Nanoparticulate Contrast Agents for Multimodality Molecular Imaging

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Molecular imaging is rapidly developing as a powerful tool in research and medical diagnostic. By integrating complementary signal reporters into a single nanoparticulate contrast agent, multimodal molecular imaging can be performed as scalable images with high sensitivity, resolution and specificity. In this review, multifunctional nanoparticles (MFNPs) are classified into four types: conjugation, encapsulation, core/shell, and co-doping. Further, new constructs of MFNPs were reported recently which have used nanoparticulate contrast agent such as quantum dots (QDs), iron oxide nanoparticles (IONPs), Upconversion nanoparticles (UCNPs), carbon based nanoparticles, gold nanoparticles (Au-NPs), Metal-Organic Frameworks (MOFs), dendrimers and porphyrins based nanoparticles. Different surface modification strategies were also developed as well as ligands are attached to those NPs to render the biocompatibility and enable specific targeting. These new development in MFNPs are expected to introduce a paradigm shift in multi-modal molecular imaging and thereby opening up an era of personalized medicine and new diagnostic medical imaging tools.

KEYWORDS: Multimodal Imaging, Molecular Imaging, Multifunctional Nanoparticles, Contrast Agents, Surface Functionalization, Theranostic.

CONTENTS

Introduction ................................................... 1553
Nanoparticle Design ....................................... 1556
Modalities to be Integrated............................... 1556
Configuration of MFNP ................................... 1556
Size ......................................................... 1557
Surface Treatment ......................................... 1557
Quantum Dots .............................................. 1557
Iron Oxide Nanoparticles ................................. 1560
Lanthanide Upconversion Particles ..................... 1562
Carbon-Based Nanoprobes ............................... 1565
Carbon Nanotubes ......................................... 1565
Graphene/Graphene Oxide ............................... 1566
Carbon Dots .............................................. 1567
Gold Nanoprobes ........................................... 1568
Metal-Organic Frameworks ............................... 1569
Porphyrins-Based Nanoprobes ........................... 1570
Dendrimer-Based Nanoprobes ........................... 1571
Nanoprobes for PET Imaging ............................ 1572

TARGETING LIGANDS ........................................ 1576
Conclusion ................................................... 1577
Acknowledgement .......................................... 1577
References and Notes ...................................... 1578

INTRODUCTION

Over the past decade, molecular imaging has emerged as a powerful tool to investigate the biological activities in the cellular and subcellular level.¹,²,³ Usually, molecular imaging utilizes a specially designed biomarker, which can target to specific cells or molecules, and generate detectable signals. Compared to the clinically available medical imaging techniques, most of them mainly focuses on macroscopic physical, physiological or functional alterations; the multimodal molecular imaging is capable of revealing the underlying molecular and cellular pathway, which is responsible for certain biological phenomena.² Novel molecular imaging applications are expected to enhance our understanding of...
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pathological development, facilitate drug discovery and development.4

Currently, multiple modalities are being applied to perform molecular imaging. However, no single modality can provide all the required information due to their inherent limitation. Table I summarizes the advantages and disadvantages of commonly used molecular imaging techniques. The idea of combining two or more complementary modalities together to obtain synergic effect is widely accepted in the research community.1,2,6,7 For example, optical imaging is highly sensitive, and can achieve a temporal resolution in the second scale, but its spatial resolution and in vivo penetration depth are limited. On the other hand magnetic resonance imaging (MRI) is capable to achieve high spatial resolution and practically infinite penetration depth.2 By combining these two modalities, High resolution and excellent sensitivity can be obtained simultaneously.6,7 While great effort has been made in designing hybrid molecular imaging instruments (e.g., PET/CT and PET/MRI system.6,8), the requirement of a single probe with multiple signaling capacities has arisen. Compared to individual single modality contrast agents, multimodality probe provides better image registration, consistent pharmacokinetics and reduced administration load for the patient.6

Among the few candidates for multimodality imaging probe, multifunctional nanoparticles (MFNPs) has been attracting most of the researchers’ interests due to a number of advantages: stronger and more stable contrast enhancement (e.g., quantum dots vs. fluorescent

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**Table I. Advantages and disadvantages for commonly used molecular imaging techniques.**

<table>
<thead>
<tr>
<th>Imaging technique</th>
<th>Contrast mechanism</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Contrast agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical imaging16–18</td>
<td>Photon emission</td>
<td>• Highly sensitive</td>
<td>• Low penetration</td>
<td>• Luciferin, Fluorescent dye, Quantum Dots, Carbon nanoparticles, Lanthanide particles</td>
</tr>
<tr>
<td>MRI19–21</td>
<td>Proton density and relaxation time</td>
<td>• Unlimited penetration</td>
<td>• Low sensitivity</td>
<td>• Gadolinium, Iron Oxide, Manganese</td>
</tr>
<tr>
<td>PET22, 23</td>
<td>γ-ray emission</td>
<td>• Unlimited penetration</td>
<td>• Low spatial resolution</td>
<td>• Non-metal Isotope: 18F,11C, Metal Isotope: 64Cu, 86Y, 68Ga.</td>
</tr>
<tr>
<td>SPECT24, 25</td>
<td>γ-ray emission</td>
<td>• Unlimited penetration</td>
<td>• Low spatial resolution</td>
<td>• Isotope: 99mTc, 123I, 111In, 177Lu</td>
</tr>
<tr>
<td>CT26</td>
<td>X-ray attenuation</td>
<td>• Unlimited penetration</td>
<td>• Limited molecular imaging application</td>
<td>• Iodine, Barium, Gold particles</td>
</tr>
<tr>
<td>Photoacoustic tomography27, 28</td>
<td>Photon absorption</td>
<td>• Deeper penetration compared to optical imaging</td>
<td>• Deeper penetration and higher resolution cannot be obtained simultaneously.</td>
<td>• Organic dye, Gold nanoparticles, Carbon Nanotube</td>
</tr>
<tr>
<td>Optical coherence tomography29–31</td>
<td>Light reflection</td>
<td>• High spatial resolution</td>
<td>• Limited tissue penetration</td>
<td>• Gold nanoparticles, Microsphere, Magnetic particles (Magnetomotive OCT)</td>
</tr>
<tr>
<td>Ultrasound32, 33</td>
<td>Acoustic echo</td>
<td>• Real-time imaging</td>
<td>• Imaging quality is operator-dependent</td>
<td>• Gas-filled microbubbles, Liposomes, Perfluorocarbon droplets</td>
</tr>
</tbody>
</table>
Nanoparticulate Contrast Agents for Multimodality Molecular Imaging

Xia et al.

Many imaging modalities have been widely used for contrast-enhanced imaging. For instance, dye9) large surface area for modification and ligand attachment,10 prolonged circulation time11 (However, this may also lead to increased cytotoxicity12), and better tumor targeting through enhanced permeability and retention (EPR) effect.13 Nanoparticles are normally 100–10000 times smaller than cells and hence can be easily internalized by cells.14 Moreover, loading ability of nanoparticles has made it possible to simultaneously load diagnostic and therapeutic moiety into one package, which initiate the idea of theranostics.15 In contrast to the conventional “diagnostic followed by therapy” approach, theranostics helps to eliminate the discrepancy in biodistribution and selectivity between separated imaging and therapeutic agent (Fig. 1). The ultimate goal of theranostics is to shift the current generalized medicine, which applies similar therapy to all patients with the same disease, into an era of personalized medicine, i.e., tailor-made therapy for an individual patient. So far, a series of attempts has been reported to integrate MFNPs with agents for Gene therapy, chemotherapy, hyperthermia therapy, photodynamic therapy, and radionuclide therapy.15

In this review, we have summarized some of the recent progress on developing MFNPs. The main focus is on integrated MRI/Optical probes while some probes that can be applied in other imaging modalities are also included. Those nanoparticles are made either by de novo synthesis or by integration of commercially available contrast agent. Most of the nanoprobe described in this review were reported in the past 5 years (from 2009). For nanoprobe that have been reported before 2009, several reviews are already available.6,7,34

**NANOPARTICLE DESIGN**

While some of the MFNPs used for molecular imaging are produced as by product or ‘happy coincidence’ from researches targeted for non-imaging application, most of the reported MFNPs are carefully designed to meet a certain requirement. The following four factors need to be determined during the design of MFNPs.

**Modalities to be Integrated**

The modalities that the MFNP can be used should be complementary to each other. The aforementioned MRI/Optical integration is one of the most widely studied combinations in MFNPs. Other combinations including MRI/PET, Optical/PET and Optical/CT have also been extensively explored. Moreover, MFNPs with triple or more signal-reporting capabilities have been synthesized with carefully designed protocols.35–38 A major challenge in the design of MFNPs is to balance the sensitivities between different modalities, which may vary up to 3 orders of magnitude.39 Luckily, the large surface area of NP can serve to overcome this problem by controlling the moiety ratio between two contrast agents. It is also important that the integration of multiple agents should not produce detrimental effects to the performance factor of each agent. Different imaging modalities rely on different properties to generate the contrast and hence their performances are evaluated accordingly. For example, MRI contrast agents enhance the contrast by accelerating the relaxation processes of neighboring water protons. There are two types of relaxation process: longitudinal relaxation \(T_1\) and transverse relaxation \(T_2\). One particular contrast agent mainly accelerates on the relaxation processes and hence MRI agents can be classified into \(T_1\) and \(T_2\) agents. The ability of one agent to affect the relaxation is defined as \(r_1\) or \(r_2\) relaxivity, corresponding to the two types of relaxations.40 For optical imaging, the quantum yield and excitation/emission wavelength are very important for the imaging resolution, signal-to-noise ratio and penetration depth.9 An MRI/Optical bi-modal probe synthesized by integrating a MRI agent and an optical agent should exhibit similar relaxivity and optical properties compared to the stand-alone contrast agent.

**Configuration of MFNP**

We classify the configuration of MFNPs into the following four categories: conjugation, encapsulation, core-shell, co-doping (Fig. 2). MFNPs with conjugation or core/shell configuration are normally synthesized through multi-step
reaction that could be more time consuming and complicated. Co-doped MFNPs can be prepared by facile one-step routes and are generally smaller in size. For different MFNPs, certain configuration may provide the best integration. Appropriate configuration can also help to reduce the interference between contrast agents. However, the actual configuration of MFNPs may be a mixture of two or more types. An encapsulation complex can be further conjugated to targeting agent, and a core/shell nanoparticle may also be doped to yield more functions.

Size

The size of MFNP plays a major role in determines its biodistribution, clearance, contrast enhancement and the cells that can be targeted. Small nanoparticles (<5.5 nm) will be quickly cleared out through renal system, while larger nanoparticles are mainly captured by the reticuloendothelial system (RES), resulting in much longer circulation time and liver accumulation. However, if the nanoparticles are too large, the chance of RES uptake will increase, leading to a shorter half-life. Longer half-life is generally desired since it gives MFNPs enough time to extravasate out of the vasculature. The size also affects the targeting efficacy of MFNPs. For instance, large NPs (>100 nm) are quickly cleared from plasma to the spleen, liver and bone marrow. Hence those NPs should be designed to target those organs. On the other hand, smaller NPs (<30 nm) should be designed to target tumor, due to their long circulation time and EPR effect. For quantum-dots based MFNP, the wavelength of fluorescent emission is also affected by its size, which give the QD based MFNPs an advantage of tunable emission through size control. For Gd\(^{3+}\) doped MFNPs, the size of NPs determines the number of surface Gd\(^{3+}\), and consequently affects the NPs’ relaxivity.

