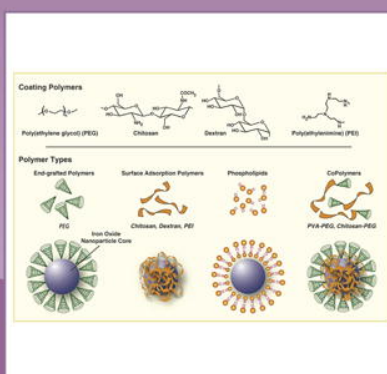




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Design and fabrication of magnetic nanoparticles for targeted drug delivery and imaging[☆]

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ABSTRACT

Magnetic nanoparticles (MNPs) represent a class of non-invasive imaging agents that have been developed for magnetic resonance (MR) imaging. These MNPs have traditionally been used for disease imaging via passive targeting, but recent advances have opened the door to cellular-specific targeting, drug delivery, and multi-modal imaging by these nanoparticles. As more elaborate MNPs are envisioned, adherence to proper design criteria (e.g. size, coating, molecular functionalization) becomes even more essential. This review summarizes the design parameters that affect MNP performance *in vivo*, including the physicochemical properties and nanoparticle surface modifications, such as MNP coating and targeting ligand functionalizations that can enhance MNP management of biological barriers. A careful review of the chemistries used to modify the surfaces of MNPs is also given, with attention paid to optimizing the activity of bound ligands while maintaining favorable physicochemical properties.

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1. Introduction

Advances in nanotechnology and molecular biology are rapidly enabling the development of nanoparticles (NPs) with specific functional properties that address the shortcomings of traditional disease diagnostic and therapeutic agents [1–3]. Brighter, tissue-specific imaging probes are being developed with NP technology to visualize and help diagnose disease at its earliest stages, in some cases, even prior to disease manifestation [4,5]. Concurrently, NPs are being developed as drug carriers thanks to careful nanostructure construction (tailored drug release characteristics, low immunogenicity, etc.) yielding improved treatment efficacy and reduction of unwanted side effects [6,7]. Significantly, these imaging and delivery facilities have been combined into unique NP formulations through clever combinations of nanoscaled materials, enabling simultaneous *in vivo* diagnostic imaging and drug delivery for real-time treatment tracking [7,8].

Among the broad spectrum of nanoscale materials being investigated for biomedical use, magnetic nanoparticles (MNPs) have gained significant attention due to their intrinsic magnetic properties, which enable tracking through the radiology cornerstone, magnetic resonance (MR) imaging [8]. This class of NPs include metallic, bimetallic, and superparamagnetic iron oxide nanoparticles (SPIONs) [8,9]. The latter of which has been widely favored because of its inoffensive toxicity profile [10–12] and reactive surface that can be readily modified with biocompatible coatings [13–16] as well as targeting, imaging, and therapeutic molecules [15–18]. This flexibility has led to SPION use in magnetic separation [19], biosensor [20,21], *in vivo* medical imaging [8,22,23], drug delivery [18,24], tissue repair [25], and hyperthermia [26] applications.

Currently, a number of SPIONs are in early clinical trials or experimental study stages [8,9,15], and several formulations have been approved for clinical use for medical imaging and therapeutic applications. Notable examples include: Lumiren® for bowel imaging [11], Feridex IV® for liver and spleen imaging [27], Combidex® for lymph node metastases imaging [28], and most recently, Ferumoxyl® for iron replacement therapy [29]. The physicochemical profiles of these SPIONs provide passive targeting, but not the higher level targeting offered by biologands. Addition of bioactive molecules to the SPION surface can increase the targeting specificity of NPs [8,9,17,30,31], producing contrast agents that specifically illuminate targeted tissue and drug carriers that don't interact with healthy tissue [8,18,19,31–34]. Development in this area represents a majority of SPION research today.

The creation of next generation SPIONs that can specifically target and eliminate or illuminate damaged tissue requires careful engineer-

ing of the size, shape, coating, and surface modifications. Thorough consideration of each design parameter must be evaluated to produce a NP that can overcome biological barriers and carry out its function. In doing so, targeting molecules must be chosen based on their physical properties in addition to their binding characteristics, and integrated into the NP system in such a way that they remain functionally active. *In vivo* use of SPION imaging preparations require attention to each of these design parameters, while SPION drug delivery systems must additionally anticipate the routes of NP uptake by target cells and the controlled release of their payloads. Herein, we will review these design considerations and fabrication strategies for the development of NPs for *in vivo* imaging and targeted drug delivery.

2. Nanoparticle design considerations

Before synthesis, MNP design requires fundamental understandings of the nature of the nanostructure as (1) a pharmaceutical construct that must navigate the body in search of its target, (2) a biocompatible entity that will not harm the patient, and (3) a contrast agent used in an external, biomedical imaging system. Here, we will consider the first of these areas, specifically looking at the physiological barriers that a MNP must overcome to gain access to its cellular target, and the NP's physical characteristics that can promote this functionality *in vivo*.

2.1. *In vivo* barriers

Intrinsic to the body's defense system are a series of “biological barriers” that serve to protect the body against foreign entities, including injected therapeutics and contrast agents, keeping them from reaching their intended destinations [1]. These barriers can restrict NP function by blocking their movement, causing physical changes to them, or by inducing a negative host response using biochemical signaling [35].

Upon intravascular administration, NPs immediately encounter blood, a high ionic strength, heterogenous solution, that can induce NP agglomeration, altering their magnetic properties and inducing particle sequestration. Additionally, NPs can nonspecifically interact with plasma proteins (which can trigger the adaptive immune system), extracellular matrices, and non-targeted cell surfaces while in the blood stream [36]. In each case, the NP is in danger of prematurely binding to or being taken up by cells before reaching its target tissue.

In addition to coping with the vascular environment, NPs must overcome various anatomical size restrictions which limit NP access

to target tissue (e.g. extravasation of lymph-targeting NPs from the blood vessels) [1]. These size limitations are especially stringent when targeting certain organs like the brain and kidney [37]. For instance, in the brain, endothelial cells and reinforcing astrocyte cells limit levels of pinocytosis and form tight junctions between cells at the blood-brain interface, yielding a structural and metabolic barrier referred to as the blood brain barrier (BBB) [38]. Here, only NPs of sufficient small sizes and appropriate physicochemical properties may pass the BBB.

Biological barriers are not unique to extracellular spaces; in fact intracellular barriers are a critical reason many drugs and drug delivery systems fail. NP systems are no exception. Once a cell-specific NP has bound to the membrane of its target, it is typically taken up by the cell through receptor-mediated endocytosis, where it is trafficked intracellularly via endosomal compartments for processing and destruction through acidification of the endosomes [39]. Most of these endosomes are then translocated into lysosomes where hydrolytic and enzymatic reactions completely metabolize macromolecules. Many therapeutics, such as DNA and siRNA, are susceptible to lysosomal degradation, rendering them ineffective upon cellular processing. However, carriers can be engineered to avoid this fate by facilitating endosomal escape prior to lysosomal trafficking [35]. NPs that are able to demonstrate endosomal escape may still be required to breach additional biological barriers, such as the nuclear membrane, as is required for effective gene therapy. Each of these obstacles illustrates a demand placed on the engineers of a given system, and must be addressed in the preparation of the core and surface properties of the NP.

2.2. Physicochemical considerations

NP pharmacokinetics and cellular uptake *in vivo*, including their ability to manage biological barriers, are largely related to NP physicochemical properties, including morphology, hydrodynamic size, charge, and other surface properties [40,41]. These properties are dictated by the types, structures, and orientations of the materials that comprise the NP. Typically, an MNP consists of a magnetically active core coated with a stabilizing shell to which targeting ligands and additional imaging modalities are anchored. Therapeutic agents can then be embedded in the shell structure or chemically bonded to its surface. At each stage of its design, the size, charge, hydrophobicity, shape, and orientation of the NP's constituent materials must be considered with regards to overall NP physicochemical properties.

2.2.1. Hydrodynamic Size

NP biodistribution appears to be significantly influenced by its physicochemical properties [37,42]. Hydrodynamic size, for instance, (1) helps govern the NP concentration profile in the blood vessel [43–45], (2) affects the mechanism of NP clearance, and (3) dictates the permeability of NPs out of the vasculature [46]. In the case of the former, Decuzzi et al produced models suggesting that smaller sized, spherical NPs observed higher diffusion rates, increasing the NP concentration at the center of a blood vessel, thus limiting interactions with endothelial cells and prolonging the NP blood circulation time [45].

Hydrodynamic size also affects NP clearance from circulation [37,47–51]. For instance, it has been reported that small NPs (<20 nm) are excreted renally [47,52], while medium sized NPs (30–150 nm) have accumulated in the bone marrow [53], heart, kidney and stomach [52], and large NPs (150–300 nm) have been found in the liver and spleen [54]. While these size ranges provide general clearance mechanisms, other physical parameters simultaneously affect NP mobility.

As previously discussed, nanoparticle size affects the ability of NPs to extravasate from the vasculature. While most endothelial barriers allow NPs <150 nm in diameter to pass, more stringent barriers, such as the BBB are far more restrictive. The BBB allows passive diffusion of

only small (<500 Da MW), neutrally charged lipid soluble molecules, prohibiting >98% of all potential neurotherapeutics and contrast agents from passing through the BBB [55,56]. In addition, a vast majority of developed NPs have been unable to breach the BBB [38]. Consequently, this has become an area of intense research [38,56–59], with broad ramifications in the development of treatment strategies for brain tumors, Parkinson's, Alzheimer's, and Huntington's diseases [1,57,60–63]. In the quest to determine the influence of NP size on BBB permeability Sonavane et al recently reported that gold NPs of 15 to 50 nm in hydrodynamic size could permeate across the BBB, while larger NPs, specifically 100 and 200 nm sized could not [64]. However, it should be noted that reviews of the literature have suggested that BBB permeability is likely influenced by all physicochemical properties discussed here and NP size may not alone dictate NP permeability across the BBB [56].

