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Inorganic nanocrystals as contrast agents in MRI:synthesis, coating and introducing multifunctionality

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Abstract

Inorganic nanocrystals have myriad applications in medicine, which includes their use as drug or gene delivery complexes, therapeutic hyperthermia agents, in diagnostic systems and as contrast agents in a wide range of medical imaging techniques. For MRI, nanocrystals can produce contrast themselves, of which iron oxides have been most extensively explored, or be given a coating that generates MR contrast, for example gold nanoparticles coated with gadolinium chelates. These MR-active nanocrystals can be used in imaging of the vasculature, liver and other organs, as well as molecular imaging, cell tracking and theranostics. Due to these exciting applications, synthesizing and rendering these nanocrystals water-soluble and biocompatible is therefore highly desirable.

We will discuss aqueous phase and organic phase methods for synthesizing inorganic nanocrystals such as gold, iron oxides and quantum dots. The pros and cons of the various methods will be highlighted. We explore various methods for making nanocrystals biocompatible, i.e. directly synthesizing nanocrystals coated with biocompatible coatings, ligand substitution, amphiphile coating and embedding in carrier matrices that can be made biocompatible. Various examples will be highlighted and their applications explained. These examples signify that synthesizing biocompatible nanocrystals with controlled properties has been achieved by numerous research groups and can be applied for a wide range of applications. Therefore we expect to see reports of preclinical applications of ever more complex MRI-active nanoparticles and their wider exploitation, as well as in novel clinical settings.

Keywords

nanoparticle; MRI; biocompatible; iron oxide; multimodality; gold nanoparticles; quantum dots; molecular imaging

1 Introduction

The NIH defines nanoparticles as particulates in the 1–100 nm size range.(1) Inorganic nanocrystals (i.e. containing elements commonly thought of as inorganic) have been known of and used by man for over 2000 years. For example, a highly ornate glass cup, known as

the Lycurgus cup, was made in the 5th to 4th century B.C. – and its glass is colored with gold nanocrystals (Figure 1).(2) Biomedical applications for inorganic nanocrystals have been proposed for centuries, as detailed with the publication in 1616 of ‘Panacea Aurea-Auro Potabile’ where drinking solutions of gold colloids were suggested as being ‘helpfully given for the health of Man in most Diseases’.(3) Nevertheless, it was not until the 20th century and the advent of medical imaging modalities that inorganic nanocrystals began to find legitimate biomedical uses, such as Thorotrast, a thorium colloid, in X-ray imaging.(4) In the 1990s, iron oxide nanoparticle formulations entered the clinic for use in MRI as contrast agents,(5) with Feridex, for example, being approved by the FDA in 1996.(6)

Since the start of the 1990s, there has been an explosion of interest in inorganic nanomaterials. The synthesis of nanostructures has been explored for many of the elements in the periodic table.(5,7–11) The development of nanochemistry has reached the extent that size, shape and other properties can be controlled synthetically for many nanostructure types. Some examples include gold nanorods, whose size and aspect ratio can be tuned with exquisite precision(12), formation of gold(13) or silver(14) triangular nanoprisms and spherical, linear or branched topologies of nanosized cadmium selenide.(15) This intense interest is a result of the unique optical, electrochemical and catalytic properties that different nanostructures possess compared to small molecules or bulk materials. In the past decade, attention has turned to applying inorganic nanoparticles to biomedical purposes.(16,17)

Inorganic nanoparticles can be used *ex vivo* as part of diagnostic devices,(18,19) but can also be used *in vivo*, via intravenous injection or other routes. Once inside the body, nanoparticles can be small enough to navigate the smallest capillary, but can also leave the bloodstream and enter tissues, if the gaps in endothelial cell junctions permit.(20) Notably, long circulating, non-targeted nanoparticles can preferentially accumulate in tumors due to the leakiness of their blood vessels and poor lymphatic drainage, a phenomenon known as the enhanced permeability and retention effect.(21,22) A recent study has elegantly shown that in rats quantum dot clearance occurs rapidly via the kidneys if the hydrodynamic radius is below 5.5 nm, while larger nanoparticles are not filtered by renal fenestrae and therefore circulate longer before removal by the organs of the mononuclear phagocyte system.(23) Via targeting or non-specific mechanisms, nanoparticles can bind to and enter cells, whereupon their cargo can be released or the contrast they generate can be detected. Targeting can be achieved via attachment of antibodies, proteins, peptides, sugars, aptamers and other molecules to the periphery of the nanoparticle. Compared with small molecule agents, nanoparticles carry high payloads, can have exceptional contrast generating properties, can have long circulation half-lives, and can be easily made multifunctional, including multiple types of contrast generating material and therapeutics in one platform.(16) These properties combined make nanoparticles very attractive as therapeutics, in diagnostic devices and as contrast agents.