Surface Treatment

Surface treatment is very important to control the MFNPs’ circulation time, biocompatibility and targeting efficiency. Many as synthesized NPs are coated with hydrophobic molecules. For bio-imaging application, those NPs need to be dispersed in an aqueous solution by hydrophilic capping agent. The capping can also provide surface functional groups to conjugate with other functional moieties. One widely used capping material is polyethylene glycol (PEG) and its derivatives. Surface PEG layer can shield the nanoparticles from reticuloendothelial system (RES) by minimizing plasma protein binding, and this kind of capped nanoparticle are often referred as stealth particle. PEGylation is widely used to increase the circulation time of NPs. PEGylation has also been used to enhance tumor targeting of NPs due to the high affinity between cancer cells and PEG cappings. Other capping agents that do the same thing have also been exploited, including poly acrylic acid (PAA), polyethylenimine (PEI), and polyhedral oligomeric silsesquioxanes (POSS). To further enhance the specific targeting and internalization of nanoprobe, biorecognition ligands, such as antibodies, peptides and aptamers, are often conjugated to the MFNPs’ surface. Due to different surface treatment, MFNPs with the same core size may have much different hydrodynamic diameter, which leads to different circulation and clearance behavior.

QUANTUM DOTS

Quantum dots (QDs) are nano-sized semiconductor crystals. Compare to conventional organic fluorescent dyes, QDs have some significant advantages including: high photostability, wide absorption, tunable emission, high quantum yield, large Stokes shift and long fluorescence lifetime. Hence a significant effort has been put on developing QD based MFNPs. Most reported QDs are synthesized with hydrophobic capping molecules; additional encapsulation by amphiphilic surfactant or ligand exchange is required before applying the QD as molecular probes. Normally, encapsulation will produce much larger QDs compared to QDs undergoes ligand exchange. The second contrast agent can be doped during QD preparation, incorporated during the encapsulation process or conjugated to the surface of those hydrophilic QDs.

In the work by Liu et al., CdTe/ZnS core/shell QDs were synthesized and capped using mPEG-phospholipid (mPEG-NH\(_2\)-2000) or Pluronic F127 (F127-NH\(_2\)). Both of those two block polymers are treated with amine to introduce the –NH\(_2\) groups. Then the QDs are conjugated with Gd-DOTA chelators. The encapsulation was achieved by mixing the hydrophobic QDs in chloroform with
mPEG-NH$_2$-2000 or F127-NH$_2$ solution. Upon heating to 70–80 °C, the amphiphilic molecules form micelle structures and the QDs aggregated inside the hydrophobic core of the micelles (Fig. 3(a)). The high luminescence was retained with a red shift of 20–40 nm (Fig. 3(b)). The different composition of the micelles resulted in different particle sizes: 10.8 ± 2.5 nm for PEG-QDs and 22.1 ± 5.7 nm for F127-QDs. However, this estimation was conducted under transmission electron microscopy (TEM), which means the estimated size is most likely the core diameter and the micelle shell is ignored. In order to chelate with Gd$^{3+}$, the QD micelles were mixed with DOTA-NHS-ester, strong amide bond formed between the surface amine and the DOTA-NHS. Then GdCl$_3$ was added into the solution and the Gd$^{3+}$ was chelated by DOTA. A difference in the relaxivities of the two nanoparticles (4.380 mM$^{-1}$ s$^{-1}$ for PEG-QD-Gd and 8.168 mM$^{-1}$ s$^{-1}$ for F127-QD-Gd, tested in 4.7T magnetic field) indicates that the amounts of conjugated Gd-DOTA chelators are different. The hydrodynamic diameters were found to be ~60 nm for PEG-QD-Gd and 90 nm for F127-QD-Gd.

In vitro cell imaging was performed on RAW264.7 macrophage cell lines and both QDs showed significant cellular uptake and intracellular luminescence. However, no MRI was conducted. Cytotoxicity of the two QDs was determined by cell viability (MTS) assay (Fig. 3(c)). F127-QD-Gd was found less toxic than PEG-QD-Gd. The author argued that both QDs could be considered as biocompatible since the concentration used in the MTT essay is much than previous reported studies.

Also using Gd-DOTA chelator, Jin et al. functionalized the hydrophobic CdSeTe/CdS QDs by coating the QD with glutathione (GSH), and then conjugated with Gd-DOTA complex. The GSH coated QD retained 60% of its luminescence. The conjugation of Gd-DOTA increased the hydrodynamic diameter from 7.0 ± 0.4 nm to 10 ± 0.2 nm. The $T_1$ relaxivity was measured to be 365 mM$^{-1}$ s$^{-1}$. The large number of Gd$^{3+}$ conjugated in one QD might cause such a high relaxivity. NIR-fluorescence imaging and MRI was performed on a mouse to demonstrate the in vivo imaging utility of the Gd-DOTA-QDs (Fig. 4).

Doping has also been used to synthesis multimodality QDs. Saha et al. reported Fe doped CdTeS quantum dots capped with N-acetyl-cysteine (NAC) ligands. Saha applied hydrothermal method to produce water dispersible QDs. Without the requirement for further surface modification, the size of the synthesized QDs is relatively small (3–6 nm). The synthesized magnetic QDs have emission in the range of 530–738 nm with quantum yields.
of 10%–67.5%. The $T_1$ relaxivity of the NIR (738 nm) emitting QD was measured to be 3.6 mM$^{-1}$ s$^{-1}$ (4.7T). Unfortunately, direct doping to the QDs’ core may cause fluorescent bleaching due to the introduced impurities. Since QDs are often coated with a protective shell to prevent environmental degradation and toxic ion leakage, doping to the shell becomes an alternative to render multimodalities. Wang et al. capped a CdSe QDs with manganese doped ZnS shell (Fig. 5). CdSe QDs were first synthesized by mixing cadmium acetate and Se into hot TOPO (Triocylphosphine oxide) solution. Then 1.5 to 6 monolayers of Zn–Mn–S shell was grown onto the surface of CdSe QDs. The Mn/Zn ratio is tuned by adjusting the amount of diethylzinc and dimethylmanganese. The manganese concentration ranges from 0.6% to 6.2% and the $T_1$ relaxivity ranges from 10 mM$^{-1}$ s$^{-1}$ to 13.1 mM$^{-1}$ s$^{-1}$ (7T). The core/shell QDs were made water dispersible by capping with octylamine-modified poly acrylic acid. The core size of the capped QDs were measured to be 4.7 nm using TEM. The quantum yield was reduced from 60% to 21% before and after organic coating. Fluorescence cell imaging and MRI of cell lysates were conducted to demonstrate the functionality imaging potential of the doped magnetic QDs.

Due to the concern about potential leakage of highly toxic cadmium ions, researchers have attempted to synthesis non-cadmium based QD (e.g., ZnO,\textsuperscript{58–60} ZnS,\textsuperscript{61,62} InP,\textsuperscript{63,64} CuInS\textsubscript{2},\textsuperscript{65}) and many of those QDs are also used to produce multimodality probes. Lin et al. reported a CuInS\textsubscript{2}/Zn\textsubscript{1−x}Mn\textsubscript{x}S Core/Shell QDs.\textsuperscript{65} It also has a manganese-doped shell, similar to Wang’s QD, but the core is composed of CuInS\textsubscript{2} instead of CdSe. The quantum yield reaches 18% in water and the $T_1$ relaxivity was measured to be 7.2 mM$^{-1}$ s$^{-1}$, comparable with the previous mentioned cadmium based magnetic QDs. Low cytotoxicity was confirmed by MTT assay with BXPC-3 Cells. In vitro fluorescence imaging showed clear internalization of the magnetic QD by the cancer cell and MR imaging was performed on the cancer cell lysate. Similarly, Ding et al. synthesized CuInS\textsubscript{2} QDs with manganese doped ZnS shell.\textsuperscript{66} The QDs were made hydrophilic by coating with DHLA-PEG through ligand exchange reaction. And in vivo fluorescence and MR imaging were conducted on nude mouse with subcutaneously transplanted tumor cells and intraperitoneally transplanted tumor, respectively. Liu et al. synthesized Gd\textsuperscript{3+} doped ZnO QDs by hydroslyzing mixed zinc acetate and gadolinium acetate in ethanol.\textsuperscript{68} Interestingly, the size of the ZnO QDs became smaller with increasing molar ratio of Gd/Zn (diameter of undoped ZnO QDs = 6 nm, diameter of Gd-doped ZnO QDs = 4 nm when Gd/Zn = 0.08). No significant lattice distortion was observed at low Gd ratio (Gd/Zn < 0.08). The doped Gd\textsuperscript{3+} also enhanced the fluorescence of ZnO QDs. The QY increased from 13.5% (undoped ZnO QD) to 34% (when Gd/Zn = 0.08). Further increasing the Gd content significantly reduced the emission intensity. The author attributed this fluorescence enhancement to the increased oxygen vacancy that is origin of ZnO QDs’ visible emission. According to a previous report, smaller ZnO QDs generally have more oxygen vacancy.\textsuperscript{59} At very high Gd\textsuperscript{3+} content, the fluorescence enhancing effect due to size reduction is overwhelmed by the detrimental effect of ZnO consumption. The $T_1$ relaxivity of the Gd-doped QDs was 16 mM$^{-1}$ s$^{-1}$ (Gd/Zn = 0.08, measured at 1.5T). Successive confocal laser-scan imaging and MR imaging was conducted on HeLa cells incubated with Gd-doped ZnO QDs. But the short excitation wavelength ($\lambda = 340$ nm) may hinder its in vivo application.

Fluorescent silicon QD is another attractive candidate for multimodality bioimaging due of its inertness and low cytotoxicity. Either doping,\textsuperscript{67–69} conjugation\textsuperscript{70} or co-encapsulation\textsuperscript{71} with magnetic moiety can induce the magnetic properties of Si QDs. A first-principle study reported by Ma et al. investigated the magnetic properties of hydrogen-passivated Si-QDs doped with different 3$d$
and 4d metals. The calculation result shows that magnetic moments of most transition metals are completely quenched after doping into Si-QDs, while certain 3d metals (V, Cr, Mn, Nb, Mo, and Tc) still retain some of their magnetic moment after doping. Among those metals, Mn is considered the optimum choice since Mn–Si bonds have similar length of Si–Si bonds.

Consistent with Ma et al.’s study, Tu et al. reported water-dispersible Mn doped Si QDs for MR and two-photon imaging of macrophage. The QDs were synthesized by refluxing Mn-doped sodium silicide in DMF with NH₄Br and allylamine added. The sodium silicide was prepared by ball-grinding of NaH, Si and Mn mixture, followed by heat treatment at 420 °C for 48 hours and at 500 °C for additional 24 hours. The surface –NH₂ groups of the as prepared QDs make it possible to coat the QD with dextran sulfate, which targets the macrophage SR-A receptor. The coating also caused particles aggregation: around 5–10 Mn doped Si nanoparticles bound together, formed nanoclusters with average size of 15–30 nm (Figs. 6(a–c)). For single-photon excitation, emission peak was observed at 441 nm under 360 nm excitation. For two-photon excitation, emission peak appeared at 478 nm under 790 nm excitation. The Si QDs also possess excellent MR properties with T₁ relaxivity of 25.50 ± 1.44 mM⁻¹ s⁻¹. The dual-modality of the Si QDs and specific targeting was further demonstrated by in vitro imaging of P388D1 murine macrophage cells.