2.2.2. Shape

In investigating the effects of NP shape on biodistribution, a limited number of comparative studies have been performed evaluating the biodistribution of non-spherical and rod shaped NPs [65–70]. It has been suggested that anisotropically shaped NPs can avoid bioelimination better than spherical NPs [67]. In one notable study by Geng et al, the authors demonstrated a relationship by which an increase in the length-to-width aspect ratio of the nanostructure correlated with increased *in vivo* blood circulation time of nanostructures [70]. High aspect ratio shaped MNPs have also been evaluated *in vivo* and found to have similarly enhanced blood circulation times over the spherical counterparts [71,72]. Although these findings are promising more studies are needed to identify exactly what aspect ratios yield most dramatic influence on NP pharmacokinetics.

2.2.3. Surface properties

NP charge and hydrophobicity can affect NP biodistribution by limiting or enhancing interactions of NPs with the adaptive immune system, plasma proteins, extracellular matrices and non-targeted cells [36]. Specifically, hydrophobic and charged NPs have short circulation times due to adsorption of plasma proteins (opsonization) which can lead to recognition by the reticuloendothelial system (RES), followed by removal from circulation [41]. Positively charged NPs can also bind with non-targeted cells (typically negatively charged) leading to non-specific internalization. In addition, hydrophobic groups on the surface of NPs induce the agglomeration of the NPs upon injection, leading to rapid removal by the RES.

To limit NP-host interactions, surface engineering has led to the development of stealth NPs. Surface modification with molecules like the hydrophilic polyethylene glycol (PEG) have been shown to reduce the potential for opsonization through steric repulsion, prolonging NP circulation times [73]. The utility of organic coatings will be properly addressed in later sections.

2.3. Directing nanoparticles *in vivo*

The specificity of NPs for select tissues is critical in both diagnostic imaging and drug-based therapies [16,74,75]. In both cases, nonspecific cell binding can place healthy tissue at risk. To limit non-specific binding, NPs have been engineered to have an affinity for target tissues through passive, active, and magnetic targeting approaches.

Passive targeting uses the predetermined physicochemical properties of a given NP to specifically migrate to a given tissue region. For example, targeting of solid tumor tissue can be achieved through passive mechanism termed enhanced permeation and retention (EPR) [76]. This phenomenon is based on the principle that tumor cells, in an effort to grow rapidly, stimulate production of new blood vessels (the neovasculature) that are poorly organized and have leaky fenestrations. This enables extravasation of small macromolecules and NPs out of the vasculature, into the tumor tissue [77,78]. Due to

inefficient lymphatic drainage, there is poor clearance of these agents, leading to selective accumulation of these agents [79,80]. However, EPR is limited to specific metastatic solid tumors, and successful implementation is dependent upon a number of factors including degree of capillary disorder, blood flow, and lymphatic drainage rate, making effective management difficult.

Because passive targeting is available for only certain *in vivo* applications and does not necessarily guarantee internalization of NPs by targeted cells, NPs can be additionally modified with molecular targeting ligands to employ active cell targeting [81,82]. NP assemblies are now decorated with targeting molecules, complementary to unique receptors on target cells, to actively target only diseased tissue. A number of SPION systems have implemented targeting ligands into their design with varying success, including: small organic molecules [81,83,84], peptides [71,85–88], proteins [89], antibodies [90–92], and aptamers [93–95]. In addition to the type of ligand used, active targeting is affected by targeting molecule density and by the size and shape of the NP.

Recent studies indicated that the density and molecular organization of bound ligands significantly influence NP binding to target cells due to the multivalency phenomenon [86,96]. Multivalency is the enhanced binding avidity phenomenon observed when multiple ligands simultaneously bind with multiple receptors between two surfaces [97–99]. Several NP systems have been engineered to achieve higher affinities to their cellular targets utilizing this principle [86,100,101]. Notably, in a study of cross-linked iron oxide (CLIO) NPs decorated with varying densities of the RGD peptide (4.1, 20, and 52 peptides per NP), it was shown that simultaneous ligand binding could be increased with higher RGD presentation, but beyond a given ligand density, multivalent interactions were sterically hindered [100]. In addition, multivalency is also affected by NP size [102]. In a notable study by Jiang et al NPs of sizes ranging from 2–100 nm were decorated with targeting herceptin antibodies and evaluated for ability to bind and be internalized by targeted cells [102]. Through a series of experiments it was revealed that NPs smaller than 25 nm

lack the ability to present multiple ligands to a cell, unlike larger variants, limiting any potential multivalency binding effects. At the same time, larger NPs are not as readily endocytosed by cells, limiting their functionality for certain applications. Notably NPs of 25–50 nm were revealed to be most suitable for multivalent binding and endocytosis. This is graphically represented in Fig. 1 where the larger NP, decorated with targeting antibodies, is able to form more multivalent interactions with the cell receptors of the targeted cell. NPs of varying size (2, 40, and 70 nm) coated with antibodies showed variable degrees of internalization by the target cell, as observed by fluorescence imaging.

NP shape has also been shown to influence NP targeting abilities. A secondary study by Decuzzi et al hypothesized that oblique-shaped particles, that have been decorated with targeting molecules, show greater cell binding affinity compared with spherical NPs [43]. This theory has been supported by several recent studies [65,66,71,103]. Most notably, in a comparative study between F3 peptide-modified spherical (5 nm) and rod shaped (5 nm × 5 nm joined cores) “nanoworm” MNP assemblies, the elongated “nanoworm” construct showed enhanced cell binding [71]. The enhanced multivalent interactions of the nanoworm are conceptually illustrated in Fig. 2. As shown the elongated shape of the MNP can bind more targeting molecules with the target cells compared with a spherical NP.

In addition to engineering NPs for tissue targeting, some researchers have used external magnetic systems to help direct MNPs localization in a strategy called magnetic targeting [18,104]. This involves focusing high field, high gradient, or rare earth magnets on the target site, inducing accumulation of the highly magnetically susceptible MNPs. Recently this technique was successfully implemented in a clinical trial to deliver the chemotherapeutic, doxorubicin, to hepatocarcinoma cells [105]. While successful, the effectivity of magnetic targeting is limited to target tissue close to the body's surface, due to loss of magnetic field strength further away from the magnetic source.

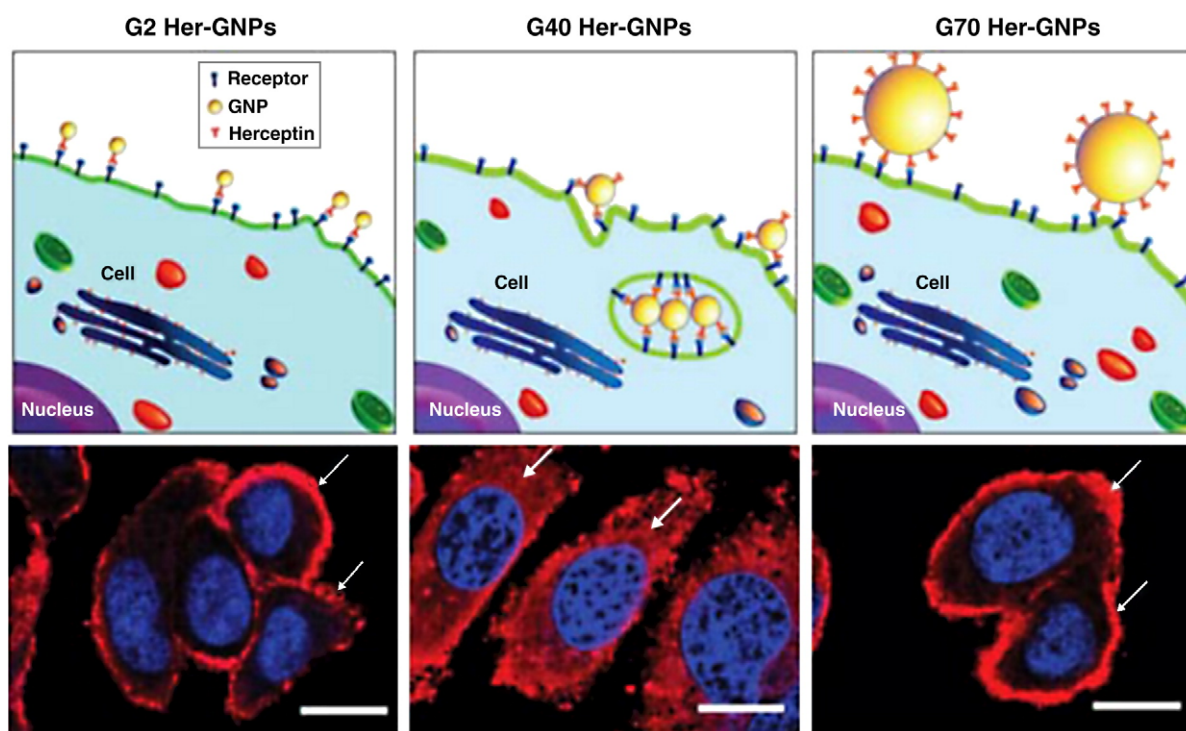


Fig. 1. Illustrations with corresponding fluorescence images of ErbB2 receptor localization after treatment with different-sized heceptin bound to gold NPs (Her-GNPs). In the fluorescence images of cells arrows indicate ErbB2 receptors, and the nucleus is counterstained with DAPI (blue) (scale bars = 10 microm). Reprinted by permission from Macmillan Publishers Ltd: *Nature Nanotechnology* [102] Copyright 2008.