Nanocrystals of gold are one of the most widely studied nanomaterials. They have been used in drug delivery, and have recently entered clinical trials as adjuvants for delivery of TNF α in patients with non-operable tumors.(24) Gold nanorods and nanoshells absorb light very strongly, and the maxima of their absorption can be tuned to be in the near infra-red window, i.e. a section of the electromagnetic spectrum where tissue absorbs light the least. Heat is emitted when gold nanorods and nanoshells absorb light and hence they have been proposed for photothermal ablation of pathological tissue.(25,26) They are also in clinical trials for this application.(27) Furthermore, gold nanoparticles have been trialed as radiosensitizers for radiotherapy of tumors.(28) Various types of gold nanoparticles have additional applications as contrast agents in fluorescence imaging,(29) surface-enhanced Raman spectroscopy,(30) computed tomography (CT) (31) and spectral computed

tomography.(32) Last, they have been FDA approved in diagnostic devices for various diseases.(33)

Quantum dots are semi-conductor nanoparticles, most commonly based on cadmium selenide, with attractive and unique fluorescent properties, such as broad absorption and narrow emission spectra.(34) Due to concerns over long-term toxicity,(35) cadmium containing QDs are likely not suitable for clinical applications, but nevertheless are very useful in preclinical applications. For example, multiple quantum dot types can be detected simultaneously, allowing multiple processes to be probed in the same setting, with seven or more quantum dot types having been imaged in tissue at the same time.(36)

The most widely known application of iron oxide nanocrystals is in MRI, as already mentioned.(5) However, iron oxides also have uses as contrast agents for magnetic particle imaging as well.(37) They have been used for magnetothermal therapy,(38) where an oscillating magnetic field causes local hyperthermia and subsequent cell death. They have also been used in magnetically guided drug delivery, where enhanced therapeutic efficacy has been observed when an external magnetic field has been used to increase nanoparticle accumulation locally.(39) Miniaturized NMR systems incorporating iron oxide nanoparticles have potential in disease diagnostics.(18)

All three of the above mentioned nanocrystal types have also been applied as platforms for delivery of nucleic acid therapeutics.(40–42) Many more nanocrystal types have biomedical applications, but a full discussion is beyond the scope of this review.

Due to these extraordinary properties and many uses, there is great interest in making biocompatible nanocrystals. Aqueous phase methods can be used to directly synthesize nanocrystals with biocompatible coatings,(43,44) but this strategy may not be possible for a variety of reasons. For example, the high temperatures and reducing conditions often used in nanocrystal synthesis could damage the desired coating material. Alternatively, synthesis using the desired coating material may result in nanocrystals of poor quality, in terms of size distribution and crystallinity.(45) Last, the nanocrystal morphology required may only be achievable under very specific synthetic conditions.(12) Therefore aqueous phase synthesis may go through an intermediate step, where the nanocrystal is synthesized using an established methodology and the biocompatible coating is subsequently applied via ligand substitution.(46) However, in many cases, the most precise and controllable methods for synthesizing nanocrystals result in structures that are not water soluble, let alone biocompatible, hence there is a need to develop procedures to alter their coating and thereby make them suitable for use in biological systems.(47) The two main strategies adopted are to either perform ligand substitution(47) or to apply an additional, amphiphilic coating.(48) Alternatively, nanocrystals can be embedded in a carrier matrix, such as a polymer, silica or an oil, which is or can be made biocompatible.(49,50) These various strategies are presented schematically in Figure 2.

2 Nanoparticles in MRI

The first step in magnetic resonance imaging is placing the subject into the bore of the scanner, where a very strong magnetic field exists. In the clinic, this field is typically 1.5–3 T, whereas in preclinical systems it is typically higher, such as 9.4 T. In these fields, the two energy levels of the hydrogen nuclei in the subject are separated and radiowaves are used to excite some of the nuclei from the lower to the higher levels. The nuclei subsequently relax to the lower energy level, emitting radiowaves in the process. The scanner records the emitted radiowaves and forms images of the subject from this data. Small local differences in the environment of water in different tissues cause their relaxation rates to differ,

providing contrast between tissues in MR images. Relaxation is defined by two parameters: longitudinal relaxation time (T_1) and transverse relaxation time (T_2).

Magnetic resonance imaging is a whole body imaging technique. It has high spatial resolution (~0.5 mm), but the temporal resolution is not high, with sequence acquisition taking several minutes. However, soft tissue contrast and anatomical information is excellent, especially compared with computed tomography. MRI is much less sensitive to contrast media than nuclear techniques such as positron emission tomography, but more sensitive than computed tomography. Recently, combined MRI-PET systems have been developed, marrying the high soft tissue contrast of MRI with the high sensitivity to contrast agents of PET.(51)

MRI-active nanocrystals can be thought of as taking one of two forms. The nanocrystal itself can be MRI-active, such as iron oxide, gadolinium oxide, manganese oxide or other core materials.(52–55) Alternatively, a non-MRI active nanocrystal can be functionalized with an MRI-active material, such as a coating of phospholipids with gadolinium chelates attached to their headgroups or embedded into a carrier matrix that also has (super)paramagnetic agents incorporated.(56–58) These nanoparticles create MR image contrast by affecting the relaxation rates of water in tissues. Nanocrystals labeled with gadolinium chelates are primarily used to reduce T_1 values via direct contact of water molecules with gadolinium ions, yielding signal brightening in the images, or positive contrast. Superparamagnetic nanocrystals, such as iron oxides or iron-platinum alloys create inhomogeneities in the local magnetic field, thus strongly reducing T_2 values, producing signal loss, i.e. image darkening or negative contrast. The ability of a contrast agent to reduce T_1 or T_2 is described by their longitudinal (r_1) and transverse (r_2) relaxivities, respectively. The higher the r_1 or r_2 values, the stronger the reduction of T_1 or T_2 and the greater the contrast produced.