As most of the as-synthesized Si QDs are hydrophobic, they can be encapsulated inside lipid micelles, and magnetic moiety can be incorporated inside the micelle core or to the lipid monolayer. Erogbogbo et al. reported co-encapsulation of hydrophobic Si QDs and superparamagnetic iron oxide NPs (SPIONs) with phospholipid polyethylene glycol (DSPE-PEG) micelles. The micelle was successfully applied for optical imaging on tumor-bearing mice. The magnetic property was determined by vibrating sample magnetometer, but no MR imaging was performed. The same group also reported Gd³⁺ chelated Si QD micelles. The Si QDs were capped with amine-terminated DSPE-PEG, and then conjugated with DOTA-GD³⁺ complex. The T₁ relaxivity of the synthesized NPs (2.43 mM⁻¹ s⁻¹) is comparable with commercial available Gd based MRI contrast agent.

**IRON OXIDE NANOPARTICLES**

Iron oxide nanoparticles (IONPs) are being widely employed as T₂ MRI contrast agent. Compare to gadolinium based contrast agent, IONPs are less toxic (LD₅₀ for iron: 450 mg/kg, LD₅₀ for Gd³⁺: 88 mg/kg). For biomedical application, IONPs need to be water dispersible and biocompatible; this is normally achieved by coating with hydrophilic organic molecules such as PEG or dextran. Further surface modification enables IONPs to be conjugated with other contrast agent and become multifunctional. Core/Shell configuration is also popular in which IONPs are functionalized with organic or inorganic shell.

A magneto-fluorescent nanoprobe with a typical conjugation configuration was reported by Ke et al. Fe₃O₄ nanoparticles were decorated by poly(acrylic acid) and became water-dispersable. This decoration also introduced free carboxyl groups to the surface. To make the NP fluorescent, Rhodamine 123 (Rh123) dye was covalently attached to the poly(acrylic acid) iron oxide (PAAIO). In addition, folic acid-linked PEG was conjugated to the PAAIO-Rh123. The folic acid helps specific targeting of cancer since folate receptor density was found increased in aggressive or undifferentiated tumors. Similar work was reported by Yen et al.: NIR IR-820 dyes were linked to Fe₃O₄ nanoparticles as specific binding ligands to human breast cancer cells (KPL-4). The nanoparticles were synthesized through a step-wised route, at each step, hydrolysis of TEOS produces a silica shell by reverse microemulsion. Functional
groups were introduced to the final surface by reaction with 3-aminopropyltrimethoxysilane (APS). The inert silica shell prevents potential heavy metal leakage. The mean size of the synthesized probe is 150 nm, which is considerably large and may cause fast clearance. However, for *in vivo* imaging, the middle VIS-QDs containing silica layer may not be required as it provides similar information with NIR QDs but less penetration depth. *In vivo* NIR and MR imaging were performed on nude mice with transplanted KPL-4 cells (human breast cancer cell).

Manganese (Mn) can be doped to IONPs to enhance its $T_2$ relaxivity. Kim et al. demonstrated a robust method to synthesis MR/NIR bimodal probe containing MnFe$_2$O$_4$ NPs and NIR QDs in a polyelectrolyte nano complex. Hydrophobic MnFe$_2$O$_4$ NPs were first prepared by thermal decomposition. Then the MnFe$_2$O$_4$ NPs were made water dispersible by capping with negatively charged poly($\gamma$-glutamic acid) ($\gamma$-PGA). Further adding of positively charged poly(L-lysine) (PLL) lead to ionic gelation and formed a nanogel with the MnFe$_2$O$_4$ NPs encapsulated inside. PEGylation was also performed to enhance the biocompatibility of the nanogel. Since the outer surface of the nanogel is positively charged, QD800 (COOH), a negatively charged QD, was attached to the nanogel, forming a magnetofluorescent polyelectrolyte nanocomposites (MagFL-PEN). The $T_2$ relaxivity of the MagFL-PEN was measured to be 436.8 mM$^{-1}$ s$^{-1}$, considerably higher than commercially available iron oxide contrast agents. NIR fluorescence was preserved after the integration. A significant size increase was observed (8 nm for MnFe$_2$O$_4$ NPs, 25 nm for QDs and >240 nm for MagFL-PEN) due the multiple encapsulation and linking process.

Besides fluorescent probes, IONPs can also be integrated with light absorbing materials to generate MRI/Photoacoustic bimodal probes. Song et al. doped ultra-small IONPs into NIR absorbing polypyrrole (PPy)
to form IONP@PPy nanocomposites, and then further modified the nanocomposites with PEG to obtain IONP@PPy-PEG nanoparticles. To prove the bimodality of the synthesized nanoparticles, tumor-bearing mice were injected with IONP@PPy-PEG nanoparticles and then imaged in MR and photoacoustic system (Fig. 7). The excellent NIR absorbance of PPy was also utilized for photothermal therapy.77

**LANTHANIDE UPCONVERSION PARTICLES**

All the previously mentioned optical imaging is based on downconversion phenomenon, that is, the emitted photons have lower energy than the absorbed photons. Upconversion luminescence (UCL) is a phenomenon that light with shorter wavelength is emitted after sequential absorption of longer wavelength light. Lanthanide doped nanoparticles (Ln-NPs) is so far the most widely studied upconversion molecular probe. Ln-NPs are commonly made by doping trivalent lanthanide ions to a host lattice.78,79 Due to the high X-ray attenuation of lanthanide elements, Ln-NPs can be readily used as Optical/CT bimodal contrast agent.80–82 In addition, Gadolinium ions are often co-doped with upconverting lanthanide ions to introduce the MR property, and since doped Gd$^{3+}$ ions are confined in a more rigid crystal matrix, it is considered much less toxic compared to the clinically available Gd$^{3+}$-chelate complexes.40 Moreover, the Gd$^{3+}$ ions may enhance the PL by acting as an intermediate which harvest the excitation energy and then transfer it to the nearby luminous Ln ions.83,84 SPION can be incorporated with Ln-NPs in a core/shell configuration.81,85 Ln-NPs have also been reported being labeled with radioactive isotopes and then used as contrast agent for PET,86,87 or SPECT.82 AREF$_4$ (A-alkali metal, RE-rare earth elements) fluorides such as NaYF$_4$ is considered one of the mostly efficient host materials for upconverting lanthanide due to its lattice consistency with Ln ions, chemical stability and low phonon vibration.79 In order to produce MRI/Optical bimodal probe, NaGdY$_4$ is often used instead of NaYF$_4$, in which the Yttrium ions are substituted by Gadolinium ions.88–91 One example of such kind of co-doped nanoprobes was reported by Zhou et al.86 The NaY$_{0.5}$Gd$_{0.8}$Yb$_{0.1}$Er$_{0.5}$F$_{14}$ nanoparticles were synthesized in organic solvent with hydrophobic oleic acid capping. Further ligand exchange replaced the oleic acid coating with hydrophilic citrate coating. The shape of the citrate capped NPs (cit-NPs) are slightly elliptical with average core size of 19–22 nm (determined by TEM) and hydrodynamic size of 28.2 nm (determined by DLS). The cit-NPs were additionally functionalized by incubation with $^{18}$F containing solution and became a PET/MR/UCL triple functional probe. However, the as-prepared cit-NPs have a green light emission when excited by a CW 980 nm laser. The short penetration of green light has limited the in vivo optical imaging capability of the tri-modality probe. The author conducted in vivo MR and PET imaging on mice but the optical imaging was performed in vitro with KB cells tissue section. Another work reported by the same group$^{88}$ synthesized Tm$^{3+}$/Er$^{3+}$/Yb$^{3+}$ co-doped NaGdF$_{4}$ NPs which demonstrated two types of UCL: NIR-to-Vis and NIR-to-NIR. The NIR emission is attributed to Tm$^{3+}$ transitions while the visible emission is caused by Er$^{3+}$ transitions. In vitro UCL imaging on mouse and ex vivo UCL imaging on the harvested organs were conducted. The synthesized NPs provided strong contrast against the background and predominantly accumulated in the liver and spleen. One problem with this UCL NPs is...
the wide size distribution of 25 and 60 nm (hydrodynamic diameter); this could lead to inconsistent biological distribution and clearance. The size of NPs also affect the $T_1$ relaxivity since smaller NPs carry more surface Gd$^{3+}$ ions, which is responsible for the relaxation enhancement.\textsuperscript{92} Li et al. reported Ln ions doped NaGdF$_4$ NPs through a microwave-assisted method.\textsuperscript{93} The emission wavelength and intensities can be tuned by manipulating the type and concentration of doped Ln ions (Yb$^{3+}$, Er$^{3+}$, Tm$^{3+}$ and Ho$^{3+}$). The NaGdF$_4$ NPs were further functionalized by encapsulation with amine terminated SiO$_2$ shell (NaGdF$_4$ core-40–65 nm, SiO$_2$ shell-3 nm) and then conjugated with folic acid. The upconversion imaging capability and specific targeting to cells with folate receptors was tested on cancer cells (HeLa and SK-OV-3 cells).

KgGdF$_4$, another AREF$_4$ type fluoride, has also been utilized for multimodal bio-imaging applications.\textsuperscript{51,83,94} Wong et al. synthesized Yb$^{3+}$, Er$^{3+}$doped KgGdF$_4$ NPs with polyethyleneimine (PEI) or 6-aminoacaproic acid (6AA) capping through a one-pot hydrothermal reaction.\textsuperscript{51} The sizes of the doped MFNPs are relatively small (14 ± 2 nm for PEI capped NPs and 13 ± 2 for 6AA capped NPs, estimated by TEM). The crystal structure of this KgGdF$_4$ MNPs remained cubic at this small size, this is considered as an advantage over the Ln doped KYF$_4$ NPs, in which the crystal phase transforms from hexagonal to cubic and dimes the upconversion luminescence.\textsuperscript{95} In vitro upconversion imaging was performed on HeLa cells incubated with the synthesized KgGdF$_4$ MNPs. The cellular uptake efficiency of PEI capped NPs was found much higher than that of 6AA capped NPs. Although no MR imaging was performed, the high magnetic mass susceptibility ($8.62 \times 10^{-5}$ emu g$^{-1}$ Oe$^{-1}$) of the PEI-MFNPs indicates the potential of this particle as optical/MRI bimodal contrast agent.