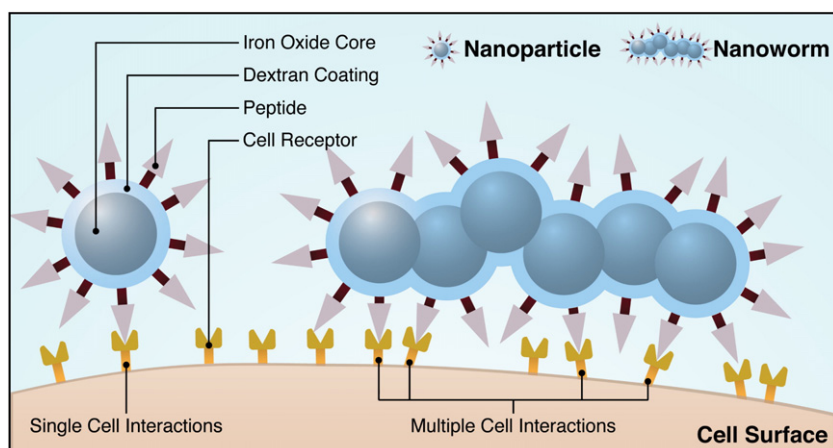


Fig. 2. Conceptual scheme illustrating the varying multivalent affinity interactions between receptors on a cell surface and targeting ligands on a nanospheres versus a nanoworm. Conceptual adaptation from the figure previously published [71].

2.4. Drug loading and Release

When loaded with a therapeutic payload, NPs that are appropriately designed, can act as efficient drug delivery systems, offering limited non-specific cell interactions, controlled therapeutic release, flexible drug loading (a variety of drugs can be loaded), and delivery tracking using a NP imaging modality. Like NPs developed for diagnostic imaging, drug-carrying NPs require careful physicochemical and targeting design, but also require additional considerations paid to drug loading, transport, and release [24,106,107]. First, the NP must be able to carry and protect a significant drug payload, typically determined by the type of coating and method of loading (e.g. covalent bonding). Second, multiple drugs can be loaded to overcome cellular drug resistance, and improve overall cell kill efficiencies, but requires careful NP planning to accommodate the different therapeu-

tics. Third, the release mechanism and rate of the therapeutic cargo unloading should be modulated for optimal therapeutic efficacy. For instance, the concentration and duration of release of drug after intracellular uptake can be predetermined, or in the case of gene therapy, the release of cargo can be adjusted to respond to the cell cycle, triggering release at an optimal time.

Additional application driven considerations are required based on the chosen therapeutics [8]. Fig. 3 describes the blueprint of multifunctional imaging/therapeutic MNPs and the local activity of several categories of therapeutic agents designed for cancer therapy. As shown MNPs developed for these dual applications may carry targeted moieties extended from NP surface via polymeric tethers (e.g. PEG), and may carry multiple imaging reporters (optical, radio, magnetic), and therapies in the forms of biotherapeutics (i.e. gene), chemotherapeutics (i.e. chemical drug formulations), and/or radiotherapeutics (i.e.

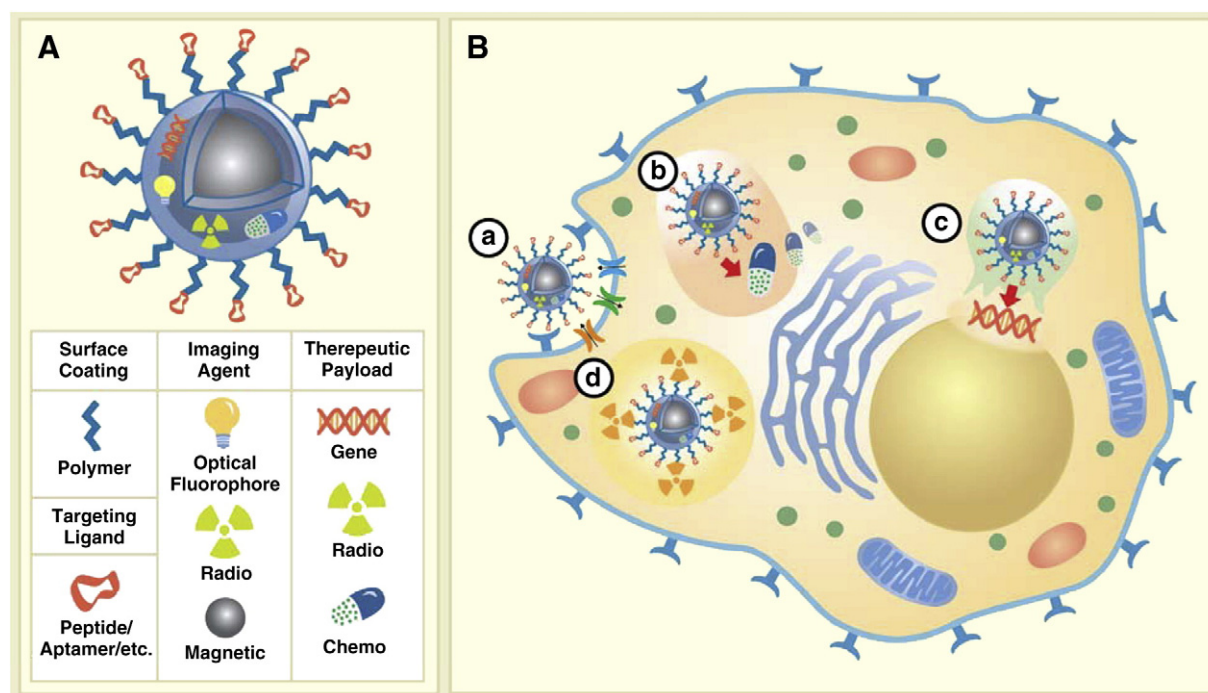


Fig. 3. Illustration of multifunctional imaging/therapeutic MNPs anatomy and potential mechanisms of action at the cellular level. (A) A multifunctional MNP modified with targeting ligands extended from MNP surface with polymeric extenders, imaging reporters (optical, radio, magnetic), and potential therapeutic payloads (gene, radio, chemo). (B) Four possible modes of action for various therapeutic agents; a) Specific MNP binding to cell surface receptors (i.e. enzymes/proteins) facilitate their internalization and/or inactivation, b) controlled intercellular release of chemotherapeutics; c) release of gene therapeutic materials post endosomal escape and subsequent targeting of nucleus; and d) intracellular decay of radioactive materials.

radionucleotides) (Fig. 3A). At the cellular level different therapeutic mechanism can then be activated depending on the choice of therapeutic integrated onto MNPs (Fig. 3B). For instance as shown in Fig. 3B, therapeutic peptides/antibodies function by binding to and inactivating cell surface receptors, possibly requiring receptor-mediated internalization (a); chemotherapeutic agents require internalization and slow intracellular drug release (b); DNA and siRNA gene therapy requires action in the perinuclear region or nucleus, necessitating cellular uptake followed by escape from endosomal compartments (c); lastly, radiotherapeutics require cellular internalization (d).

2.5. Toxicity

To ensure a developed MNP system poses no threat to the patient after administration, toxicity of the individual components and NP as a whole must be evaluated. When evaluating NP toxicity it is necessary to both consider how the assembled NP system will interact with the body during its functional lifetime, and how the independent components will affect the body during biodegradation and liver processing [10]. Nanotoxicology is an emerging area of research, but additional studies are needed to better understand the body's response to nanoparticulates. For an in-depth discussion of nanotoxicology concerns please refer to recent articles on this area published by Longmire et al. and Vega-Villa et al. [37,108], or for a more comprehensive review please refer to the book published by Zhao et al. [109]. Typically, MNPs are not excreted from the body as a construct, necessitating the use of components that can individually be biodegraded by the body.

3. Fabrication of target-specific magnetic nanoparticles (MNPs)

In order to take advantage of our knowledge of the NP bioresponses and targeting techniques detailed above, to control the physicochemical properties of NPs, we need to understand and implement controlled synthesis and coating processes. In the following sections we will discuss some of the techniques used in the MNP field and detail the significant design parameters that can assist in synthesizing target-specific NPs.

3.1. MNP core fabrication

Typically comprised of an organic coating and multiple functional molecules at its surface, the magnetic functionality of MNPs for MR imaging is dictated by the composition, size, and shape of its magnetic core. These NP cores have been made from different materials and with varying sizes, shapes, uniformities, and magnetic properties [8,13,14,110,111]. Specifically, MNPs have been formed from pure iron and cobalt metals [69,112], alloys such as CoPt₃ [113], FePt [114], FeZn [115], and from iron oxides [15], including magnetite (Fe₃O₄) and maghemite (γ -Fe₂O₃) [15,116,117]. The iron oxides have also been doped to enhance their magnetic properties to form MFe₂O₄ structures where M is a +2 cation such as Mn, Fe, Co or Ni [30,118]. While these MNPs make excellent MR contrast agents, those containing cobalt, nickel, and manganese are potentially toxic, making them poor candidates for clinical use until advanced coatings and chelating agents are developed [119]. Alternatively, the non-doped iron oxides degrade to their non-toxic iron and oxygen components, making them particularly attractive as NP cores [120]. Of the two, magnetite is typically preferred due to its superior magnetic properties and will be the focus of the remainder of this section [15].