The specific advantages of nanoparticle agents compared with small molecule agents in MRI are as follows. Grafting of gadolinium chelates to the surface of nanoparticles results in higher r_1 values than free chelates, at clinical fields. For example, the r_1 of gadolinium chelates adsorbed onto the surface of indium phosphide quantum dots was found to be roughly twice that of free chelates at 60 MHz (Figure 3).(59) This enhanced relaxivity is attributed to the slower rotation of the nanoparticle as compared to the free chelate.(60) These higher r_1 values produce enhanced sensitivity of detection. In the case of iron oxide nanoparticles, due to their superparamagnetism and high payload they generate contrast that is not available with small molecules. Furthermore, nanoparticles facilitate targeted imaging in MRI, as the low sensitivity of MRI necessitates the delivery of large amounts of contrast media to targets.

MRI active nanoparticles have found a variety of uses. Gadolinium labeled nanoparticles have been used in vascular imaging and various types have been used for imaging organs such as liver.(5,44) Targeted nanoparticles are used for MR molecular imaging, the “*in vivo* characterization and measurement of biologic processes at the cellular and molecular level”.(61) Cell tracking is often accomplished with iron oxide nanoparticles.(62) A recent trend in nanoparticle strategies for delivery of drugs or nucleic acids has been to develop platforms that incorporate diagnostically active nanocrystals, to facilitate tracking of accumulation of the therapeutics in target tissues, otherwise known as image-guided drug delivery, a form of theranostics.(63)

In the subsequent sections, we will briefly cover some specific nanocrystal synthesis methods, before discussing various routes to biocompatible and MRI active nanoparticles, highlighted with examples from our own work and the work of others.

3 Nanocrystal synthesis

Interested readers can find extensive discussions of nanocrystal synthesis in these reviews. (2,7–12) Here we will highlight a few prominent examples. Any synthesis starts with a mixture of metal salts and capping ligands, after which a change in the conditions is induced, such as the addition of a reducing agent or an increase in pH, causing the nanocrystals to form. Many aqueous phase syntheses of gold nanocrystals are based on the Turkevich method.(64) Here, a solution of chloroauric acid is heated to boiling point and sodium citrate is added, reducing the gold ions and forming nanocrystals. The reaction is complete after only a few minutes. The resulting citrate capped nanocrystals are in a reasonably narrow size range and the conditions can be controlled to produce diameters from 15 nm up to 150 nm.(65) The disadvantages of this method are that citrate capped gold nanocrystals have poor stability,(2) are not biocompatible(66) and the scale of synthesis is quite small.(64) Nevertheless, the citrate capping ligands can be easily displaced with the desired coating material to make stable, biocompatible nanoparticles (Figure 2A).(46) Gold nanoparticles synthesized using variants of this method can be used to grow a variety of shapes such as nanorods or stars.(67,68)

The Brust method of gold nanocrystal synthesis starts with the solubilization of chloroauric acid in toluene via use of a phase transfer agent.(69) Dodecanethiol is added and gold nanocrystal synthesis is induced by addition of the reducing agent, sodium borohydride. Dodecanethiol capped nanocrystals result, which are purified by precipitation from methanol as a waxy black solid.(69) This product is highly stable, but only soluble in organic solvents. The particle size achievable is only in the 1–5 nm range,(70) but, in our experience, the procedure is easy to perform and can be carried out on a multigram scale.

Molday et al. reported an aqueous phase synthesis of dextran coated iron oxides, which results in amorphous nanocrystals with a relatively wide size range.(43) In this synthesis, FeCl_3 and FeCl_2 salts are mixed with a concentrated solution of dextran. The pH is increased to 10–11 and the mixture is heated at 60–65 °C for 15 min. This can be considered an example of direct, aqueous phase synthesis of biocompatible nanocrystals, as depicted in Figure 2B. Massart reported a method for synthesizing iron oxide nanocrystals capped with a loose coating of ions(71) that can be subsequently substituted with biocompatible molecules,(72) i.e. the process represented in Figure 2A. In this process, a mixed solution of FeCl_3 and FeCl_2 in a 2:1 ratio is poured into ammonia resulting in a precipitate. This precipitate is isolated and then dispersed with either tetramethylammonium hydroxide or perchloric acid. Transmission electron microscopy (TEM) reveals that iron oxide nanocrystals produced in this manner to be irregular in shape and diameter (Figure 4A).(73)

Various hydrophobic phase based methods exist for synthesizing iron oxides and they can result in highly crystalline nanocrystals with controllable and monodisperse diameters (Figure 4B).(47) For example, in the procedure reported by Hyeon,(74) iron chloride is reacted with sodium oleate to form iron oleate. This complex is then dissolved in octadecene and slowly heated to 320 °C. The longer the solution is held at that temperature, the larger the resulting nanocrystals, ranging from 5 nm if the reaction is stopped immediately, to 13 nm if the solution is heated for 1 hour.(74) The nanocrystals are formed due to thermal decomposition of the iron oleate complex. These nanocrystals are oleic acid coated and are thus dispersible in organic solvents. The reaction can be carried out on a large scale.(75)

The optical properties of quantum dots depend on their composition, but are also highly dependent on their size, which allows fine-tuning of the emission wavelength. Therefore the need for monodisperse particles is high in order to achieve narrow emission spectra. This is typically achieved via a ‘hot injection’ method,(78) for example when the precursor salts,

dimethyl cadmium and trioctylphosphine selenide, are injected into the hot (300 °C) trioctylphosphine oxide (TOPO) solvent.(79) The swift injection of precursors results in a super saturated solution and 'burst' nucleation, which quickly reduces the precursor concentration so that no new nanocrystals are nucleated and solely nanocrystal growth occurs, creating a very monodisperse nanoparticle size distribution. The resulting quantum dots are capped with TOPO and are dispersible in organic solvents.