To further enhance the photo-stability, Chen et al. synthesized Ln-NPs with a core/shell configuration.\textsuperscript{91} The Tm$^{3+}$ doped NaYbF$_4$ core was synthesized first to provide the NIR-to-NIR upconversion photoluminescence. Then a shell of NaGdF$_4$ was grown on the NaYbF$_4$:Tm$^{3+}$ core to protect the PL and induce MR property. Compared to bare NaYbF$_4$:Tm$^{3+}$ NPs, the luminescence intensities of core/shell NaYbF$_4$:Tm$^{3+}$/NaGdF$_4$ NPs are 3 times higher. This PL enhancement is caused by the suppression of quenching effect and the activation of upconverting ions in the core surface (the Yb$^{3+}$ and Tm$^{3+}$ ions in the core surface are provided a similar lattice environment with those inside the core matrix). In addition, the PL decay of the core/shell NPs was much slower than that of the bare NPs. The $T_1$ relaxivity was measured to be 2.6 mM$^{-1}$ s$^{-1}$. The same group also reported another similar core/shell NP with a NaGdF$_4$:Er$^{3+}$/Yb$^{3+}$ core and a NaGdF$_4$ shell.\textsuperscript{95} The bimodal capability of this core/shell NPs was demonstrated by wide-field cell imaging on breast cancer cells incubated with the core/shell NPs and MR imaging on the respected cell pellet. Their works provided a good example of combined doping and core/shell strategy. A more detailed protocol on how to synthesize core/shell, Ln-doped NaGdF$_4$ NPs has been reported by Wang et al. recently.\textsuperscript{96} The core NaGdF$_4$ was first synthesized by co-precipitation of lanthanide fluoride and long-chain hydrocarbon, unsaturated fatty acids was also added as the capping agent to control the particle growth. Then the pre-synthesized core NPs was used as template according to which the outer shell grew epitaxially. Lanthanides doping was achieved concomitantly with particle growth, and different Ln ions can be doped into different compartments to achieve multi-color emission.

Cheng et al. integrated Ln-NP with ultrasmall SPION through a layer-by-layer self-assembly process.\textsuperscript{85} PAA coated hexagonal Ln-NPs (NaYF$_4$:Yb$^{3+}$/Er$^{3+}$) were mixed with dopamine modified ultrasmall SPIONs (5 nm). The SPIONs were attached to the Ln-NPs by electrostatic attraction. Then negatively charged Au seeds were added and formed an Au shell on the surface of the SPION-LnNP complexes. The synthesized nanoparticles were further functionalized with PEGylation through gold-thiol bonding. Hydrodynamic diameter of the PEGylated MNPs was measured to be 230 nm. Folic acids were also conjugated to the MNPs in order to target cancer cell specifically. The MNPs have a high transverse ($T_2$) relaxivity of 352.8 mM$^{-1}$ s$^{-1}$ (7T). Successful UCL and MR imaging were performed in vitro and in vivo (Fig. 8). What’s more, the gold shell, which has excellent surface plasmon absorption, can be utilized for photo thermal therapy (PTT). Cancer cells incubated with the MNPs were effectively destructed after exposure to NIR laser. However, the gold shell also causes quenching to the upconversion luminescence. Luckily, due to the intermediate SPION layer, the quenching effect is much weaker compared to Ln-NPs with directly-attached gold shell.\textsuperscript{97} Fe$_3$O$_4$ can also be integrated as core particles. In the work by Zhu et al., Fe$_3$O$_4$ NPs with were first encapsulated with SiO$_2$ shell through an Stöber method and then further capped with a lanthanide carbonate layer (Lu, Yb, Er/Tm(OH)$_2$CO$_3$). After calcination 600 °C for 6 h, the Ln carbonate layer was transformed into Yb$^{3+}$/Er$^{3+}$/Tm$^{3+}$ doped Lu$_2$O$_3$ layer. To get the final MNPs, HF and NaF were added to convert the lanthanide oxide to lanthanide fluoride, and also etch out the SiO$_2$ layer.\textsuperscript{11}

Fluorides in simpler form (i.e., CaF$_2$, GdF$_3$, and SrF$_2$) have also been doped with various Ln ions to produce multifunctional Ln-NPs. Passuello et al. synthesized Ln-doped (Er$^{3+}$/Yb$^{3+}$ and Tm$^{3+}$/Yb$^{3+}$) GdF$_3$ NPs by hydrothermal treatment on mixed lanthanide chloride/NH$_3$F solution.\textsuperscript{45} PEG was also added as the capping agent. Rod-shape NPs (average size: 85 × 150 nm) were produced while uncapped GdF$_3$ NPs were spherical (average size: 65–75 nm). The PEG layers reduce the water molecules around Ln ions that could quench
performed on mice to demonstrate the potential of GdF3 NPs as $T_2$ MRI contrast agent.

Gadolinium oxide NPs can be directly used as an MRI/Optical bimodal probe. They are also very good host material to upconverting lanthanide ions and have been more widely exploited as bimodal probe ($\text{Gd}_2\text{O}_3, \text{Gd}_2\text{O}_2\text{S}_{80}$, $\text{Gd}_2\text{O}_3$). Paik et al. reported an $\text{Er}^{3+}/\text{Yb}^{3+}$ doped $\text{Gd}_2\text{O}_3$ NPs as potential bimodal imaging probe. What is particularly interesting about Paik’s work is that, through the addition LiOH as shape directing agent, the shape of the $\text{Gd}_2\text{O}_3$ NPs can be tuned from tri-podal to triangular shape. The morphology of NPs has been reported to have influence on cellular uptake, biodistribution, and cytototoxicity. As most multimodal probes are in spherical shape, Paik’s work represents an alternative perspective to design target-specific, biocompatible nanoparticles by shape controlling. Apart from doping, $\text{Gd}_2\text{O}_3$ NPs can also be conjugated with other imaging probes. Recently, Li et al. conjugated PEG-lyted $\text{Gd}_2\text{O}_3$ NPs with aptamer functionalized silver nanoclusters (aptamer-Ag NCs). The conjugation was achieved by covalent bonding between the carboxylic groups on the surface of PEG-$\text{Gd}_2\text{O}_3$ NPs and the amino groups from the aptamer. The fluorescence of aptamer-Ag NCs and the relaxivity of $\text{Gd}_2\text{O}_3$ NPs were well retained after the conjugation. Subsequently, MR/Optical imaging was conducted on MCF-7 cells to demonstrate the dual-modal imaging capability of PEG-$\text{Gd}_2\text{O}_3$/aptamer-Ag nanoprobe. Enhanced cells uptake of the nanoprobe was also observed due to the presence of aptamers as targeting ligands.

Currently, the clinical MRI community is paying more and more attention on high magnetic field strength (>3T) MRI as it can provide image with higher signal-to-noise ratio. At this strong magnetic field, $\text{Gd}^{3+}$ based contrast agents exhibit lower relaxivity and magnetization. Effort has been invested to explore the potential of other paramagnetic lanthanide ions, including $\text{ Dy}^{3+}$, $\text{Pr}^{3+}$, $\text{Sm}^{3+}$, $\text{Ho}^{3+}$, $\text{ Er}^{3+}$, and $\text{Yb}^{3+}$, as MRI contrast agents. Compare to $\text{Gd}^{3+}$ ions, those paramagnetic ions have shorter electronic relaxation time and mainly affect $T_2$ contrast. Among those lanthanides, $\text{ Dy}^{3+}$ is attracting most of the research interest due to its short relaxation and high magnetic moment. And just like other lanthanide ions, the optical property of $\text{ Dy}^{3+}$ can be manipulated through doping in order to be applied for optical imaging. One good example was reported by Das and his colleague. $\text{ Dy}_2\text{O}_3$ nanocrystals with an average diameter of 3 nm were synthesized through a stepwise thermolysis of $\text{Tb-Dy-oleate}$ complex at 280 °C. $\text{Tb}^{3+}$ ions were doped to enhance the luminescence. The transverse relaxivity ($T_2$) was measured to be 2.17 $\text{mM}^{-1}$ $\text{s}^{-1}$ at 7T magnetic field. The contrast enhancement is demonstrated by phantom imaging (Fig. 9(a)). A clear dose-dependent contrast can be observed, which indicates the good sensitivity of the $\text{ Dy}_2\text{O}_3$ nanocrystal.
Ever since its discovery in 1991, Carbon Nanotubes (CNTs) have become one of the hottest materials in the research community due to its unique physical, chemical and biological properties. In the bio-imaging field, CNTs have been proved to have very low toxicity by various in vitro and in vivo studies. SWNTs exhibit strong fluorescence emission in the NIR region. The high NIR absorbance of CNTs makes it a good contrast agent for photoacoustic imaging. SWCTs have also been utilized in Raman imaging due to its unique Raman signature and large scattering cross-section. The good echogenicity of multi walled CNTs (MWNTs) can be utilized for contrast enhancement in ultrasound imaging. Metal catalysts are often used in the fabrication CNTs and the residual metal ions enable CNTs to function as MRI contrast agents. Besides exploiting the intrinsic properties of CNTs, additional imaging modalities can be integrated with CNTs through conjugation. The large surface area of CNTs can be functionalized and then linked with other imaging probes, including radio-isotopes, iron oxide, and lanthanide nanocrystals and QDs. By sealing the endings of CNT, a large internal cavity can be created, in which a great amount of imaging and therapeutic agents can be loaded.

Recently, Chao et al. demonstrated human mesenchymal stem cells (hMSCs) labeling with protamine-conjugated SWNTs and successfully conducted in vivo Raman/PAT/MRI triple modality imaging. The SWNTs were synthesized by high-pressure CO disproportionation method, facilitated by metal catalyst. To make the SWNTs water dispersible, they were further PEGylated through sonication with C18PMH-PEG-NH2, a PEG-grafted amphiphilic polymer. Protamine was also conjugated to the PEG-SWNTs to enhance the cellular uptake. A considerable amount of ferric nanoparticles was found remain in the functionalized SWNTs, which brings a high $T_2$ relaxivity of 482.6 m M$^{-1}$s$^{-1}$. In vivo stem cell mapping was performed on a nude mouse model. hMSCs with and without SWNTs labeling were injected to a mouse subcutaneously. And then the mouse was imaged by Raman spectroscopic mapping, MR imaging and photoacoustic imaging. In all three imaging modalities, the site with SWNT-labeled stem cells injection shows notable differences compared to the control site (Fig. 10).

Instead of working as contrast agents, Chen et al. utilized CNTs as a nano-platform to which other imaging probes are attached. The internal cavity of CNTs was filled with Fe$_3$O$_4$ nanoparticles while the exterior surface was modified with dispersing agent poly(sodium 4-styrenesulfonate) (PSS). CdTe QDs was encapsulated with a thin SiO$_2$ layer (HQDs) and then capped with poly(allylamine) (PAH). By conjugating the HQDs and CNTs, a magnetofluorescent nano-complex was created. Transferrin was attached to the CNTs to enhance the tumor targeting capability. Doxorubicin hydrochloride (DOX) was also loaded to the CNTs, making the nano-complex a theranostic ensemble (Fig. 11). The drug delivery and fluorescent imaging functionalities were demonstrated in HeLa cell. However, the Fe$_3$O$_4$ nanoparticles were only utilized for magnetically guided drug delivery, no MR imaging was conducted.
Graphene/Graphene Oxide

Graphene is a carbon monolayer in which the atoms are arranged in hexagonal (honeycomb) lattice,\textsuperscript{133} it can be described as an unzipped SWNT. Graphene shares many similar features with CNTs, including large surface area for drug/probe loading and high NIR absorbance.\textsuperscript{134} Therefore graphene and its derivative (e.g., graphene oxide (GO) and reduced graphene oxide (RGO)) have also been studied extensively in the bio-imaging community. Due the presence of large amount of reactive oxygen groups, GO and RGO are more commonly used for biological applications as a versatile nanoplatform. Those reactive groups can be utilized to crosslink with hydrophilic capping molecules to prevent aggregation and to reduce cytotoxicity.\textsuperscript{135} Then additional therapeutic/diagnostic agents can be then conjugated with the functionalized GO/RGO.