SPIONs can be fabricated by either top-down (mechanical attrition) or bottom-up (chemical synthesis) approaches. However, chemical routes are better suited to produce nanoparticles with uniform composition and size (typical deviations of >10% in less than 10% of the nanoparticle batch) [121]. The solution chemical methods include standard iron chloride co-precipitation, co-precipitation in

constrained environments, thermal decomposition and/or reduction, hydrothermal synthesis, and polyol synthesis [8,15,111]. An excellent review of these methods has been presented by Lauret et al [15].

While each method has its own specific advantages, the most common preparation method is that of co-precipitation of Fe²⁺/Fe³⁺ salt solutions with the addition of base under an inert atmosphere. Here, NPs are formed by a nucleation and growth mechanism that typically allows for good monodispersity of the end product by optimization of the conditions that yield a short nucleation event followed by a slower growth phase. Type of salts employed (e.g. chlorides, sulfates, nitrates), ratio of Fe²⁺/Fe³⁺, temperature, pH and ionic strength all affect the properties of the synthesized SPIONs [111]. While adequate, standard co-precipitation methods have difficulty yielding consistent SPION size, shape, and polydispersity, and can introduce impurities and surface defects into the particles, compromising their magnetic properties [13].

To help improve the uniformity and stability of SPIONs, modifications of the standard co-precipitation approach have been investigated. Specifically, several studies added polymers or polyelectrolytes to the iron chloride solution during co-precipitation to specifically tune the size, shape, and crystallinity of the SPIONs [13,122–125]. In one study, the SPION core was tuned between 7–14 nm by changing the concentration of poly (acrylic acid) in solution [123], while in another, graft PEG-g-poly(glycerol monoacrylate) polymers were used to modulate SPION size [125]. Importantly, these polymers may act as surface coatings after the nucleation and growth processes are complete. These coatings are sometimes referred to as “in situ” because they are present during nanoparticle synthesis. Unfortunately, this approach can limit the crystallinity of the formed SPIONs, which may negatively affect their magnetic susceptibility.

Recently, new synthesis techniques have been developed using high-temperature decomposition methods and organic iron precursors [30,116]. In one notable study by Sun et al. high-temperature reaction of iron (III) acetylacetonate, Fe(acac)₃, in phenyl ether in the presence of alcohol, oleic acid, and oleylamine, yielded monodisperse, hydrophobic magnetite NPs with tunable sizes of 4–20 nm [116]. The limitation of these synthesis approaches is that additional steps are required to remove the hydrophobic coating, or to modify the surfaces with an amphiphilic surfactant to render the NPs usable for biomedical applications. Though these new techniques yield more uniform NPs with superior magnetic properties, the co-precipitation method continues to be most widely used for biomedical applications because of ease of implementation and need for less hazardous materials and procedures.

3.2. Coating of SPIONs

After synthesis, unmodified SPIONs are stable in high and low pH solutions, but use *in vivo* requires that SPIONs be coated. These surface coatings, typically comprised of small organic molecules and polymers, function to (1) protect against iron oxide core agglomeration, (2) provide chemical handles for the conjugation of drug molecules, targeting ligands, and reporter moieties, and (3) limit non-specific cell interactions. Additionally, polymeric coatings have been engineered to enhance SPION pharmacokinetics, endosomal release, and tailored drug loading and release behaviors. To serve these coating functions, a diverse group of polymers have been investigated including PEG [81,126–132], dextran [133–136], chitosan [137–140], PEI [104,141–145], and phospholipids [145–147].

SPION coating can be achieved via a number of approaches, including *in situ* coating, post-synthesis adsorption and post-synthesis end grafting [15] (Fig. 4). *In situ* and post synthesis modification with polysaccharides and copolymers lead to coatings that uniformly encapsulate cores. Alternatively, end grafted polymers (e.g. PEG) are anchored to the NP surface by the polymer end groups, forming brush like extensions. Liposome and micelle-forming

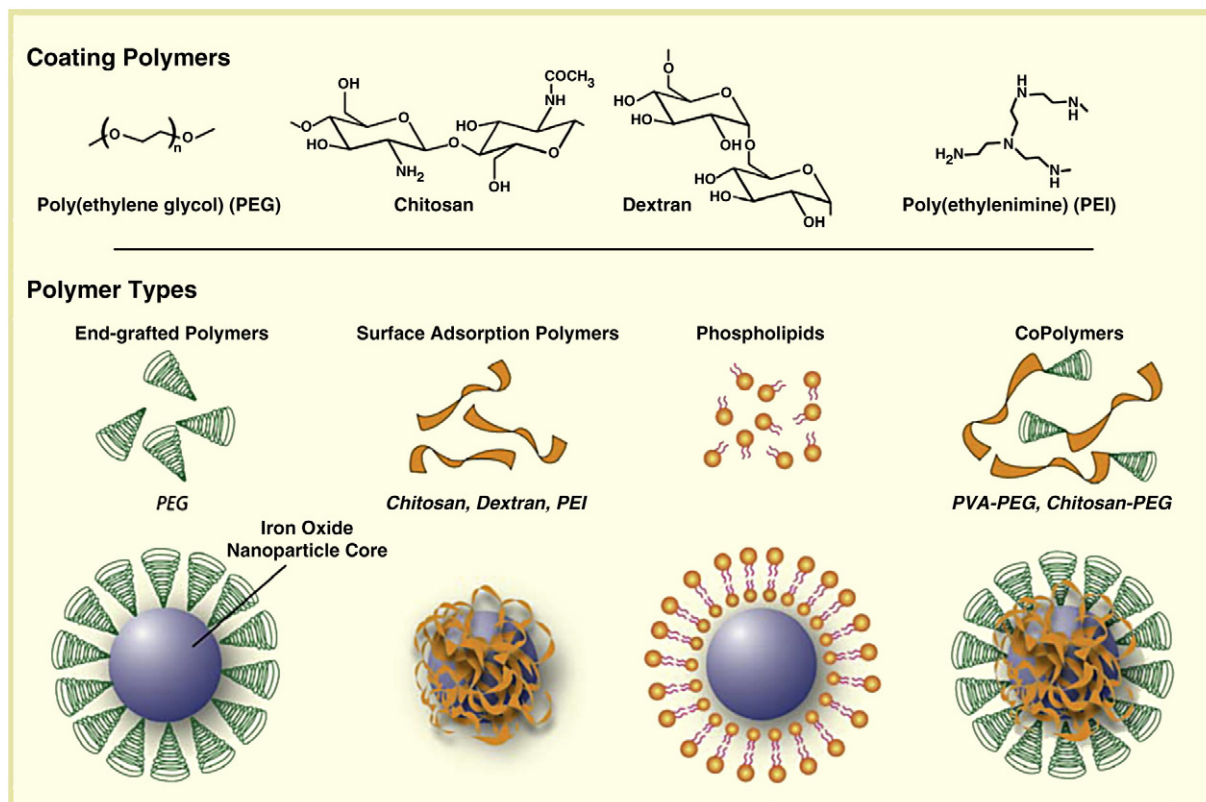


Fig. 4. Illustration depicting the assembly of polymers onto the surface of magnetic nanoparticle cores.

molecules create a shell around the SPION core. These structures retain hydrophobic regions that can be used for drug encapsulation. Each technique retains specific advantages and disadvantages depending upon the polymer employed (e.g. ease of coating, number of functional groups, etc.).

Coating materials and immobilization strategies each influence the magnetic properties of MNPs in different ways. Several studies have revealed that the coating thickness, and hydrophobicity can drastically affect the magnetic properties of MNPs [145,148]. In particular, LaConte et al showed that thicker coatings lowered R2 relaxivities [148], while Duan et al determined that polymer coatings of decreasing hydrophobicity (e.g. PEI versus octadecene coating) caused higher R2 relaxivities [145]. This illustrates the combinatorial effect that these different parameters play on the final properties of a given nanoparticle system. In the proceeding sections, we will detail some of the most common organic coatings, including their methods of attachment, functionality, and specific examples of use.

3.3. Organic surface coatings

3.3.1. Poly(ethylene glycol) PEG

PEG is a biocompatible linear synthetic polyether that can be prepared with a wide range of sizes and terminal functional groups [149]. For decades variations of this polymer have been used clinically as excipients in FDA approved pharmaceutical formulations [150]. They are neutral, hydrophilic molecules in biological fluids, which helps to improve the dispersity and blood circulation time of the SPIONs they are bound to [51,81,130,131,151–153]. PEG-coated (or PEGylated) SPIONs are commonly regarded to as “stealth” nanoparticles because they are not readily recognized by the RES [73]. This limits their use in imaging macrophages or other RES-related cells [154], but the same characteristic makes them ideally suited for use in target-specific cell labeling after modification with targeting ligands [155–157].