For any of the syntheses that result in hydrophobically capped nanocrystals it has been shown that either ligand substitution or amphiphile coating can be used to render these nanocrystals biocompatible (Figure 2C/D).(32,47,80–84) In our laboratory, we have found that gold or iron oxide nanocrystals can be easily synthesized using relatively cheap and simple chemistry equipment. Nevertheless, in addition to performing the synthesis oneself, good quality nanocrystals can be purchased from a variety of commercial sources.(85–88)

4 Aqueous phase synthesis of MRI-inactive nanocrystals coated with MRI-active materials

The two routes available to synthesize MRI-inactive nanocrystals with an MRI-active coating are: A) synthesis of a nanocrystal core by a known method, followed by substitution of the coating with an MRI-active one(46) or B) synthesizing the nanocrystal in the presence of MRI active coating materials resulting in the product in one step (Figure 2A/B).(44) The use of either route may depend on the stability of the coating material to the reaction conditions – nanocrystal syntheses often require high temperatures or strongly reducing conditions. Established syntheses have the advantage of yielding nanocrystals of defined dimensions and properties and therefore may be considered preferable to syntheses that directly use the coating material, where the resulting nanocrystal properties can be unpredictable or may be difficult to tune. For example, gold nanoparticles produced by the Turkevich method and subsequently given an MRI-active coating are homogeneous in size (Figure 4C), whereas gold nanoparticles directly synthesized from a gold salt in the presence of an MRI-active coating material have a heterogeneous size distribution (Figure 4D).(73,77)

An example of route A) was reported by Park et al., where citrate-coated 12 nm gold nanocrystals were first synthesized by the Turkevich method. Diethylene triamine pentaacetic acid (DTPA), a well known gadolinium chelate, was modified with cysteine and was subsequently complexed with gadolinium. As sulphur binds to gold very strongly, incubation of this gadolinium ligand with the gold nanocrystals resulted in displacement of citrate and formation of Gd-DTPA coated gold nanoparticles (Figure 5A).(73) The authors demonstrated this compound to have a longitudinal relaxivity (r_1) of $17.9 \text{ mM}^{-1}\cdot\text{s}^{-1}$ at 1.5 T and 20 °C, and to act effectively as a blood-pool and liver imaging agent in mice (Figure 5B). In comparison, the relaxivity of Gd-DTPA under these conditions is much lower at $3.3 \text{ mM}^{-1}\cdot\text{s}^{-1}$. As gold cores provide CT contrast, the mice were imaged with CT as well, with strong contrast generated *in vivo* (Figure 5C). A similar approach has been taken by others, for example Song et al. used Gd-labeled DNA to coat gold nanocrystals to create a platform for cell labeling.(89)

Several groups have directly created MRI-active gold nanoparticles by reduction of chloroauric acid in water in the presence of a thiol-containing gadolinium ligand.(44,77,90–93) Alric et al. synthesized aminoethanethiol modified DTPA and subsequently formed gold nanoparticles in the presence of this ligand. Incubation with GdCl_3 at room temperature resulted in gadolinium complexed in the DTPA surface coating.(44) These nanoparticles were also shown to be effective MRI and CT contrast agents in mice. Interestingly, Lim et al. coated gold nanoshells with an anti-HER2 antibody-Gd-DTPA conjugate through binding

of the antibody to protein G adsorbed on the surface of the gold nanoshells.(91) These nanoshells were shown to target HER2-expressing cancer cells via MR imaging experiments. In addition, these nanoparticles were used to induce cancer cell death through a photothermal effect, where the gold nanoshells heated up after irradiation with a laser light.

Oostendorp et al. have used an approach where quantum dots are first coated with a polymer, which has streptavidin attached to the distal end.(94) The streptavidin group is used to further functionalize the nanoparticle through non-covalent binding with biotinylated ligands. For example, biotinylated Gd-DTPA dendrimer wedges and biotinylated peptide targeting ligands can be bound to the streptavidin on the quantum dot surface to make a targeted, MRI-active nanoparticle. The peptide used was specific for CD13, a protein overexpressed by angiogenic blood vessels, so this agent has been applied as a molecular imaging contrast agent to investigate angiogenesis in tumors and infarcted hearts with MRI. (94–96) It is worth noting that the translational potential of nanocrystals loaded with Gd-DTPA chelates may be limited. This is due to concerns surrounding the possibility of inducing a condition known as nephrogenic systemic fibrosis (NSF) from Gd-chelates that are not swiftly excreted.(97)

5 Direct synthesis of biocompatible MRI-active nanocrystals

As mentioned in section 2, Molday developed a synthesis of iron oxide nanocrystals by precipitation of FeCl_3 and FeCl_2 in the presence of dextran.(43) This precipitation is achieved by raising the pH to 10–11 via the addition of ammonia. This process results in iron oxide cores ranging in diameter from 10–20 nm, covered in a dextran coating, with an overall diameter of 40 nm. Dextran is a polysaccharide that has uses in the clinic as an anti-coagulant and is biocompatible. Therefore, the iron oxide nanoparticles resulting from this synthesis are biocompatible and the clinically approved agents ferumoxide and ferumoxtran-10 are made in a process based on this method.(5) Ferucarbotran, another clinically approved agent, is made in a similar method where carboxydextran is used as the coating agent. The r_2 values at 37 °C and 1.5 T are 120, 65 and 189 $\text{mM}^{-1}\text{s}^{-1}$ for ferumoxides, ferumoxtran-10 and ferucarbotran, respectively. The pH induced precipitation of iron oxide nanoparticles in the presence of a polymer is an adaptable method and has been used to produce iron oxide with a variety of coatings, such as poly(oligo(ethylene glycol) methacrylate-*co*-methacrylic acid), polyglycidyl methacrylate or poly(vinylalcohol). (98–100)