In the work reported by Shi et al., A MRI/CT dual-modality probe was synthesized by crosslinking graphene oxide with IONPs and gold nanoparticles (AuNPs).\textsuperscript{136} Here, graphene oxide was used as a nanoplatform to integrate IONPs and AuNPs. The IONPs and GO was first integrated by a one-step hydrothermal reaction and then AuNPs are attached to the GO through seeded crystallization. The as prepared AuNPs-IONPs-GO nanocomposite was further functionalized with lipoic acid modified PEG (LA-PEG) and folic acid conjugated LA-PEG (LA-PEG-FA). \textit{In vivo} MRI/CT dual-model imaging was conducted with tumor-bearing BALB/c mice. Clear contrast enhancement can be observed in both MR and CT.
images. In addition, the high NIR absorbance of nanocomposite was utilized to perform photothermal therapy (PTT). The mice were irradiated under an 808-nm laser at the power density of 0.75 W/cm² for 5 min. The photothermal effect was evidenced by the IR thermal images of the mice and the temperature change of tumor. The efficacy of the PTT treatment was evaluated by post-treatment tumor growth. In another similar work reported by the same group, IONPs were also anchored to RGQ, in order to offer additional functions. The difference is that the high NIR absorbance of graphene is used not only for PTT but also for photoacoustic imaging. The RGO-IONPs were functionalized with PEG and then labeled with ¹²⁵I and Cy5. Blood circulation, biodistribution studies and multimodal imaging were then conducted on 4T₁ tumor-bearing mice (Fig. 12).

Another interesting derivative from graphene is graphene quantum dots (GQDs). GQDs are small luminescent fragment (<20 nm in size) of graphene, produced by either fragmentation of graphene sheet or chemical synthesis. It is often confused with carbon dots (CDs, which will be discussed later) due to the similar properties. So far there is no strict boundary to differentiate those two types of nanoparticles. One practical method is to differentiate GQDs with CDs by their morphologies. Produced from graphene sheet, GQDs appears more “flat” while CDs are generally spherical. The early studies of GQDs mainly focus on its physical properties. Recently, due to its excellent optical properties and low cytotoxicity, more and more researchers started to investigate the potential of GQDs as bio-imaging probe.

**Carbon Dots**

Carbon dots (CDs) are quantum sized (<10 nm) photoluminescent (PL) carbon particles. Due to its excellent biocompatibility, abundant nature and potential for large-scale green synthesis, it has got significant attention in the bioimaging community as an alternative to heavy-metal-containing QDs. The synthetic methods of CDs are generally categorized into two types: top-down approach, including arc discharge, laser ablation, and electrochemical oxidation, where CDs are “peeled off” from a bulk carbon source; bottom-up approach, including thermal/acidic oxidation, hydrothermal reaction, and microwave/ultrasonic methods. A clear understanding of the luminescent origin is still missing. However, it is believed that the PL property is strongly related to the surface passivation/oxidation. Sun et al. argued that surface energy traps cause the luminescence. Those energy traps are stabilized by quantum confinement effect and become emissive after surface passivation. This hypothesis is supported by the fact that many reported CDs require a passivation layer to exhibit PL. However, some CDs produced from bottom-up methods can exhibit PL properties without any additional passivation agent. This may be argued that those passivation-free CDs can only exhibit PL with the presence of solvent; the solvent molecules are providing the passivation effect to the suspending CDs. Some researchers attribute the luminescence to the surface carboxylate group. Since most of the as-synthesized CDs are made of particles with different size, the emission spectra of CDs are wide band spectra, characterized by an emission peak in short wavelength and a long “tail”.

![Figure 12. The blood circulation (a) and biodistribution (b) of ¹²⁵I-RGO–IONP–PEG. Particles were found accumulating in tumor. (c–e) Multimodal imaging of mice after intravenous injection of RGO–IONP–PEG: (c) Fluorescence imaging using Cy5-RGO–IONP–PEG; (d) T₂-weighted MR imaging, and (e) photoacoustic imaging using RGO–IONP–PEG. The images obtained from various modalities further proved the high tumor uptake of RGO–IONP–PEG particles. Reprinted with permission from [137], K. Yang, et al., Multimodal imaging guided photothermal therapy using functionalized graphene nanosheets anchored with magnetic nanoparticles. *Adv. Mater.* 24, 1868 (2012). © 2012, John Wiley and Sons.](image_url)
extends into longer wavelength region. Further separation of CDs can narrow down the emission spectra and also increase the quantum yields.\textsuperscript{148,149} On the other hand, the wide emission spectra can be exploited for multi-color imaging. The emission spectra of CDs are also excitation dependent, and the peak position has a red-shifter when exited with longer-wavelength light. One drawback for CDs as in vivo imaging probe is that the emission peaks of currently reported CDs are limited in the UV-Vis region, which will be strongly scattered by biology tissue. Some CDs exhibit detectable emission at VIS-IR region, and might be suitable for in vivo imaging. Tao et al. synthesized CDs by treating CNTs and graphite with mixed acid.\textsuperscript{150} The CDs were used for in vivo imaging of mice under different excitation. As show in Figure 13, the CDs’ fluorescence can still be well distinguished with background autofluorescence at 704 nm excitation (770 nm emission).

Although CDs are being investigated as optical probe, not many CD based multimodality probes has been reported. Since doping has been used to enhance the quantum yield of CDs, magnetic moieties (Gadolinium,\textsuperscript{151–153} iron oxide\textsuperscript{154}) may be doped into CDs to produce magnetofluorescent CDs. The surface functional groups also possess potential for covalent conjugation with other imaging or therapeutic agent.\textsuperscript{155}

One example of such kind of doped magnetofluorescent CDs was reported by Bourlinos et al.\textsuperscript{151} The CDs are synthesized by thermal decomposition of tris(hydroxymethyl)aminomethane and betaine hydrochloride. Gadopentetic acid was added before the pyrolysis to incorporate the Gd\textsuperscript{3+} ions (Fig. 14(a)). The core size of the Gd-QCDs is $\sim$3 nm while the average hydrodynamic diameter is 4 nm. The Gd-QCDs have a wavelength-dependent emission in the UV-VIS region, with an emission peak in 445 nm. The author did not measure the relaxivity of the Gd-QCDs, but proved the MRI contrast enhancing capability of the Gd-QCDs by comparing the MR image of Gd-QCDs with a commercial $T_1$ contrast agent Gadovist (Fig. 14(b)).

**GOLD NANOPROBES**

Nano-scaled (1–100 nm) gold particles (AuNPs) possess many distinct properties compared to bulk gold. Particularly, for bio-imaging application, the following features are considered most interesting: The absorbance and fluorescence of gold nanostructures is significantly enhanced with respect to bulk gold, and just like QDs, the absorption and emission wavelength can be tuned by manipulating the size and morphology of the nanoparticles.\textsuperscript{156} Rod-shaped gold nanoparticles exhibit strong multi-photon fluorescence in NIR range and have been already applied to image cancer cells\textsuperscript{157} and cerebral vasculature.\textsuperscript{158} The heavy atomic weight of gold element makes it an ideal contrast agent for X-ray CT. Gold nanorod shows higher contrast enhancement compared to the commercial iodine CT contrast agent.\textsuperscript{159} AuNPs are also holding the potential as MRI contrast agents due to their paramagnetic nature.\textsuperscript{160} The strong electromagnetic enhancement of AuNPs can be utilized in surface-enhanced Raman scattering (SERS) essays.\textsuperscript{161} At their plasmon wavelength, AuNPs show strong light scattering. This feature can be used for contrast enhancement in various optical imaging techniques. Au-NPs with various shapes, including nanoshells,\textsuperscript{162} nanocages\textsuperscript{163} and nanorods,\textsuperscript{164} have been applied for OCT imaging. Besides, dark field microscopy imaging\textsuperscript{165} and reflectance confocal microscopy (RCM) imaging\textsuperscript{166} using Au-NPs have been reported. The excellent light absorption and efficient light-heat conversion of Au-NPs have been exploited for photothermal\textsuperscript{167} (direct detection of generated heat) and photoacoustic\textsuperscript{168} (detection of generated phonons) imaging. The absorption of AuNPs decreases as the 3rd power of diameter while scattering decreases as the 6th power of diameter. This means that below certain size, absorption will be the predominant

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\caption{(A) In vivo fluorescence images of a mouse injected with carbon dots. The images were captured at various excitation wavelengths (455, 523, 595, 605, 635, 661, and 704 nm). Red represents fluorescent signals of CDs and green represent signals from tissue autofluorescence. (B) Spectra difference between carbon dots fluorescence (1) and tissue autofluorescence (2) under 704 nm excitation. Reprinted with permission from [150]. H. Tao, et al., In vivo NIR fluorescence imaging, biodistribution, and toxicology of photoluminescent carbon dots produced from carbon nanotubes and graphite. Small 8, 281 (2012). © 2012, John Wiley and Sons.}
\end{figure}
event. Therefore, small Au-NPs are often used for photothermal imaging while larger AuNPs are more suitable for photoacoustic imaging.\textsuperscript{161} The laser induced heat effect is also often used for photothermal therapy.

The first scientifically reported synthesis of colloidal gold particles can be traced back to the year of 1857, when Michael Faraday obtained a “ruby fluid” by stabilizing reduced gold chloride with carbon-disulfide.\textsuperscript{169} Today, the similar principle is still followed in many nanoparticle preparation methods: gold salts are reduced with the addition of capping agents. The capping agents will prevent aggregation of particles and therefore the particles sizes are restricted in the nano range. With the development of nano-synthesis technology, controlled synthesis of AuNPs with various shapes and sizes are possible.\textsuperscript{170} For bio-imaging application, this means researcher can choose the optimal Au-NPs which suit the imaging targets.

Before applying AuNPs to bio-imaging applications, one essential step is to functionalize the particles surface. Many capping agents used in the synthesis of AuNPs are not biocompatible. For example, Cetrimonium bromide (CTAB), which is often used for morphology control during the Au-NPs synthesis, is found to be toxic.\textsuperscript{172} A variety of functionalization methods, including ligand exchange, PEGylation, silica/polymer coating and biomolecule capping, has been developed to in order to enhance the biocompatibility of AuNPs.\textsuperscript{173} Additionally, the functionalized Au-NPs can be further conjugated with other imaging probe or therapeutic agents.