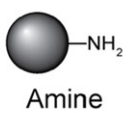
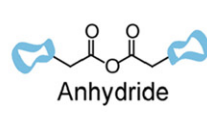
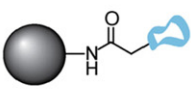
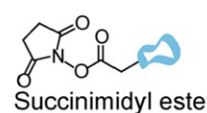
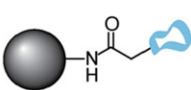
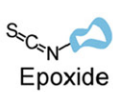
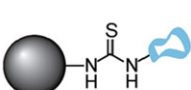

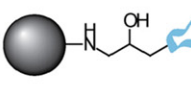
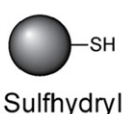
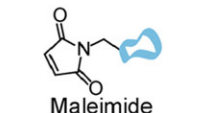
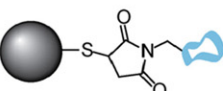
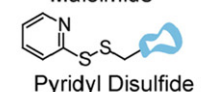
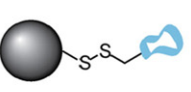
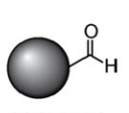

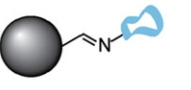
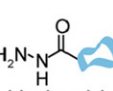
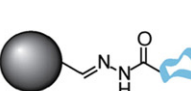
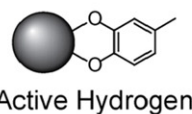

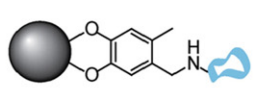
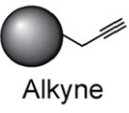

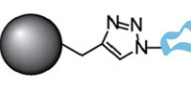
At sizes below 100,000 Da, PEG polymers are considered amphiphilic and are soluble in water as well as in many organic solvents, including methylene chloride, ethanol, toluene, acetone, and chloroform. This allows PEG assembly at the SPION surface using a variety of chemistries that require use of either aqueous or organic solvents. For example, Lutz et al demonstrated in situ coating of PEG onto SPIONs precipitated under aqueous conditions [158], while Kohler et al grafted PEG to SPIONs via a silane group in the organic solvent, toluene [131]. Significantly, in the latter study a hetrobifunctional PEG was prepared that could covalently attached to the SPION surface by one end and then functionalized with targeting ligands, imaging reporter molecules, or therapeutic agents by the other end [84,85,89,155,156,159]. This represents a polymeric design strategy that is conscious of both efficient surface coating and functionalization after PEG attachment.

3.3.2. Dextran

Dextran is a branched polysaccharide comprised of glucose subunits that can be prepared with sizes ranging from 10 to 150 kDa. The polymer's wide use in SPION coatings has been attributed to its biocompatibility and its polar interactions (chelation and hydrogen bonding) that give dextran a high affinity for iron oxide surfaces [160]. As such many of the clinically approved SPION preparations are dextran coated [120,161–165]. Typically, these coatings are prepared by in situ techniques, as was first described in 1982 by Molday and Mackenzie [134]. Since then, various forms of dextran polymers, including carboxydextran and carboxymethyl dextran, have been used to coat SPIONs with varying hydrodynamic sizes [15].

Conventional dextran coatings are based on hydrogen bonding, making the polymer susceptible to detachment, but the polymers have been crosslinked after SPION attachment using epichlohydrin and ammonia, forming a CLIO [166]. CLIOs, have become a versatile platform that has demonstrated a high circulation half-life in blood

Table 1
Examples of various SPION surface modification chemistry.

<i>Direct nanoparticle conjugation</i>				
Nanoparticle	Reactive ligand	Conjugate	Typical ligands	Notes
 Amine	 Anhydride		Activated small molecules, dyes, peptides, proteins or antibodies	Stable linkages (except isothiocyanate)
	 Succinimidyl ester			
	 Epoxide			
	 Isothiocyanate			
 Sulfhydryl	 Maleimide		Activated dyes, peptides, proteins or antibodies	Interparticle disulfide linkage may form
	 Pyridyl Disulfide			
 Aldehyde	 Amine	 Schiff-Base Condensation	Amine-containing or hydrazide activated small molecules, dyes, peptides, proteins or antibodies	Cleavable under acidic conditions
	 Hydrazide	 Schiff-Base Condensation		
 Active Hydrogen	 Amine	 Mannich Reaction	Amine-containing small molecules	May lead to crosslinking
 Alkyne	 Azide	 Click Chemistry	Activated small molecules, dyes, peptides, proteins or antibodies	Stable linkage, highly specific, high product yields

with no acute toxicity [167]. However, due to the use of epichlorohydrin and an inability to degrade and clear from the body, their use in a clinical setting is unlikely [9]. To replace the need for cross-linking, Duguet et al created a multistep process to covalently bind dextran to the SPION surface using silane chemistry, and is expected to yield increased interest [136,168].

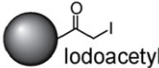

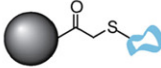

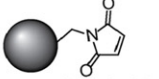

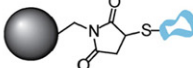
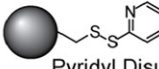

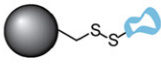


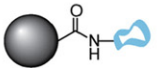
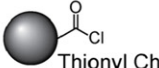

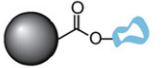



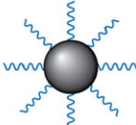

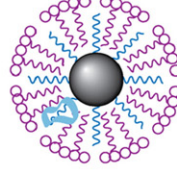

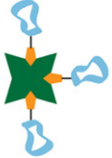
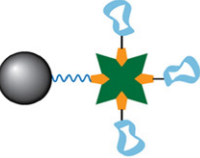
3.3.3. Chitosan

Chitosan is a cationic, hydrophilic polymer that is also nontoxic, biocompatible, and bioabsorbable, which has made it a popular

material for drug delivery applications in recent years [169,170]. This is primarily due to its large abundance in nature, biocompatibility, and ease of functionalization. For decades chitosan and its derivatives have been used to form polymeric nanoparticles through electrostatic complexation with nucleic acids and various pharmaceutical formulations [169], only recently being used in combination with magnetic nanoparticles [140,171–176].

To coat SPIONs with chitosan, it has been found that direct, in situ coating is problematic because of its poor solubility at pH values necessary to precipitate SPIONs [170]. Chitosan-coated SPIONs have

Table 1 (continued)

Linker chemistry conjugation					
Starting nanoparticle	Linker chemistry	Reactive ligand	Conjugate	Typical ligands	Notes
	 Iodoacetyl	+ HS- 			Stable linkage
 Amine	 Maleimide	+ HS- 		Activated small molecules, peptides, proteins, antibodies or aptamers	Stable linkage, sulfhydryl selective
	 Pyridyl Disulfide	+ HS- 			Sulfhydryl selective, cleavable under reducing condition
	 Carboxyl	+ H ₂ N- 		Amine-containing molecules	Stable linkage
	 Thionyl Chloride	+ HO- 		Hydroxyl-containing small molecules	Reacts under anhydrous condition, cleavable under alkaline conditions
Physical interaction attachment					
Starting nanoparticle	Ligand	Functionalized nanoparticle	Typical ligands	Notes	
 Charged surface	+ 	Electrostatic Interaction → 	Modified peptides, proteins or antibodies, DNAs or RNAs	Sensitive to the environment	
 Hydrophobic surface	+ 	Hydrophobic Interaction → 	Hydrophobic molecules	Sensitive to the environment	
 Biotinylated	+ 	Biotin-Avidin Interaction → 	Modified small molecules, dyes, peptides, proteins or antibodies	Strongest non-covalent binding interaction	

been produced, though, by physically adsorbing chitosan onto oleic acid-coated nanoparticles yielding spherically shaped SPIONs (15 nm diameter) [175]. The cationic nature of the polymer allows complexation with genetic material making it suitable for use as a gene delivery carrier, even when used as a SPION coating. For example, Bhattarai et al. loaded chitosan-coated iron oxide nanoparticles with anionic adenovirus vectors through electrostatic interactions [174]. These SPIONs were used to enhance gene transfection. In addition to its bioadsorptive properties, chitosan possesses both amino and hydroxyl functional groups, which can be used for SPION functionalization with targeting, imaging, and therapeutic agents.

3.3.4. Polyethyleneimine (PEI)

PEI is another water soluble cationic polymer that can take both linear and branched forms [177]. For decades PEI-based polymers has been used for gene delivery thanks to their ability to complex with DNA, facilitate endosomal release via the “proton sponge effect”, and

guide intracellular trafficking of their cargo into the nucleus [177,178]. To capitalize on these properties, PEI has been integrated into SPION coatings in recent years [141–144,179]. Naturally, the most common application of these constructs has been for *in vitro* cell transfection with either DNA or siRNA nucleotides [141,180].

SPION attachment has been performed by *in situ* coating [179], post-synthesis adsorption [145], and post-synthesis grafting [143,144]. While successful binding has been shown, several problems remain, including PEI's intrinsic toxicity [177] and low colloidal stability of SPION constructs in biological solutions [143,181].

3.3.5. Liposomes and Micelles

Liposomes and micelles, spherical aggregates of amphiphilic molecules, can be used to coat SPIONs in two ways: post-synthesis incorporation or by synthesizing SPIONs directly within their open core. In the first case, water-soluble SPIONs have been confined to the aqueous center of the liposome [135,182], or alternatively, hydrophobic

SPIONs can be coated with micelles around the structure [183–185]. In the second case, SPIONs can be precipitated in the liposomal core, which yield highly uniform NPs with sizes as small as 15 nm in diameter [184].