Dextran coated iron oxides have been extensively explored for targeted MR imaging. For example, Ruehm et al. showed that macrophages could be detected in a rabbit model of atherosclerosis via injections of ferumoxtran-10.(101) The Weissleder group has used iron oxide whose dextran coating is cross-linked and aminated via use of epichlorohydrin and ammonia(102) for imaging a variety of targets in atherosclerosis. The amination of the coating facilitates attachment of targeting ligands and other functionalities such as fluorophores or radioactive metals.(103,104) This methodology has been used to image a variety of targets such as VCAM-1, E-selectin and apoptotic cells.(105–107) Recently, several groups have reported dextran-coated iron oxides that have been modified with radioactive nuclei to allow their detection with nuclear-based techniques. For example, Nahrendorf et al. modified dextran coated iron oxides with DTPA molecules, which were used to chelate Cu-64 for PET imaging.(103) Such nanoparticles can also be labeled with Cu-64 by doping the nanocrystal core with copper.(108) De Rosales et al. labeled dextran-coated iron oxides with Tc-99m via bisphosphonate linkers for single-photon emission computed tomography imaging.(109)

6 Ligand substitution of MRI-active hydrophobic nanocrystals

Nanocrystals possessing monodisperse and tunable diameters are highly desirable.(110) However, the synthetic methods that yield such nanocrystals, such as the iron oleate thermal decomposition method, result in iron oxide nanoparticles with hydrophobic coatings (e.g. oleic acid).(74) To allow use in biological settings, one strategy is to substitute the hydrophobic ligands with ones that confer water solubility. Such ligands are typically bifunctional at a minimum, possessing a functional group that can effectively bind to the iron oxide surface and another that faces the solvent and provides biocompatibility. The functional groups that have been reported to stably bind to the iron oxide surface are limited in number and are typically oxygen containing, such as carboxylates,(47) diols,(111) catechols/dopamines,(112) hydroxamic acids,(113) phosphine oxides(114) or silanes.(115) There can be many different groups on the water facing end of the capping ligand, including polyethylene glycol (PEG), amines, thiols, carboxylates, sugars, zwitterionic polymers and metal chelating groups (to allow attachment of a radioactive or fluorescent metal).(114–118) Examples of such ligands are depicted in Figure 6A. Interestingly, several groups have attached initiator molecules to iron oxides and synthesized biocompatible polymers on the nanoparticle surface.(119) Others have used polymers containing many carboxylate groups, such as polyacrylic acid, as coating materials.(120) Furthermore, the groups on the water facing end of the ligand can be used to attach targeting moieties or other functional groups, thus producing a nanoparticle with the desired properties.(47,118,121)

For example, Jun et al. mixed oleic acid coated iron oxide nanocrystals in toluene with 2,3-dimercaptosuccinic acid (DMSA) in dimethylsulfoxide. This resulted in displacement of the oleic acid coating with DMSA and water soluble nanocrystals (Figure 6B). The thermal decomposition synthesis method allowed the production of highly monodisperse nanocrystals whose average diameter ranged from 4–12 nm (Figure 6C). The changing size of the nanocrystals had a marked effect on their MR contrast properties, with stronger contrast observed for nanocrystals with larger cores, despite holding the iron concentration constant (Figure 6C). Figure 6D illustrates the solubility of DMSA-coated nanocrystals in aqueous media. Herceptin, an antibody specific for the HER2/neu receptor, which is overexpressed on breast cancer cells, was conjugated to 9 nm iron oxide nanocrystals via the thiol functional groups of DMSA. SK-BR3 cells that express this receptor, were incubated with the Herceptin targeted nanocrystals, control antibody conjugated nanocrystals or without nanocrystals. MR images of pellets of the incubated cells revealed strong contrast in the cells incubated with Herceptin-nanoparticles, compared to the controls, indicating that the targeting was successful (Figure 6E).

Notably, Lee et al. found that substituting one manganese(II) ion for a iron(II) ion in Fe_3O_4 (i.e. to make MnFe_2O_4), led to a roughly $100 \text{ mM}^{-1}\cdot\text{s}^{-1}$ increase in r_2 values at 1.5 T and room temperature.(52) Also, increasing the core size from 6 to 12 nm also led to a $100 \text{ mM}^{-1}\cdot\text{s}^{-1}$ increase in r_2 , with a 12 nm MnFe_2O_4 nanoparticle having an r_2 value of over $350 \text{ mM}^{-1}\cdot\text{s}^{-1}$, while that of 6 nm Fe_3O_4 is little more than $100 \text{ mM}^{-1}\cdot\text{s}^{-1}$.