Without integrating with other imaging probes, the intrinsic properties has already enable AuNPs to function as nanoprobes in multimodal imaging. Recently, Hembury et al. synthesized gold-silica quantum rattles (QRs) and applied them in NIR fluorescence, photoacoustic, and MR imaging.\textsuperscript{175} The Au–SiO\textsubscript{2} QRs were synthesized by restricted gold nucleation within the mesoporous silica nanoparticles. The as synthesized QRs exhibit well-defined hollow spherical shape (∼150 nm diameter) with mesoporous silica shells (∼25 nm). Two types of gold nanoparticles were produced and separately distributed: small Au-NPs (<2 nm) inside the silica shell and large AuNPs (∼7.3 nm) in the macrocavity (Fig. 15). The tri-modal imaging potential of the Au–SiO\textsubscript{2} QRs was investigated by NIR/photoacoustic/MR imaging in an LS174T-luc tumor bearing mouse model. In addition, the Au–SiO\textsubscript{2} QRs were also tested in photothermal therapy with HeLa cells and tumor bearing mouse. Efficient and localized tumor ablation was observed after NIR irradiation (671 nm, 38 W·cm\textsuperscript{−2}, 10 min). Drug loading efficiency was tested by conjugating DOX with the QRs.

It is also very common to integrate AuNPs with other imaging probes, such as QDs,\textsuperscript{175,176} IONPs,\textsuperscript{177} Gd\textsuperscript{3+} chelates\textsuperscript{178} and radionuclide,\textsuperscript{179} to produce multimodal probes. Besides, gold has been widely used as shell materials to produce multimodal nanoprobes with Core/Shell configuration.\textsuperscript{180,181} Recently, Coughlin et al. conjugated gadolinium chelates with gold nanoshells and successfully applies this nanocomplex in multimodal imaging.\textsuperscript{178} The ~16 nm gold shells were grown on the surface of ~120 nm silica nanoparticles. The Au-nanoshells were further functionalized with PEG and then conjugated with Gd-DOTA chelates. The synthesized gadolinium-nanoshell (Gd-NS) complexes exhibit a high $T_1$ relaxivity of 37 mM\textsuperscript{−1} S\textsuperscript{−1}. In vivo $T_1$-MR and CT imaging were conducted on a tumor-bearing mouse after intratumoral injection of Gd-NS. Compared to images of control groups, the Gd-NS produced significant contrast enhancement (Fig. 16).

**METAL-ORGANIC FRAMEWORKS**

Metal-Organic Frameworks (MOFs) are a type of materials formed by crosslinking inorganic ions or clusters with organic ligands. By changing the metal/ligand combination, the physicochemical properties can manipulated in a
Nanoparticulate Contrast Agents for Multimodality Molecular Imaging

Xia et al.

Figure 15. The morphology of Au–SiO₂ QRs. (A) Schematic illustration of a QR. The gray shell represents the mesoporous silica shell, the red dots inside the mesopores represent the small Au-NPs while the yellow dots in the macrocavity represent the large AuNPs. (B) An ultrathin section of the resin-embedded QRs, imaged by Bright field TEM, the large AuNPs in the macrocavity of the QRs can be clearly visualized. (Scale bar, 200 nm.) (C) Bright field TEM image of a QR with large AuNPs inside the cavity of the silica shell. (Scale bar, 20 nm.) (D) QR’s silica shell image by higher magnification TEM, The red arrows point out the small AuNPs with the mesopores. (Scale bar, 20 nm.) (E) The gold nanostructures within a QR are highlighted by HAADF-STEM imaging (Scale bar, 20 nm.) (F) QR’s silica shell image by higher magnification HAADF-STEM. The red arrows point out the small AuNPs with the mesopores. (Scale bar, 20 nm.) Reprinted with permission from [174], M. Hembury, et al., Gold–silica quantum rattles for multimodal imaging and therapy. Proceedings of the National Academy of Sciences 112, 1959 (2015). © 2015, National Academy of Sciences.

Figure 16. T₁-weighted MR images and X-ray CT images of B16-F10 melanoma tumor bearing mice. 50 μL of Gd-NS (6.3 x 10¹² particles/mL) was injected intratumorally for A and B. Compared to the images taken on mice without Gd-NS injection, the Gd-NS produced significant contrast enhancement. The red circle indicates the tumor position. Reprinted with permission from [178], A. J. Coughlin, et al., Gadolinium-conjugated gold nanoshells for multimodal diagnostic imaging and photothermal cancer therapy. Small 10, 556 (2014). © 2014, John Wiley and Sons.

wide range. Generally, MOFs can be characterized by their ultra-high porosity and exceptionally large surface area, which makes them a promising material in gas storage, separation and catalysis. Recently, nanoscale MOFs (NMOFs) have been attracting more and more attention in the bio-imaging field. First, the diversity of NMOFs makes it very convenient to tailor NMOFs for different bio-imaging application. Second, by crosslinking organic and inorganic moieties, the strengths of the types of materials can be combined. And last but not the least, MOFs is generally biodegradable due to the relatively weak metal-ligands bonds, which reduced the risk of long-term biological retention and accompanying side effect.

MOFs based nanoprobes can be prepared by two different strategies: to incorporate the imaging moieties during the synthesis of MOFs or to load imaging probes by post-synthesis treatment. The direct incorporation methods normally involve metal ions or ligands that can function as imaging probes. MOFs contain magnetic ions have been reported as MRI contrast agent. Lanthanide-based MOFs possess the potential as optical imaging probes. MOFs with iodinated ligands can be used for contrast enhancement in X-ray CT. For the post-synthesis strategy, imaging probes can be loaded to the pores of MOFs through covalent conjugation or noncovalent encapsulation.

To synthesize multimodal MOF probes, different imaging probes can be integrated in the same MOFs. In a very recent work reported by Tian et al., a MRI/CT bimodal MOF probe was synthesized by applying both of the aforementioned loading strategies. Gadolinium MOFs (GdMOFs) were first synthesized through a surfactant (CTAB) facilitated method with Gd³⁺ as the coordinating ions. Then Poly(acrylic acid) (PAA) was used to functionalize the GdMOFs. Next, gold ions were bonded to the carboxylic acid groups of PAA. Finally, by reducing the Au ions, gold nanoparticles (average size 4 ± 2 nm) were produced at the surface of PAA-GdMOFs (Fig. 17). The bimodal imaging performance of the nanoprobes was only evaluated by CT/MR imaging of Au-PAA-GdMOFs solution with various concentrations. Further in vitro and in vivo imaging applications are expected.

PORPHYRINS-BASED NANOPROBES

Porphyrins are a groups of aromatic molecules that are widely occurring in nature. Porphyrin structures play important roles in many vital metabolism processes including photosynthesis reaction (porphyrin presented in chlorophyll) and oxygen transportation (porphyrin
Figure 17. Nanostructures during the synthesis of Au-PAA-GdMOF nanoprobe. (a) PAA functionalized GdMOF, (b) PAA-GdMOFs loaded with Au$^{3+}$. (c) Au nanoparticles produced by Au$^{3+}$ reduction. (d) A structure illustration of hybrid Au-PAA-GdMOF nanocomplex. The blue core represents GdMOF, the blue chains represent PAA molecules and golden dots represent AuNPs. Reprinted with permission from [186], C. Tian, et al., Poly(acrylic acid) bridged gadolinium metal-organic framework-gold nanoparticle composites as contrast agents for computed tomography and magnetic resonance bimodal imaging. ACS Applied Materials and Interfaces (2015). © 2015, American Chemical Society.

presented in hemoglobin). Intrinsic porphyrin-containing hemoglobin has already been used as contrast agent for phocoustic imaging$^{189}$ and functional MRI.$^{190}$ On the other hand, exogenous porphyrins are also holding a potential as molecular imaging probes due to its NIR fluorescence.$^{191}$ The metal chelating capability can be utilized to load magnetic ions$^{192}$ or radionuclides.$^{193}$ In addition, due to the high singlet oxygen yield, porphyrins and their derivatives have become one of the most popular photosensitizer in photo dynamic therapy (PDT).$^{194}$ After delivery to the tumors site, porphyrins generate cytotoxic singlet oxygen to kill the tumor cells. One problem of porphyrin derivatives is that they are often hydrophobic. Therefore, porphyrins are often incorporated with phospholipid to form porphyrin liposome (porphysome) or porphyrin-lipid nanocomplex.$^{195}$

While mostly applied in optical/photonic imaging and therapy, porphyrin based nanoparticles can also be integrated with other imaging probes. Taking advantages of the porphyrin’s intrinsic metal chelating properties, Liu et al. incorporated$^{64}$Cu to porphysome with an exceptionally high loading capacity (2800 Ci/μmol per particle).$^{196}$ The$^{64}$Cu chelation was found stable in vivo for at least 30 hours, which is essential for the co-registration of optical/PET image. The$^{64}$Cu-phorphysome was applied for PET/optical dual-modal imaging on prthotopic prostate tumor bearing mouse models.$^{197}$ Similar to liposomes, porphysomes can also be used to conjugate or encapsulate other imaging probes. Rieffel et al. encapsulated UCNPs (core–shell NaYbF$_4$:Tm$^{3+}$-NaYF$_4$) with$^{64}$Cu labeled porphyrin-phospholipid (PoP).$^{198}$ After functionalization with PEG-lipid, the$^{64}$Cu-PoP-UCNPs were applied for hexamodal imaging: down-conversion fluorescence (FL), up-conversion luminescence (UC), PET, CT (the dense electron content of UCNPs provides high X-ray attenuation), Cerenkov luminescence (CL) and photoacoustic imaging. (Fig. 18)

**DENDRIMER-BASED NANOPROBES**

Dendrimers are defined as highly branched synthetic macromolecules with tree-like configuration. Dendrimers are considered as excellent nanoplatform due to their branched interior, globular shape and abundant surface functional groups that enables further modifications.$^{199}$ Multimodal dendrimer probes can be prepared by conjugating intrinsically multimodal contrast agents with dendrimer or by conjugating different contrast agents with same dendrimer. The Raymond group developed a series of bifunctional lanthanide (Ln) chelators, and those chelators can be further conjugated to functionalized dendrimers. In 2012, Andolina et al. from the Raymond groups chelated Dy$^{3+}$, Eu$^{3+}$, Gd$^{3+}$/Sm$^{3+}$, and Yb$^{3+}$ with TREN-bis(1-Me)-3,2-HOPO-TAM-NX (X = 1, 2, 3) ligands and subsequently conjugated to the esteramide dendrimer (EA). The intrinsic fluorescent and magnetic nature of Ln ions makes the EA-Ln conjugates potential MRI/Optical bimodal probes.$^{200}$ Also using chelating method, Harrison et al. conjugated two contrast agents, Gd$^{3+}$ and fluorescein isothiocyanate, to a phenolic dendrimer core. The phenolic core was modified with amine-functionalized linkers in order to form stable bond with the fluorescein molecules (Fig. 19).$^{201}$ Dendrimers have also been used to encapsulate other nanoparticles, such as Au-NPs$^{202}$ and IONPs,$^{203}$ to form dendrimer entrapped nanoparticles (DENPs). The dendrimer entrapment renders those nanoparticles with enhanced biocompatibilities and tunable surface functionalities. Recently, Chen et al. reported a stepwise method to prepared gold DENPs with Gd chelation.$^{204}$ First, amine-terminated generation 5 poly(amidoamine) dendrimers (G5-NH$_2$) were
conjugated with DOTA-NHS chelators. Next, the G5-DOTA nanocomplexes were functionalized by PEGlyted RGD peptides. Subsequently, Au-NPs were generated in the interior of RGD-G5-DOTA templated nanocrystallization. After chelation with Gd\(^{3+}\) ions, the final step is to transfer the residual terminal amine groups to acetamide groups through acetylation (Fig. 20). The as prepared Gd–Au DENPs-RGD probes have an average core size of 3.8 nm (determined by TEM). The hydrodynamic diameter is much larger (71.7 ± 0.8 nm, determined by DLS), but still within the appropriate range for biomedical application. After confirming the biocompatibility with MTT essay, the Gd–Au DENPs-RGD probes were applied for CT and \(T_1\) weighted imaging on U87MG tumor bearing mice. Significant contrast enhancement can be observed by comparing the mouse injected with Gd–Au DENPs-RGD and the mouse injected with free RGD peptides.