SPION coating with either liposomal or micellar structures provide SPIONs with important advantages, especially when using the construct in drug delivery applications, including: (1) simple and easy surface modification, (2) convenient encapsulation of pharmaceuticals inside the amphiphilic substructures, and (3) sequestration and protection of pharmaceuticals from the body until degraded in target cells [186]. However, when applying these coatings, there is a danger in coating agglomerates rather than discrete SPION cores in micellar or phospholipid structures, leading to poor physicochemical and magnetic properties [187].

3.3.6. Copolymers

Copolymers have been developed to take advantage of the distinct functionalities derived from its constituents. For example, Veisoh et al demonstrated that by joining PEI and PEG polymers, a new polymer structure is created that can both complex DNA to facilitate cell transfection (PEI functionality), and enable a stealth profile necessary for molecular targeting of cancer cells (PEG functionality) [188].

The advantages these copolymers provide can be applied to SPION coatings. For instance, Kievit et al recently developed a SPION coated with a copolymer of PEG-g-chitosan-g-PEI and demonstrated its use as a DNA delivering nanovector [189]. This study demonstrated that a coating comprised of three polymers grafted together was ideally suitable for DNA complexation, stabilization of NP for in vivo use, and gene transfection. Guo et al developed a triblock PEG-poly(methacrylic acid)-poly(glycerol monomethacrylate) copolymer that was used to coat SPIONs via in situ coating (23 nm diameter) [190]. This yielded a unique pH sensitive coating with a hydrophobic center layer that could encapsulate drug molecules, preferentially releasing the therapeutics in the acidic environment of the cellular endosome. Similar copolymers are being investigated that can attach to the SPION surface by layer-by-layer deposition directed by matching of electrostatic interactions [191], hydrophilic/hydrophobic interactions [183,184], and covalently grafting polymer layers to base coatings [192].

3.4. Surface modification chemistry

A number of chemical approaches have been used for the conjugation of targeting, therapeutic, and imaging reporter molecules with NP surfaces. These can be categorized into covalent linkage strategies (direct nanoparticle conjugation, click chemistry, covalent linker chemistry) and physical interactions (electrostatic, hydrophilic/hydrophobic, affinity interactions). The choice of chemistry is dictated, in part, by the chemical properties and functional groups found on the SPION coating and ligand to be linked. The primary goal is to bind the targeting, imaging, or therapeutic moiety without compromising its functionality once attached. Functionality in such assemblies is dictated by the nature of the ligand (e.g. conformation of biomolecules) and the manner in which it is attached. For example, if an antibody is bonded to the NP such that its recognition site is shielded, it may lose its ability to bind a target. Furthermore, engineering the attachment of therapeutics carries the additional burden of integrating a release mechanism into the NP. Table 1 lists several examples of chemistries that can be used at the SPION surface, reflecting a broad spectrum of approaches that have been evaluated in the literature. The reactions have been categorized by conjugation strategy, NP functional group, and the reactive functional group on the ligand to be attached. Also listed are unique features of each linkage formed. For detailed information on each of these agents and conjugation strategies, readers are directed to refer to G. Hermanson's handbook [193]. In the following sections we will review unique advantages and drawbacks of each strategy described in Table 1, and highlights example application of the strategy in bioconjugation.

3.4.1. Covalent linkages

Covalent linkages are strong and stable bonds, which can be specifically formed between functional groups, typically amino, carboxylic acid, and thiol groups found on the NP surface and conjugated ligands. Usually, these functional groups are added to the NP surface via its polymer coating, which can dictate both the type and number of functional groups on each NP. These chemical handles are found either on the body of the polymer (chitosan, PEI, dextran) or at their terminal ends (PEG). More binding sites can be added per polymer chain, on its body, thus affecting the total number of reactive groups available. For example dextran-coated SPIONs (38 nm) have been reported with 62 reactive amino groups per NP [194], while a larger PEG-coated SPION (64 nm) was reported to have 26 reactive amino groups per NP [89]. These same chemical groups are also found on the targeting, optical, or therapeutic agent to be covalently attached. To link the functional groups a host of chemistries are available (Table 1), which are subdivided into direct reaction, click chemistry, and linker strategies.

3.4.1.1. Direct nanoparticle conjugation. In direct reaction strategies functional groups at the NP surfaces are either directly bonded to reactive ligands, or a linkage reaction is facilitated with the aid of catalysts. As listed in Table 1, NP surfaces functionalized with amine, sulfhydryl, aldehyde, and active hydrogen functional groups can be targeted. These strategies are particularly suitable for small molecule conjugation. In one notable study, 46 different NP systems were prepared from the same dextran-coated base NP, each functionalized with a different small molecule [194]. However, the efficiency of these chemistries is variable, and with the exception of amine-functionalized NPs, direct conjugation methods are susceptible to intercalating or cross-linking. Specifically, NPs may crosslink due to disulfide linkage formation between NPs, or when multiple NPs bind a single ligand bearing multiple amino functional groups. Moreover, biomolecules are not natively reactive with NPs, requiring initial modification prior to conjugation. This can be a challenging task because many biomolecules can lose bioactivity through modification, and often as demonstrated in a study by Shellenberger et al, only precise, limited modifications can be tolerated [195]. Therefore, chemical modification must be controlled to limit loss of biofunctionality. However, it is typically difficult to perform with direct conjugation agents such as glutaraldehyde. This amine group cross-linking reagent can denature proteins and peptides (abundant with amine groups), and thus has limited applicability for biomolecule-NP attachment.

3.4.1.2. Click Chemistry. "Click" chemistry is a relatively new approach of direct conjugation, developed by Sharpless et al almost a decade ago [196]. Representing a set of Cu-catalyzed azide-alkyne chemistries (Table 1), click chemistry was developed to make conjugations between bioactive surfaces easier and less harsh to biomolecule ligands [197,198]. Specifically, click reactions are fast, efficient, require mild reaction conditions (aqueous environment, relatively neutral pH), and create water-soluble and biocompatible linkages (electron configuration similar to amide bonds) [198]. Compared to other direct conjugation strategies this method of attachment offers several unique features. First, azide and alkyne reactive groups are highly specific for one another, and unreactive with most functional groups, ensuring specific conjugation at the desired location(s) on the reactive moiety. Second, the formed bonds are highly stable. This is in contrast to amide bonds, which can be cleaved by hydrolysis reactions, and disulfide linkages that are susceptible to cleavage under reducing environments. Third, the formed linkages are extremely rigid, which helps to maintain conformation of reacted moieties at the MNP surface and prevents their cross interactions. Combined, these features enable the production of highly oriented linkages engineered to ensure optimal reaction activity and efficiency. This technique appears to be specially suited for attachment of targeting moieties where orientation and stability of

Table 2
Example molecular targeting strategies combined with SPIONs, their cellular targets, applications, and functionality for therapeutic applications.

	Name	Target	Application	Internalized?	Therapeutic?	Published Reports
Small Molecules	Folic acid	Folate receptor	Breast cancer imaging	Yes	No	[84]
	Methotrexate	Folate receptor	Brain tumor imaging and therapy	Yes	Yes	[83,209]
	Non-peptidic RGD mimetic	av β 3 integrin	Integrin positive cell imaging	No	No	[210]
	Mimetic of the sialyl Lewis ^x	E-selectin	Inflammatory disease imaging	Unclear	No	[88]
Peptides	RGD	av β 3 integrin	Breast cancer imaging	No	No	[86,100]
	Chlorotoxin	MMP-2	Brain tumor imaging and therapy	Yes	Yes	[85,155,156,159]
	Synaptotagmin I, C2 domain	Phospholipids	Apoptosis imaging	No	No	[211]
	VHSPNKK	Endothelial vascular adhesion molecule-1	Cardiovascular disease imaging	Yes	No	[212]
Aptamers	EPPT1 (YCAREPPTRTFAFWG)	Underglycosylated mucin-1 antigen	Multiple tumor type imaging	Yes	No	[213]
	A10 RNA aptamer	Prostate-specific membrane antigen	Prostate cancer imaging	Yes	No	[204]
	Thrm-A and Thrm-B DNA aptamers	Human alpha-thrombin protein	Serum protein detection	N/A	No	[95]
Proteins	Annexin V	Phosphatidylserine	Apoptosis imaging	No	No	[203,214]
	Luteinizing hormone releasing hormone (LHRH)	LHRH receptor	Breast cancer imaging	Yes	No	[74]
Antibodies	Transferrin	Transferrin receptor	Breast cancer imaging	Yes	No	[75]
	Monoclonal antibody A7	Colorectal carcinoma	Colon cancer imaging	No	No	[215]
	Herceptin (Trastuzumab)	Her2/neu (Breast cancer)	Breast cancer imaging and therapy	No	Yes	[216,217]
	Rituxan (Rituximab)	CD20 antigen (B-cell non-Hodgkin lymphoma)	Lymphoma imaging therapy	No	Yes	[218]

linkages are particularly important. As such this chemistry has been implemented with SPIONs, effective at binding targeting biomolecules to the NP surface with >90% efficiency under mild reaction conditions and reaction times of 5–8 hours [199,200].

Though this technique has many unique advantages there are limitations to its implementation. First, even though the formed linkages are biocompatible, the Cu catalyst needed to drive the reaction can lead to problems in vivo if not properly purified before use. Excessive Cu consumption has been linked to a number of disorders such as hepatitis, neurological disorders, kidney diseases, and Alzheimer's disease [198]. Therefore, extensive measures are required to remove all of the catalyst from MNP solutions post-conjugation. Second, the highly stable linkages formed may inhibit MNP biodegradation. Further investigation is needed towards the long-term toxicity of MNPs developed using click chemistries.