Iron oxides are a very commonly used nanoparticle type, so the chemistry of ligand substitution is relatively well developed. Other types of MRI-active nanocrystal are less often studied, but ligand substitution is a possibility for them also. For example, FePt nanocrystals, synthesized with an oleic acid coating, which was substituted by incubation with cystamine.(122) The thiol moiety of cystamine bound to the nanocrystal surface, leaving the amine group facing the water, free to be coupled to targeting ligands. Ligand exchange with iron oxides synthesized in the aqueous phase is possible, but has not frequently been studied in recent years.(123)

7 Amphiphile coating of hydrophobic nanocrystals

An alternative route to making hydrophobic nanocrystals biocompatible is via coating with amphiphiles, molecules containing a hydrophilic and a hydrophobic part. Amphiphilic molecules can self-assemble in a number of morphologies, including micelles, vesicles, rod-like micelles and bilayered vesicles. The relative ratio between the hydrophilic and the hydrophobic moieties determines the morphology adopted by the aggregate. For example, micelles form when the hydrophilic fraction is above 50%. To obtain a tightly packed shell, the amphiphilic molecule mix used for nanocrystal coating should, under the right conditions, self-aggregate into a micellar conformation. The protective shell around the nanoparticles is maintained through preferential interactions between the amphiphile's hydrophobic moieties with the nanoparticle's capping molecules and the amphiphile's hydrophilic moieties with the aqueous environment.

Phospholipids and polymer-containing phospholipids are amphiphiles that are frequently used to coat nanocrystals. Dubertret and coworkers were the first to demonstrate this principle by encapsulating quantum dots inside phospholipid micelles.(83) The phospholipid coating rendered nanoparticles that were stable under biological conditions. This was accomplished using PEG functionalized lipids for which their PEG moiety causes an increase in the size of the hydrophilic part, promoting the formation of micelles. Also, PEG protects the nanoparticles from non-specific interactions with biological molecules and thereby increases the circulation time of the particle.

We have found this methodology to be very versatile, allowing numerous combinations of nanocrystals and lipids to be incorporated in a modular fashion to form the overall nanoparticle.(48) For example, we modified the abovementioned method of Dubertret to render quantum dots with a paramagnetic lipid coating for MRI.(57) This was achieved by first co-dissolving the quantum dots, PEG lipids and gadolinium labeled lipids in a 20:1 chloroform:methanol solvent mixture (Figure 7A). The solvents were evaporated to form a lipid film, which was hydrated at 70 °C with HEPES buffer, to form a mixture of micelles with and without quantum dots in their core. Centrifugation was used to isolate micelles with quantum dots in their core. Variants of this procedure include adding the lipid/nanocrystal solution to buffer to create an emulsion and subsequently evaporating the solvents or adding the solution dropwise to hot buffer, instead of forming a lipid film and hydrating it. We have found slow dripping of the lipid/nanocrystal solution to more reliably produce individually coated nanocrystals. The r_1 value of the gadolinium labeled quantum dots produced was found to be $12.4 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 37 °C and 1.5 T, three times greater than Gd-DTPA under these conditions.(57)

Inclusion of a small fraction of maleimide functionalized PEG-lipids allowed reaction with a thiol labeled arginine-glycine-aspartic acid (RGD) peptide. This peptide is a ligand for the $\alpha_v\beta_3$ -integrin, a receptor over-expressed on angiogenic endothelial cells. RGD targeted (RGD-pQDs) and non-targeted (pQDs) paramagnetic quantum dots were incubated with human umbilical vein endothelial cells (HUVEC), a commonly used model of angiogenic cells. Fluorescence microscopy of these cells and MR imaging of pellets of these cells (Figure 7B–D) revealed higher uptake of the RGD-pQDs. Contrast in MR images was observed at the tumor rim of mice injected with RGD-pQDs (Figure 7E–G), indicating accumulation of the nanoparticles in angiogenic areas of the tumor.(124) Fluorescence microscopy of the tumor showed the quantum dots to be associated with the tumor vasculature, confirming angiogenesis targeting (Figure 7H).

An alternative method to create bimodal MRI and fluorescent contrast agents is by encasing hydrophobic superparamagnetic nanoparticles in a fluorescent lipid containing shell.

(125,126) The presence of a functional group in the shell allows covalent coupling with targeting molecules. As well as directly coating nanocrystals, we have used PEG-, fluorescent-modified and/or paramagnetic lipids to coat nanocrystals embedded in silica. (127,128) In these studies it was also shown that QDs coated by a 10–20 nm layer of silica allow an increased number of Gd atoms per particle to overcome the poor MRI detection sensitivity of Gd-based contrast agents. Moreover, we showed that a trimodal contrast agent for MRI, CT and fluorescence imaging could be created by encasing a gold/silica particle in fluorescent and paramagnetic lipids.(129)

Amphiphilic block copolymers are synthetic molecules composed of two or more chemically different polymers covalently bound together. In the presence of aqueous solvents (i.e. a solvent selective for the hydrophilic block), amphiphilic block copolymers undergo phase separation resulting in the formation of nano-sized aggregates, such as micelles. Several procedures have been developed for the synthesis of block copolymers with different chemical compositions and characteristics. Biodegradable, biocompatible or stimuli responsive block copolymers of different lengths are found in the literature.(130) Block copolymers offer excellent control of the number of particles encased per micellar structure.(84) For iron oxide probes to have control over the number of nanocrystals inside each aggregate is of great importance since assemblies containing multiple nanoparticles have shown r_2 values much greater than micelles containing single nanoparticles.(131) Multifunctional nanoparticles for targeted drug delivery containing aggregated nanoparticles in the core have been used for *in vitro* and *in vivo* applications.(132,133) The position of each component plays an important role in the overall characteristics of the assembly. Block copolymers can be custom made to compartmentalize diverse agents at specific positions inside the same nanostructure. Hu and coworkers showed that selective aggregation of magnetic nanoparticles in the corona and hydrophobic drugs in the core renders multifunctional hybrid micelles with enhanced T_2 relaxivity.(134)

