

- i. The overall hydrodynamic size of the nanoparticles must be minimized to allow renal filtration which will minimize the accumulation of functionalized particles in organs.
- ii. Biocompatible long lasting surface coating, mainly but not exclusively using PEG, is needed to protect the particles from RES capture and degradation. This will provide sufficient time for the particles to be cleared from the body after performing their task (e.g. targeting and delivery).
- iii. Minimal quantities of nanoparticles must be used for *in vivo* imaging, targeting and drug delivery.
- iv. Low non-specific binding of QDs to the blood plasma components.
- v. Blood and tissue samples and analysis must be collected for expression of potential serum proinflammatory cytokines such as interferons, tumor necrosis factor α (TNF α), etc., as well as organ histopathology (e.g. H&E staining).

The increase in use of nanoparticulate materials in both biological and other applications necessitates elucidation of adverse effects of these nanomaterials, whether through upon intentional or unintentional exposure. Given the potential societal benefits offered by the nanoparticle technology, realizing the mechanisms and sources of nanoparticles toxicity will help to avoid the pitfalls encountered by the misapplication of some previous technologies. Development of nanotoxicology information defining the mechanisms of their toxic action will be vital in aiding research labs, health care centers, and industry in the production and safe use of nanoparticles.

15. Summary and Future outlook

To advance the application of nanotechnology to clinical trials, the National Cancer Institute (NCI) is currently funding eight Centers of Cancer Nanotechnology Excellence (CCNEs) and twelve Cancer Nanotechnology Platform Partnerships. It is envisioned that fusing the development efforts in nanotechnology, biotechnology, and cancer research will effectively aid the prevention, diagnosis, and treatment of human cancer in the future. The use of nanoparticle technology in cancer research provides many unprecedented opportunities for

early diagnosis and effective treatment of human cancer. For instance, in those patients where early cancer detection is achieved, successful surgical intervention is possible. This necessitates the removal of all cancerous cells surrounding the tumor site. Bioconjugated nanoparticles can be used as sensitive biomarkers to label these cancerous cells and aid the surgeons in visualizing and safely resecting the tumors while reducing damage to healthy tissues. In addition, nanoparticles offer a novel method for delivering anticancer drugs to the tumor sites, which can significantly improve the efficacy and decrease the toxicity of chemotherapy drugs. In the future, the use of nanoparticles technology will have dramatic impact in disciplines ranging from biological research to oncology.

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Figure 1.
Emission spectra for different sizes of CdSe QDs. The CdSe QDs size decreases as the emission peak blue shifted.

Figure 2. TEM image of CdSe/CdS/ZnS QDs. The size is ~6 nm. The scale bar is 100 nm.

Figure 3.
TEM image of InP/ZnS QDs.

Figure 4.
TEM image of CdTe/ZnTe QDs

Figure 5.
Emission spectra for different sizes of CdTe/ZnTe QDs. The CdTe/ZnTe QDs size increases as the emission peak red shifted.

Figure 6. Size-dependent optical properties of aqueous dispersion of CdTe/ZnTe QDs, demonstrating quantum confinement and size-tunable luminescent emission.

Figure 7.
HTREM image of silicon QDs.

Figure 8.
TEM image of PbSe QRs. The scale bar is 200 nm.

Figure 9.
TEM image of CdS QRs. The scale bar is 100 nm.

Figure 10.
TEM image of CdSe/CdS/ZnS QRs. The scale bar is 70 nm.

Figure 11. TEM image of monodispersed SiO₂ coated QR nanoparticle. The average size is 40 nm. The scale bar is 100 nm.

Figure 12.
Confocal image of macrophage cells treated with SiO₂ coated QR particles.

Figure 13.
TEM image of gold NRs.

Figure 14.
Visible absorption spectra of gold NRs with different aspect ratio.

Figure 15.
Scattering image of gold NRs with different aspect ratio.

Figure 16. Confocal images of Panc-1 cells treated with rhodamine-B-conjugated ORMOSIL NPs, without (a) and with (b) bioconjugation with anti-claudin-4.

Figure 17.

Confocal microscopy images of MiaPaCa cells labeled with unconjugated (lysine-coated) (a, b, and c), and transferrin-conjugated (d, e, and f) CdSe/CdS/ZnS QRs. The images to the left (a and d) show transmission images. The corresponding fluorescence images are shown in (b) and (e), and overlays of the two are shown in (c) and (f).

Figure 18. Confocal image of pancreatic cancer cells labeled with anti-mesothelin conjugated CdSe/ZnTe QDs (overlay of transmission and fluorescence images).

Figure 19. Confocal image of pancreatic cell lines labeled with CdSe/CdS/ZnS quantum rod bioconjugates.

Figure 20.

Cytotoxicity studies of Panc-1, HeLa, and KB cells treated with drug-QRs probe. MTT assays illustrating cell viability upon exposing the cells with different concentration of drug bioconjugates for 24 hours.

Figure 21. *in vivo* fluorescence image of a mouse treated with phospholipid micelle encapsulated near-IR CdTeSe QDs. The NIR QDs were injected sub-Q under the brain area of the mouse. The red and green colors correspond to the luminescence from the QDs and autofluorescence from the mouse, respectively.

Figure 22.

In vivo fluorescence image of a tumor-bearing mouse using anti-claudin-4 conjugated CdSe/CdS/ZnS QRs as early cancer detection probe.

Figure 23. Dark-field scattering images from Panc-1 cells following (a) treatment with anti-mesothelin-conjugated gold NRs and (b) no treatment. The wavelength selective scattering (orange/red) associated with the NRs can be clearly distinguished from the background, and corresponds to the longitudinal surface plasmon oscillation in the red region of the optical spectrum.

Figure 24. TEM images of Panc-1 cells following (a) treatment with anti-mesothelin-conjugated gold NRs and (b) no treatment.