3.4.1.3. Linker Chemistry. These linker molecules offer control over the molecular orientation of bound ligands, critical when protecting targeting ligand functionality. Additionally linkers can be selectively cleaved for use in ligand quantification, controlled release and other applications. SPION surfaces with amine or carboxylic acid functional groups have been modified with heterobifunctional molecules and further reacted with ligands as shown in Table 1. The most common approach here is the use of a linker molecule that binds the amine group of a SPION surface with the sulfhydryl of a biomolecule. In the case of proteins and peptides cystine amino acid residues can be targeted for reaction. If no reactive cystine amino acids are present, lysine, or terminal primary amine groups can be thiolated with trauts reagent (2-Mercaptoethylamine•HCl reagents) [156], or SATA (*N*-Succinimidyl *S*-Acetylthioacetate) [85]. In the latter case the introduced sulfhydryl group is initially protected to prevent undesired crosslinking prior to reaction with NPs. Oligonucleotide based molecules such as siRNA biomolecules have been chemically synthesized to contain sulfhydryls which then could be conjugated to SPIONs using linker chemistry [201]. This chemistry method is especially suitable for reactions with complex biological molecules where multiple reactive sites and sensitivity to over-labeling are a concern.

The superiority of linker chemistry over direct conjugation strategies was highlighted in a notable study by Hogman et al where SPIONs decorated with the transferring (Tf) protein were prepared using pyridyl disulfide (PD) heterobifunctional linker chemistry or direct conjugation through Schiff base catalyzed reaction [202]. The comparison revealed that conjugation using PD linker chemistry allowed approximately a 4-fold increase in the number of Tf molecules attached per SPION, and a 10-fold improvement of binding and uptake by cells, resulting in a 16 \times more sensitive SPION for imaging. Linker chemistry offers better control over the binding sites used in ligand conjugations, increasing the number of active Tf proteins at the NP surface. In addition, a milder reactive condition of this chemistry limits the oxidative conditions that may harm the bioactivity of the protein during conjugation.

Pyridyl disulfide (PD) heterobifunctional linkers are also interesting because they produce cleavable disulfide linkages and a quantifiable reaction byproduct, which can be used to evaluate efficiency of reaction. Schellenberger et al demonstrated this utility in the preparation of SPIONs decorated with annexin V targeting ligands [203]. Though the bonds formed via PD linker chemistry are sensitive to reducing environments, more stable linkers, like iodoacetyls or maleimides, can be used if the application demands it. Linker chemistry does have its drawbacks, including covalent complexation between NPs or ligands, requiring stepwise NP modification prior to ligand attachment, and some linker chemistries require long reaction times and purifications between each step. This also may contribute to the low product yield, as loss of desired product can occur at each purification stage.

SPIONs decorated with carboxylic acid groups can be covalently bonded to biomolecules bearing primary amines through EDC/NHS linkers, which form amide linkages. This approach has been used in the attachment of aptamers [204] and folic acid [131] to SPIONs. While effective for the attachment of molecules that have only one amino group, it is difficult to control the binding orientation of ligands with multiple amines, often leading to inactivation of the ligands. Therefore, amino-decorated NPs conjugated to ligands using sulfhydryl-based linker chemistry, as described above, is the preferred conjugation approach for the attachment of peptides, proteins, antibodies, and enzymes to SPIONs.

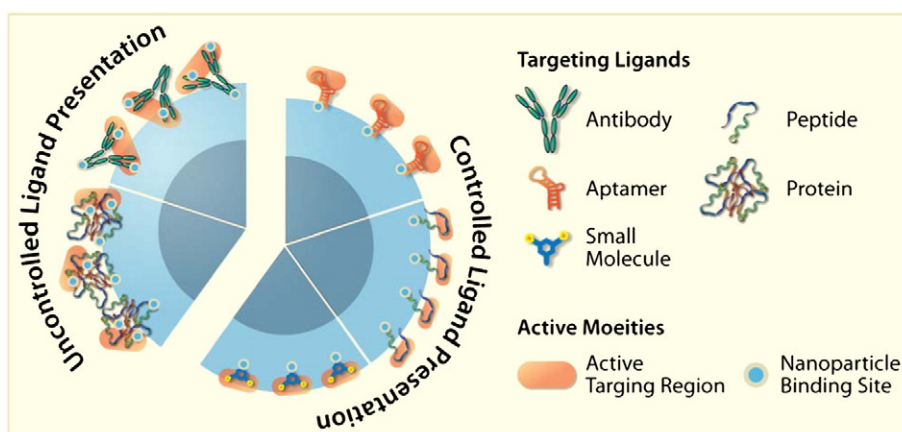


Fig. 5. Illustration of the supermolecular assembly and presentation of targeting antibodies, proteins, peptides, aptamers and small molecules on the surfaces of SPIONs. Note that protein and antibody assembly is difficult to control. Small organic molecules do assemble well but their small size may cause their active targeting regions to be sterically blocked by polymeric coatings. Peptides and aptamers assembly can be controlled through their engineering, and can be modified to assemble in a manner that ensures their active sites are available for interaction with targets on cell surfaces.

3.4.2. Physical interactions

Physical interactions include electrostatic, hydrophilic/hydrophobic, and affinity interactions, as highlighted in Table 1. There are several unique advantages of this chemistry, including: rapid speed of binding, high efficiencies, and no need for intermediate modification steps. Electrostatic interactions have particularly proved useful in the assembly of plasmid DNA onto SPIONs. Several research groups have demonstrated this utility by creating SPIONs coated with cationic polymers of PEI, which are then complexed with negatively-charged plasmid DNA molecules [141–144,180]. A recent study also demonstrated the feasibility of using electrostatic interactions for binding cationic proteins to an anionic SPION surface [205].

Hydrophobic/hydrophilic interactions have proved highly useful when adsorbing hydrophobic drugs onto SPIONs. For this application, SPIONs are engineered with hydrophobic layers that can adsorb hydrophobic drugs that are then triggered for release intracellularly when the coating degrades [32,206,207]. This strategy has drawbacks, which include NP sensitivity to environmental conditions and low control over molecular orientation of bound ligands. Thus while suitable for drug delivery applications where the attached molecule is

released for functionality, attachment of targeting ligands through these strategies are unattractive.

Affinity interactions on the other hand have shown to be very effective for bioconjugation of targeting ligands to SPIONs [89,208]. As shown in Table 1 SPION surfaces can be modified with streptavidin, which specifically binds biotinylated molecules. The linkage formed is highly stable and the strongest of all non-covalent linkages chemistries. Unlike hydrophobic and electrostatic interactions, affinity binding is not sensitive to environmental conditions such as changes in pH, salinity, or hydrophilicity. Using this strategy Gunn et al produced high affinity multivalent display of targeted SPIONs for immunotherapy applications [89].

3.5. SPION targeting strategies

Targeting agents, including antibodies, proteins, peptides, aptamers and small organic molecules, have been used in SPION systems as targeting agents against specific surface markers on target cells.

Table 2 describes several examples for each targeting agent type. These agents have been subcategorized into small organic molecules, peptide, aptamers, and antibodies. Listed is the name of targeting ligand

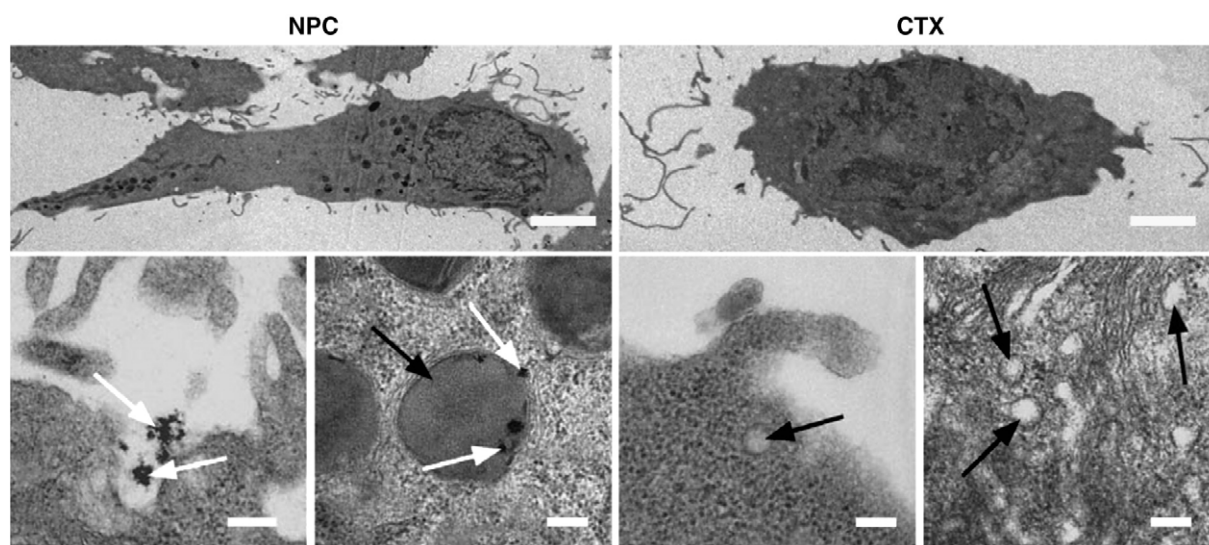


Fig. 6. TEM images showing increased membrane uptake subsequent to NP-CTX (NPC in figure) binding. Scale bars represent 5 mm for whole cell images (first row) and 200 nm for high magnification images (second row). White and black arrows identify NP-CTX and endosomes, respectively. Reproduced with permission from Wiley-VCH Verlag GmbH & Co. KGaA: Small [159] Copyright 2009.

used, its target, and application. It should be noted that some of these agents serve dual purposes, such as chlorotoxin (CTX), which can act as both targeting agent and brain tumor therapeutic. In addition, some of these agents can help initiate endocytosis of the NPs to which they are bound, making them particularly attractive for drug delivery applications.