**NANOPROBES FOR PET IMAGING**

Positron emission tomography (PET) has now been widely applied in both clinical and research. The basic principle of PET was proposed in the 1950s, when researchers start to realize that the high-energy photons produced by positron annihilation can be utilized to determine the 3-D position of certain substance. After mid-1980s, PET devices had become available for diagnostic imaging. Since then, significant advances in PET instrumentation, method and computational algorithm have been witnessed, which makes PET imaging a powerful imaging technique as we see it today.\(^{205}\)
Generally, the PET protocol begins with injection of a radio labeled tracer. One of the most widely used radiotracers is 2-deoxy-2-\(^{(18)}\)fluoro-D-glucose (FDG), an analog to glucose in which the normal hydroxyl group is substituted by positron emitting \(^{18}\)F isotope. After the injection, the radiotracer will accumulate in certain regions, to which the radiotracers have affinity. For example, the FDG will most probably accumulate in brain or tumor, which consumes a large quantity of glucose. The radionuclides will then decay and emit positrons. The ejected positrons will immediately combine with nearby electrons and undergo annihilation process. Two high-energy photons with same speed but opposite directions will be generated by this annihilation process. By detecting the generated photons, the position of the annihilation event can be determined.\(^{206}\)

While most of the clinical PET radiotracers are small molecules, there is an increasing interest to develop nanoparticle based PET probe in order to combine the quantitation of PET with specific targeting and multifunctionality of nanoparticles. The structure of nanoparticles and the wide variety of functionalization agent give nanoparticles a high freedom when choosing the radioligand. Chelating agent such as DOTA plays an important role to conjugate PET isotopes with nanoparticles.

Positron emission isotopes can be conjugated to QDs to produce Optical/PET bimodal probes. Compared to magnetofluorescent QDs, the smaller sensitivities discrepancy makes it practically easier to combine PET and Optical probes.\(^{39}\) PET imaging has higher penetration depth and is more quantitative, while optical imaging can provide real time visualization. This kind of bimodal probe has been used to image cancer\(^{207,208}\) and to study the biodistribution of QDs.\(^{208}\) Another way to combine optical and PET imaging is to utilize the Cerenkov radiation (CR) phenomenon. CR occurs when charged particles travel in an insulating

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**Figure 19.** Fluorescein was conjugated to Gd-dendrimer complex through isothiocyanate-amine coupling. Reprinted with permission from [201], V. S. R. Harrison, et al., A multimeric MR-optical contrast agent for multimodal imaging. Chem. Commun. 50, 11469 (2014). © 2014, Royal Society of Chemistry.

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**Figure 20.** Synthetic route of Gd−Au DENPs-RGD nanoprobes. The red circles indicate the Au-NPs generated inside the G5 dendrimers. Reprinted with permission from [204], Q. Chen, et al., Multifunctional dendrimer-entraped gold nanoparticles modified with RGD peptide for targeted computed tomography/magnetic resonance dual-modal imaging of tumors. Anal. Chem. 87, 3949 (2015). © 2015, American Chemical Society.
Nanoparticulate Contrast Agents for Multimodality Molecular Imaging
Xia et al.

Figure 21.  (a) Optical, (b) microPET and (c) MR images of 4T1 tumor bearing mouse with \(^{124}\text{I}\) labeled IONPs injection from paws (indicate by the capital ‘I’). The tumor was implanted in the shoulder (yellow arrow). Red dashed circle: sentinel lymph node, white arrow: fiduciary markers, and red arrow: bladder. (d) \textit{Ex vivo} luminescence images (upper) and microPET (bottom) images of the dissected lymph nodes. (e) Schematic illustration of the injection route of nanoparticles. Reprinted with permission from [37], J. C. Park, et al., Facile preparation of a hybrid nanoprobe for triple-modality optical/PET/MR imaging. Small 6, 2863 (2010). © 2010, John Wiley and Sons.

material with a speed higher than speed of light in that material. And this radiation can be detected by optical imaging system.\(^{209,210}\) Positron-emitting nuclides, including fluorine-18, oxygen-15, gallium-68 and iodine-124, have enough energy to cause CR and hence can be used in CR imaging.\(^{211}\) That is to say, NPs synthesized with only PET and MR imaging agents may be readily detectable by optical imaging system. Therefore, the development of CR imaging provided an easier strategy to prepare Optical/MRI/PET probes. However, the light produced by CR is mostly in the UV-VIS range, which may limit its \textit{in vivo} application due to the high absorbance in biological tissues. Researchers have been trying to overcome this limitation by utilizing a process termed Cerenkov radiation energy transfer (CRET), in which a second fluorophore (such as QD) was conjugated with the PEI isotope.\(^{212}\) The second fluorophore absorbs the short wavelength CR light and re-emit light in a much shorter wavelength, which is easier to detect.

Apart from PET/Optical integration, MRI/PET multimodal agent may be more promising in clinical application. Both MRI and PET have unlimited \textit{in vivo} penetration depth and combined PET/MRI instrument has been reported.\(^{213}\) One proposed strategy to apply PET/MRI imaging is to use PET whole body screen to identify the region of interest and then use MRI to obtain the high-resolution image of that specific region. This strategy is expected save a lot of time that is otherwise required for whole body MRI screen.\(^{6}\) Tu et al. reported a MRI/PET probe made by conjugating \(^{64}\text{Cu}-\text{DOTA}\) complex with dextran coated iron oxide (DIO) nanoparticles.\(^{214}\) This bimodal probe was specially modified with maleic anhydride to obtain a high negative surface charge. The increased anionic charge helps the probe to target as the scavenger receptor type A (SR-A) which is commonly expressed in macrophage events. The core size of the maleylated DIO (MDIO) was determined around 7–8 nm, similar to that of non-maleylated DIO, but the maleylation significantly increased the hydrodynamic size (DIO-38.1 nm, MDIO-62.7 nm). The \(T_2\) relaxivity of MDIO is measured to be 95.8 mM\(^{-1}\)s\(^{-1}\) (1.7T). In order to attest the specific binding ability of MDIO, the author further labeled the MDIO with fluorescent dye 5(6)-TAMRA-SE. The fluorescent image on P388D1 cells (mouse macrophage cell line) and \(T_2\) MR image on cell lysate proved the enhanced cell uptake of MDIO. This also suggests that the MDIO can be functionalized to become a triple functional probe. Xie et al. reported a similar PET/Optical/MRI triple nanoprobe by labeling human serum albumin (HAS) encapsulated IONPs with \(^{64}\text{Cu}-\text{DOTA}\) and NIR dye Cy5.5.\(^{38}\) The HSA matrices prolonged the NPs’ circulation time. \textit{In vivo} PET/MR/NIR imaging on U87MG (glioblastoma cell) xenografted mouse model revealed significant NP accumulation in the
Table II. Summary of the discussed multimodal probes and their imaging modalities.

<table>
<thead>
<tr>
<th>Probe category</th>
<th>Integrated contrast agents</th>
<th>Integrated modalities</th>
<th>Integration strategy</th>
<th>Surface functionalization</th>
<th>Size (nm)</th>
<th>Therapeutic agent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>QDs</td>
<td>CdTe/ZnS QD and Gd-DOTA</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>mPEG-NH₂, 2000/Fl127-NH₂</td>
<td>10.8 ± 2.5 (PEG-QDs)/22.1 ± 5.7 (F127-QDs)</td>
<td>–</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>CdSeTe/CdS QDs and Gd-DOTA</td>
<td>Fluorescence and MRI</td>
<td>Doping</td>
<td>Glutathione</td>
<td>10 ± 0.2</td>
<td>–</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>CdTeS QD and Fe³⁺</td>
<td>Fluorescence and MRI</td>
<td>Doping</td>
<td>N-acetyl-cysteine</td>
<td>3–6</td>
<td>–</td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td>CuInS₂/ZnS QD and Mn²⁺</td>
<td>Fluorescence and MRI</td>
<td>Shell doping</td>
<td>PAA</td>
<td>4.7</td>
<td>–</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td>CuInS₂/ZnS QD and Mn²⁺</td>
<td>Fluorescence and MRI</td>
<td>Shell doping</td>
<td>CTAB</td>
<td>4</td>
<td>–</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>ZnO QD and Gd³⁺</td>
<td>Fluorescence and MRI</td>
<td>Doping</td>
<td>Amido</td>
<td>4</td>
<td>–</td>
<td>[58]</td>
</tr>
<tr>
<td>Si QD and Mn²⁺</td>
<td>Fluorescence and MRI</td>
<td>Doping</td>
<td>Dextran sulfate</td>
<td>4.3 ± 1.0 Dextran sulfate</td>
<td>–</td>
<td>[68]</td>
<td></td>
</tr>
<tr>
<td>Si QD and SPION</td>
<td>Fluorescence and MRI</td>
<td>Co-encapsulation</td>
<td>DSPE-PEG</td>
<td>50–100</td>
<td>–</td>
<td>[71]</td>
<td></td>
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<tr>
<td>Si QD and Gd-DOTA</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>DSPE-PEG</td>
<td>90</td>
<td>–</td>
<td>[70]</td>
<td></td>
</tr>
<tr>
<td>IONPs</td>
<td>Fe₃O₄ NP and Rh123</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>Poly(acrylic acid) ~10</td>
<td>Folic acid</td>
<td>[12]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe₃O₄ NP and CdSe/ZnS QD</td>
<td>Fluorescence and MRI</td>
<td>Multilayer encapsulation</td>
<td>APS 150 Anti-HER2 antibodies</td>
<td>–</td>
<td>[75]</td>
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<td></td>
<td>MnFe₂O₄ NP and CdSe/ZnS QD</td>
<td>Fluorescence and MRI</td>
<td>Gel encapsulation</td>
<td>PEG 240</td>
<td>–</td>
<td>[76]</td>
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<tr>
<td></td>
<td>USPION and polypyrrole</td>
<td>Photoacoustic and MRI</td>
<td>Encapsulation</td>
<td>PEG 100</td>
<td>–</td>
<td>[77]</td>
<td></td>
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<tr>
<td></td>
<td>IONP and ⁶⁴Cu-DOTA and 5(6)-TAMRA-SE</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>Dextran 7–8 Maleic anhydride</td>
<td>–</td>
<td>[214]</td>
<td></td>
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<tr>
<td></td>
<td>IONP and ⁶⁴Cu-DOTA and CyS₅</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>Dopamine/ HAS 29.4 ± 1.2</td>
<td>–</td>
<td>[38]</td>
<td></td>
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<tr>
<td></td>
<td>IONP and ¹²⁴I</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>Tyramine 39 ± 8</td>
<td>–</td>
<td>[37]</td>
<td></td>
</tr>
<tr>
<td>Ln-NPs</td>
<td>NaYb₄Gd₆Yb₁₈E₅₂F₄₊₁₈⁺ and ¹⁸F</td>
<td>PET and MRI and UCL</td>
<td>Doping</td>
<td>Citrate 19–22</td>
<td>–</td>
<td>[86]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaGdF₄ NP and Tm³⁺/Er³⁺/Yb³⁺</td>
<td>UCL and MRI</td>
<td>Doping</td>
<td>–COOH 25–60</td>
<td>–</td>
<td>[88]</td>
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<tr>
<td></td>
<td>NaGdF₄ NP and Yb³⁺/Er³⁺/Tm³⁺/Ho³⁺</td>
<td>UCL and MRI</td>
<td>Doping</td>
<td>Aminated SiO₂ shell 43–68</td>
<td>Folic acid</td>
<td>[93]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KGdF₄ NP and Yb³⁺/Er³⁺</td>
<td>UCL and MRI</td>
<td>Doping</td>
<td>PEI or 6AA 14 ± 2 (PEI-NPs)</td>
<td>–</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaYbF₄/Tm³⁺ and NaGdF₄</td>
<td>UCL and MRI</td>
<td>Doping and core/shell</td>
<td>PAA 12</td>
<td>–</td>
<td>[91]</td>
<td></td>
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<tr>
<td></td>
<td>NaGdF₄/Er³⁺/Yb³⁺ and NaGdF₄</td>
<td>UCL and MRI</td>
<td>Doping and core/shell</td>
<td>PEG 20/31/41</td>
<td>–</td>
<td>[89]</td>
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<tr>
<td></td>
<td>NaYF₄/Yb³⁺/Er³⁺ and USPION</td>
<td>UCL and MRI</td>
<td>Core/Shell</td>
<td>PEG 230 Folic acid and Au shell</td>
<td>–</td>
<td>[85]</td>
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</table>