In addition to the above described approaches, nanocrystals can be rendered water soluble and target-specific with lipoprotein-based coatings.(135–137) To that end, we and co-workers have developed a natural lipid/protein amphiphile coating, derived from high density lipoprotein (HDL) isolated from serum, to create a variety of MRI-active nanocrystals. The resulting hybrid nanoparticles can be used for multimodal imaging purposes. Gold incorporated HDL nanoparticles can be detected by MRI, CT, fluorescence techniques and TEM, while quantum dots with a paramagnetic HDL shell or magnetic nanoparticles in fluorescent HDL shell served as trimodal MRI/optical/TEM contrast agents. (32,56,138) Importantly, as lipoproteins play a crucial role in cardiovascular disease, multimodal nanocrystal HDL can be used as tracer material to probe these conditions with *in vivo* (MRI, CT), as well as *ex vivo* (TEM, optical) imaging techniques.

8 Embedding into carrier materials

An alternative approach to either directly coating nanocrystals or using amphiphiles to confer biocompatibility is to embed the nanocrystal in a carrier matrix, such as silica, an oil or a polymer, which itself is biocompatible or can be made biocompatible. This can have certain advantages over the other methods. For example, it may be easier to make the carrier material biocompatible than the nanocrystal itself. A carrier matrix typically allows multiple nanocrystals to be encapsulated together in the same nanoparticle, leading to a high payload per particle, or multiple nanocrystal types, such as iron oxides and quantum dots, can be included so that the particle produces contrast for MRI and fluorescence techniques.(39) Furthermore, a matrix can facilitate inclusion of drugs (or other agents). As mentioned above, inclusion of diagnostically-active nanocrystals in drug delivery platforms can be valuable to track accumulation in target tissues, i.e. a ‘theranostic’ approach.(139)

We have recently developed an oil-in-water nanoemulsion platform, where oleic acid coated iron oxide nanocrystals are suspended in a soybean oil core, which is coated with phospholipids (Figure 8A).(49) Near infrared fluorophores such as Cy5.5 or Cy7 were incorporated in the phospholipid coating to allow fluorescence imaging of the nanoparticles, while the incorporation of a hydrophobic glucocorticoid, prednisolone acetate valerate (PAV), provided therapeutic effects.(139) The synthesis of such nanoemulsions is quite simple: the oil, iron oxides, drug and phospholipids are co-dissolved in chloroform and then slowly dripped into hot (70 °C) water. The chloroform evaporates swiftly, leaving a crude emulsion, which is homogenized via sonication, concentrated and may be further functionalized with targeting ligands – the $\alpha_v\beta_3$ specific RGD-peptide was used in this case. (139) The platform is flexible, allowing for the inclusion of alternative hydrophobically capped nanocrystals, drugs or functional phospholipids.

The diagnostically active components of the nanoparticle allowed detection of accumulation in the tumors of mice. Comparison of MR images of mice uninjected and 48 hr after injection with iron oxide nanoemulsions revealed strong contrast (signal loss) in the tumors of injected mice (Figure 8B and C). Similar results were observed in fluorescence imaging experiments, confirming that the drug was being delivered to the tumor. Tracking tumor volumes over time revealed that injections of the nanoemulsions loaded with PAV slowed tumor growth compared to drug-free controls (Figure 8E). Furthermore, we have recently applied this platform for cell labeling.(140)

Methods to coat nanocrystals in silica are now well established.(141) For example, iron oxide nanocrystals can be first synthesized and then subsequently coated with silica. Lu et al. synthesized oleic acid coated iron oxide nanocrystals by thermal decomposition of iron acetylacetonate.(142) They coated these nanocrystals using a water-in-oil reverse micelle method, where the water droplets include tetraethyl orthosilicate, which polymerizes to form a silica coating around the particle. Silane tagged FITC was incorporated into the silica coating to create fluorescence contrast. These silica coated iron oxides were used to label and track stem cells.(142) One point to note is that using silica coatings of over 30 nm results in a marked reduction in relaxivity of iron oxides, so overly thick silica coatings should be avoided.(143) Silica alone confers biocompatibility, but the surface can be further modified to improve biocompatibility, with coatings such as PEG, amines or phosphonates. (128,144,145) If silica nanoparticles are synthesized in the presence of the surfactant cetyltrimethylammonium bromide a mesoporous structure is formed, whose pores can be loaded with drugs or other cargos.(10) Kim et al. reported a method to coat iron oxide nanocrystals with mesoporous silica.(145) The silica coating was loaded with ibuprofen as a model drug and the surface chemistry of the silica was found to influence the rate of drug release.

Polymeric matrices have frequently been used in biomedicine as drug delivery systems, (146) but can also be used to deliver nanocrystals. Such an approach has the advantage of allowing multiple types of nanocrystals in the same nanoparticle, as well as allowing drug delivery to be studied via non-invasive imaging methods such as MRI. One of the most commonly used polymers for drug delivery is poly(D,L-lactic-co-glycolic acid) (PLGA), as its biodegradable nature facilitates controlled release of encapsulated drugs.(147) For example, PLGA has been used to co-encapsulate iron oxide, quantum dots and doxorubicin. (39) As PLGA is not very hydrophilic itself, a surfactant (Pluronic F127) whose hydrophilic portion is polyethylene glycol, was used as a coating. The nanoparticles were further modified with polylysine-polyethylene glycol-folate molecules to provide targeting to cancer cells that over express the folate receptor. The authors observed enhanced uptake in such cells with MRI and confocal fluorescence microscopy of the targeted formulation as compared to a non-targeted formulation. Furthermore, an enhanced reduction in cell

viability was observed with the targeted nanoparticles, as compared to the free drug. The Shapiro group has used PLGA to encapsulate iron oxide or manganese oxide nanocrystals, to form particles ranging from 0.1–2 μm .(55,148) These particles were used to label cells, for the purpose of cell tracking. In the case of the manganese oxide nanocrystals, degradation of the PLGA matrix at low pH resulted in release of manganese ions and a consequent increase in MR contrast, so the platform has exciting uses as a ‘smart’ contrast agent.(55)