lesion. Different from the dual-labelling strategy, Park et al. reported a more facile preparation of triple functional IONPs with single labelling. Thermally cross-linked IONPs were labeled with positron emitting $^{124}$I. $^{124}$I decays with a strong $\beta^+$ mean energy, leading to strong Cerenkov radiation, which can be used for Cerenkov luminescence imaging. The strong tissue absorption of CR light is partially offset by the high emission intensity. Optical/microPET/MRI triple modality imaging was conducted on 4 $T_1$ tumor bearing mouse (Fig. 21).

**TARGETING LIGANDS**

So far we have introduced a variety of MFNPs and many of those particles are equipped with targeting ligands. Several excellent reviews on targeting ligands have already

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**Table II. Continued.**

<table>
<thead>
<tr>
<th>Probe category</th>
<th>Integrated contrast agents</th>
<th>Integrated modalities</th>
<th>Integration strategy</th>
<th>Surface functionalization</th>
<th>Size (nm)</th>
<th>Targeting/Therapeutic agent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaLuF$_4$:Yb$^{3+}$/Er$^{3+}$/Tm$^{3+}$ and Fe$_3$O$_4$ NP</td>
<td>UCL and MRI and CT</td>
<td>Core/Shell</td>
<td>N.A.</td>
<td>$\sim 330$</td>
<td>–</td>
<td>[97]</td>
<td></td>
</tr>
<tr>
<td>GdF$_3$ NP and Er$^{3+}$/Yb$^{3+}$ and Tm$^{3+}$/Yb$^{3+}$</td>
<td>UCL and MRI</td>
<td>Doping</td>
<td>PEG</td>
<td>$85 \times 150$ (Rod shape)</td>
<td>–</td>
<td>[43]</td>
<td></td>
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<tr>
<td>Gd$_2$O$_3$ NP and Er$^{3+}$/Yb$^{3+}$</td>
<td>UCL and MRI</td>
<td>Doping</td>
<td>PEI</td>
<td>$\sim 30$ (Triangular and Tripodal)</td>
<td>–</td>
<td>[104]</td>
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<td>Gd$_2$O$_3$ NP and Ag NCs</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>PEG</td>
<td>3.2</td>
<td>Aptamer</td>
<td>[110]</td>
<td></td>
</tr>
<tr>
<td>Dy$_2$O$_3$ NP and Tb$^{3+}$</td>
<td>Fluorescence and MRI</td>
<td>Doping</td>
<td>APS</td>
<td>$3.0 \pm 0.4$</td>
<td>–</td>
<td>[111]</td>
<td></td>
</tr>
<tr>
<td>CNT SWNT and Ferric NPs</td>
<td>Raman and photoacoustic and MRI</td>
<td>Doping</td>
<td>PEG</td>
<td>N.A.</td>
<td>Protamine</td>
<td>[124]</td>
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<tr>
<td>CdTe/SiO$_2$ QD and Fe$_3$O$_4$ NP</td>
<td>Fluorescence and MRI</td>
<td>Dual-conjugation</td>
<td>PSS/PAH</td>
<td>N.A.</td>
<td>Transferin and DOX</td>
<td>[133]</td>
<td></td>
</tr>
<tr>
<td>Graphene IONP and AuNPs</td>
<td>MRI and CT</td>
<td>Dual-conjugation</td>
<td>Conjugation</td>
<td>LA-PEG</td>
<td>200–500</td>
<td>Folic acid</td>
<td>[136]</td>
</tr>
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<td>Carbon dots</td>
<td>CD and Gd$^{3+}$</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>Betaine</td>
<td>3–4</td>
<td>–</td>
<td>[151]</td>
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<td>AuNPs</td>
<td>AuNP and SiO$_2$ NP</td>
<td>Fluorescence and photoacoustic and fluorescence</td>
<td>Encapsulation</td>
<td>CTAB</td>
<td>$\sim 150$</td>
<td>DOX</td>
<td>[174]</td>
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<td>MOFs</td>
<td>Au nanoshell and Gd-DOTA</td>
<td>MRI and CT</td>
<td>Conjugation</td>
<td>PEG</td>
<td>$\sim 136$</td>
<td>–</td>
<td>[178]</td>
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<tr>
<td>Porphyrins NP</td>
<td>GdMOF and AuNPs</td>
<td>MRI and CT</td>
<td>Conjugation</td>
<td>PAA</td>
<td>$155 \pm 30 \times 30 \pm 11$</td>
<td>–</td>
<td>[186]</td>
</tr>
<tr>
<td>Dendrimer</td>
<td>Gd$^{3+}$ and fluorescein Au DENP and Gd-DOTA</td>
<td>Fluorescence and MRI</td>
<td>Conjugation</td>
<td>PEG</td>
<td>3.8</td>
<td>RGD</td>
<td>[204]</td>
</tr>
</tbody>
</table>
been published,\textsuperscript{53, 215–217} so here we will just make a brief summary. Targeting ligands are often used in targeted drug delivery. To differentiate pathological cells with healthy cells, those targeting ligands are designed to bind with receptors that are over expressed in pathological cells but much less expressed in normal cells. Through receptor-mediated endocytosis, these additional targeting ligands can enhance the specific cell binding and internalization. Without targeting ligand, NP internalization is mainly determined by non-specific interaction with cell membrane, which can be relatively slower, especially when the NPs are shielded with PEG layer.\textsuperscript{51} For nanoprobe, targeting ligands can also facilitate the particle localization in lesion.\textsuperscript{218} In terms of particle morphology, targeting ligands are normally presented at the outer surface, on top of the capping layer, to achieve maximum receptor binding. According to their molecular constitution, targeting ligands can be classified into five groups: Full antibody, antibody fragment, peptide, aptamer and small molecules. Each group has its own advantages and limitations on targeting efficacy, stability and ease of preparation.\textsuperscript{217} Significant effort has been put on monoclonal antibodies as targeting ligands, and several successful therapies have been reported based on engineered antibodies.\textsuperscript{219} Despite those successes, the large molecule size, complex modification process and high batch-to-batch variation of monoclonal antibodies have driven researcher to search for alternative ligands. Full sized antibodies can be decomposed into different fragments using proteolytic digestion. Some of those fragments retain the receptor-binding capability. Compare to the full antibody, antibody fragment are smaller and easier to manipulate.\textsuperscript{220} Peptide is another attractive candidate for targeting ligands. Thanks to the development of different peptides library and screen technology, a variety of peptides have been discovered to have high affinities with certain receptors.\textsuperscript{221} Other than proteomic ligands, small nucleic acid ligands, referred as aptamers, have also been attracting many interests. Aptamers can bind with a wide range of receptors with an affinity comparable to antibodies. More excitingly, aptamers can be synthesized with large scale and high batch-to-batch consistency, using an \textit{in vitro} chemical technique known as systematic evolution of ligands by exponential enrichment (SELEX).\textsuperscript{222, 223} Some small molecules have also been found to have targeting capability. One of the promising tumor targeting ligands is folic acids, which target to over-expressed folate receptor in cancer cells.\textsuperscript{224}

The quantity of targeting ligands affects the targeting efficacy. Generally, more targeting ligands lead to better targeting effect. Nanoparticles which are conjugated with multiple ligands (multivalency) normally possess enhanced affinity, slower disassociation and better biodistribution, compared to stand-alone targeting ligands (monovalency).\textsuperscript{225} However, for PEGlytated nanoparticles, too many surface ligands may be detrimental to the stealth capability. Studies have shown that high density of surface ligands leads to shorter circulation and lower tumor localization compared to nanoparticles conjugated with less ligand.\textsuperscript{226, 227} On the other hand, the morphology of the PEG chains also affects the targeting and stealth capability. At moderate concentration, the lateral pressure between PEG chains forces them to extend into a brush conformation. If the PEG density is too high, the PEG chain will tangle itself, forming a random-coil conformation, also known as the mushroom conformation.\textsuperscript{228} Brush PEG layer provides better stealth effect than mushroom PEG chains due to its greater protein repulsion.\textsuperscript{229} Targeting ligands that are grafted on the extended PEG chains have more chance to interact with receptors. To achieve optimal combination of specific targeting and stealth effect, the concentrations of targeting and stealth agents should be carefully balanced.

**CONCLUSION**

The development of MFNPs as molecular imaging contrast agent is expected to help reveal the molecular pathways of disease development, enable early detection and intervention for many diseases that are hard to deal with using the conventional generalized approaches. A variety of interrogation, integration and surface modification methods have been proposed by researchers in the recent past to combine different imaging modalities and enhancing targeting capabilities. In this review, we introduced several representative works of MFNPs preparation (Listed in Table II). However, the applications of MFNPs are still limited in research community. MFNPs have been extensively exploited in small animal imaging and \textit{in vitro} imaging. But many challenges remain to be overcome before MFNPs can be applied in clinical arena as a diagnostic tool. The accuracy and reproducibility of different MFNPs need to be systematically determined, the synthetic methods need to be standardize and the biological fate needs to be followed to ensure their biocompatibility. Theoretical modeling of material integration is also needed to guide the search for optimum MFNPs. All those challenges cannot be resolved without an inter-disciplinary collaboration between chemist, biologist and clinician. It is envisaged that the new research thrust area combining these MFNPs including carbon dots can lead to new generation multimodal molecular probes for introducing a paradigm shift in clinical diagnostics.

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Nanoparticulate Contrast Agents for Multimodality Molecular Imaging

Xia et al.


Xia et al.  

1582  