9 Conclusions

Chemical methods to synthesize a wide variety of functional nanocrystals are currently available, and fine control of nanocrystal shape and size is available for selected core compositions. As we have described herein, several different routes to make these nanocrystals biocompatible exist, including direct synthesis, ligand substitution methods, amphiphile coating and embedding in a carrier matrix. Once made biocompatible, further modification of the nanoparticle surface is possible to introduce targeting moieties or additional functional materials such as fluorophores or drugs. These approaches facilitate the deployment of MRI active nanocrystals such as iron oxides in biomedical applications. MRI inactive nanocrystals such as gold or quantum dots may be made MRI active by, for example, the application of a coating that contains gadolinium chelates. Such nanoparticles have applications in MR imaging of the vasculature, liver and other organs, as well as molecular imaging, cell tracking and theranostics. Iron oxide nanocrystals have been used in the clinic and we anticipate that MRI-active inorganic nanocrystals will continue to find new applications in advancing our knowledge of disease as well as providing improved diagnoses.

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Abbreviations

CT	computed tomography
DMSA	2,3-dimercaptosuccinic acid
DTPA	diethylene triamine pentaacetic acid
NSF	nephrogenic systemic fibrosis
PAV	prednisolone acetate valerate
PEG	polyethylene glycol
PET	positron emission tomography
PLGA	poly(D,L-lactic-co-glycolic acid)
pQD	paramagnetic quantum dot
RGD	arginine-glycine-aspartic acid
TEM	transmission electron microscopy
TOPO	trioctylphosphine

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Figure 1.
The Lycurgus cup, which was made around 2500 years ago. Due to gold nanocrystals in the glass, it appears green in reflected light (A) and red in transmitted light (B). Images reused with permission courtesy of the Trustees of the British Museum.

Figure 2.

Inorganic nanocrystals are typically synthesized from metal salts. In the aqueous phase, a precursor nanocrystal can be synthesized, after which the coating is substituted for a more biocompatible one (A) or biocompatible nanocrystals can be directly synthesized (B). In hydrophobic phase syntheses, precursor nanocrystals are synthesized, which are soluble only in non-polar solvents. These precursor nanocrystals can be made biocompatible via ligand substitution (C), by applying a second coating of amphiphiles (D), or by embedding in a carrier matrix that can be made biocompatible (E).

Figure 3. Relaxivity across a variety of magnetic field strengths of two types of free gadolinium chelates, Gd.1 (open circles) and Gd.2 (crosses) compared with Gd.2 absorbed onto the surface of InP quantum dots (black circles). Reproduced with permission from (59).

Figure 4.

TEM images of: A) iron oxide synthesized in an aqueous phase by the Massart method, B) iron oxide synthesized in a hydrophobic phase with the iron oleate thermal decomposition method, C) gold nanoparticles synthesized by the Turkevich method and subsequently modified with an MRI-active coating, D) gold nanoparticles directly synthesized with an MRI-active coating present. Reproduced with permission from (73,74,76,77).

Figure 5.

A) Schematic of the synthesis of Gd-DTPA coated gold nanoparticles. B) MR images of a mouse before and after injection with the gold nanoparticles. C) CT images of a mouse before and after injection with the gold nanoparticles. H, heart; L, liver; K, kidney; A, aorta; B, bladder. Reproduced with permission from (73).

Figure 6.

A) Chemical structures of examples of ligands used to confer biocompatibility on iron oxide nanocrystals.(47,113–115,118) The iron oxide binding functional groups are in dashed boxes. B) Exchange of oleic acid capping ligands with another carboxylate ligand, DMSA. C) Effect of size on the MRI contrast produced by DMSA capped iron oxide nanocrystals. D) Photograph indicating the water solubility of DMSA-capped nanocrystals. E) MR images of cell pellets incubated with either herceptin antibody-conjugated DMSA-capped iron oxides or controls. Parts of this figure reproduced, with permission, from (47).

Figure 7.

A) Synthesis and purification of paramagnetic, lipid coated quantum dots. B, C) Fluorescence microscopy of HUVEC cells incubated with either RGD-pQDs or pQDs. D) MR image of cell pellets. E–G) MR images of a tumor mouse injected with RGD-pQDs. Contrast is highlighted in color in G). H) Fluorescence microscopy of tumor tissue from a mouse injected with RGD-pQDs (blue-nuclei, green-QDs). Reproduced with permission from (57) and (124).

Figure 8.

A) schematic depiction of an oil-in-water nanoemulsion carrying oleic acid coated iron oxide nanocrystals. T₂*-weighted MR images of the tumors (red dashed circle) of mice B) uninjected and C) 48 hr after injection with iron oxide nanoemulsions. D) Fluorescence images of tumor bearing mice uninjected (left) and 48 hr after injection with iron oxide nanoemulsions (right). E) Tumor growth profiles of various nanoemulsion formulations. PAV=prednisolone acetate valerate. Reproduced with permission from (139).