While each targeting agent enables SPION binding specificity, the type of ligand and method of NP attachment can significantly affect its targeting capabilities. Fig. 5 gives a graphical representation of how these molecules are typically organized onto the NP surface, and depict some of the inherent advantages and disadvantages of each ligand. For instance, relatively bulky proteins and antibodies are difficult to assemble onto a surface. This can be due to the lack of consistent covalent bonding orientation of the protein (there are often *many* active functional groups per molecule, and it's difficult to regulate which gets bonded), or due to non-specific physical interactions by a protein's charged, hydrophobic or hydrophilic regions with the NP surface before being covalently attached. Importantly, this loss of control can limit the presentation of protein binding sites outward, lowering its binding activity.

Alternatively, smaller peptides and nucleic acid-based aptamers can be engineered to have only one active molecular handle per molecule, to ensure consistent linking to the NP surface, and no loss of binding activity. This has been shown to provide significant multivalent binding activity [9,96,100]. In addition, small organic molecules can also be engineered to assemble in high densities, but may require long linker molecules to ensure that the NP coating does not obscure the active region.

In addition to ligand bioactivity, these molecules also affect NP stability and immunogenicity. For instance, antibodies and proteins are often derived from non-human animal sources, which create the possibility of unwanted immune responses. Alternatively, peptides and aptamers can be chemically synthesized and have been shown to be non immunogenic [219].

4. MNP drug delivery vehicles

4.1. Chemotherapeutic agents

Chemotherapeutics encompass a broad category of small molecule drug formulations, which have been developed to initiate a therapeutic response via cytotoxic, cytostatic, or antineoplastic effects. Most chemotherapeutics do not have cell-targeting capabilities (notable exceptions are highlighted in Table 2) and can elicit unwanted side effects when internalized by healthy cells. However, their integration into target-specific NP formulations can limit unwanted side effects, while increasing the dosage at the diseased tissue.

Successful NP drug delivery devices have a prolonged circulation time (small particles <200 nm hydrodynamic size), are internalized by targeted cells, can carry a chemotherapeutic payload, and can be engineered to release its drugs after cell internalization. Currently, several chemical drug formulations have been combined with MNPs, including paclitaxel, doxorubicin, and methotrexate (MTX), all specifically developed for cancer therapy [206,209,220,221].

To successfully integrate a drug into a NP system, several design strategies can be explored, including physical complexation with hydrophobic drugs [190,206,207,220,222], or covalent bonding with cleavable linkages for intracellular release [209]. Drugs loaded through hydrophobic interactions are typically encapsulated within the NP coating, limiting non-specific cell interactions. This approach is advantageous in applications where a drug being delivered could seriously harm non-targeted tissue.

Alternatively, in applications where the drug, such as MTX has an affinity for the target cell, it can be advantageous to graft the drug to the surface of the NP [83,156,209]. Kohler et al. first demonstrated this utility in a study where MTX was covalently attached to the surface of a PEG-coated SPION via a cleavable amide linkage [209]. Recently, Sun et al further modified the same SPION system with CTX to enhance the NP's targeting abilities against brain tumor cells [156].

4.2. Radiotherapeutics

Radionuclides (particularly β -emitters) can function as therapeutic agents, because their localized decay in target cells generates DNA damaging free radicals, which can induce apoptosis. Over the past few decades various targeting strategies including conjugation with antibodies and peptides, have been investigated to help direct radionuclide away from healthy tissue, paralleling chemotherapeutic development [223].

SPIONs and other nanocarriers have recently been evaluated as radionuclides [224]. Compared to the integration of chemotherapeutics into a NP system, radionuclides pose a unique engineering challenge, because they are continuously decaying. In addition, while biotherapeutics and, to some extent, chemotherapeutics can be engineered to illicit therapeutic effects only on to their target cells, radiotherapeutics can damage practically any cell of the body. To limit the possibility of non-specific cellular damage, the SPION-radionuclide complex must remain intact, even after cell uptake, during the radiation decay. This can limit the potential for radionuclide interaction with non-targeted cells.

Most of the radionuclide-containing SPIONs developed so far have been prepared using ^{188}Re radioactive isotopes (17 hour half-life) [225–228]. Histidine decorated SPIONs are known to chelate these radionuclides enabling attachment. These studies include ^{188}Re -enabled SPIONs that were functionalized with albumin [225], or with specific antibodies for liver cancer targeting. The latter study successfully demonstrated the ability to specifically induce cell death in the targeted liver cancer cell line *in vitro* [227].

4.3. Biotherapeutics

4.3.1. Therapeutic peptides/antibodies

Peptides and antibodies function in a cell-specific manner, eliciting therapeutic effects by inhibiting or stimulating various cellular pathways making them attractive therapeutic agents. These biotherapeutics can be used against a number of cell mechanisms, including: activation of apoptotic/necrotic pathways, function blocking (e.g. interfering with cell adhesion, cell surface receptors, angiogenesis, or inhibiting protease and kinase action), and immune response stimulation [229].

SPIONs can be used as biotherapeutic carriers for peptides, and more importantly, these NPs can leverage the multivalent display of the biotherapeutics to improve its potency as a therapeutic agent, itself. At the same time, some biological pathways may require that the SPIONs be internalized to be effective. Here, SPIONs must have an appropriate size (~25–50 nm hydrodynamic size [102]) and coating (e.g. PEG, which can facilitate internalization [81]) to induce uptake. In a recent report, Veiseh et al demonstrated the enhanced potency of the CTX peptide at the SPION surface [159]. The CTX peptide has a high affinity for a set of lipid raft-anchored complexes that contains matrix metalloproteinase-2 (MMP-2) and chloride ion channels which are necessary to sustain the glioma cancer cell's invasive nature. These NPs showed improved internalization via receptor-mediated endocytosis, subsequently impeding the target cell's ability to invade neighboring tissue.

In this study, CTX-decorated SPIONs (NP-CTX) exhibited substantially enhanced cellular uptake, and showed an increased invasion inhibition rate compared to free CTX (98% versus 45%). As shown in Fig. 6, TEM studies of glioma cells treated with NP-CTX (Fig. 6 a) or free CTX (Fig. 6 b) revealed that NP-CTX treatment facilitated internalization of larger volumes of MMP2 containing lipid rafts compared to free CTX. Additional assays were performed, illustrating that NP-CTX, owing to multivalency, was more efficient at limiting glioma cell invasion by promoting the internalization of cell surface-bound peptidases and volume regulating channels.

SPIONs decorated with antibodies have also been evaluated for therapeutic applications. A notable example includes the use of the antibody Herceptin (commercially marketed as Trastuzumab), which targets the Her2/neu receptor [230]. Her2/neu is characterized to be a

growth factor, upregulated on cell surfaces of 20–30% of early stage breast cancer tumors, and essential for cell proliferation. Clinical evidence has revealed that the interaction of Herceptin with this receptor leads to its inactivation, and subsequent inhibition of cell proliferation. As such SPIONs have been decorated with Herceptin, and evaluated as targeting/biotherapeutic NPs [216,217].

4.3.2. Gene therapy

Gene therapy describes the use of DNA and antisense RNA (siRNA) technologies for therapeutic gene expression, and expression silencing of defective genes, respectively. These therapeutics have been integrated into MNP preparations, which have helped to protect the nucleic acids against enzymatic degradation and facilitate cellular internalization and endosomal release [104,231]. To accomplish this, most MNP systems have been engineered using cationic polymers such as PEI [143], polyamidoamine [232], or chitosan [174]. These coatings can complex negatively charged nucleic acids, and assist endosomal release by inducing acidification of endosomal vesicles (“the proton sponge effect”) [180]. While these approaches have shown great success *in vitro*, their applicability *in vivo* has been limited because of toxicity and stability concerns. Highly cationic MNPs, specifically PEI coated ones, have shown poor stability in biological solutions, and potential for *in vivo* toxicity [181]. New preparations that can address these problems are still needed.

One alternative to cationic coatings was offered in a study by Medarova et al [201]. Here, antisense RNA was bonded to CLIO NPs by covalent linkages, while a cell penetrating peptide was used to facilitate transfection. This strategy proved to be highly successful for the delivery of therapeutic siRNAs to human colorectal carcinoma tumors *in vivo*, and represents the first targeted/siRNA MNP used for therapeutic application.

5. Applications of SPIONs for *in vivo* imaging

5.1. Imaging modalities

SPIONs were developed specifically as MR imaging contrast agents, but new preparations are being developed that incorporate multiple

imaging moieties onto the MNP for use in integrated imaging systems [233]. These multimodal agents can assist investigators to visualize the MNP across different platforms, including MR, optical, or nuclear imaging systems [234]. This offers clinicians the ability to obtain a variety of pathologic information using the unique imaging capabilities of each system, with a common contrast agent [233].

5.1.1. MR Imaging

MR imaging, one of the most effective tools in medicine, offers clinicians the ability to non-invasively obtain anatomic, and metabolic/functional information with high spatial and temporal resolution [28]. This imaging technique uses high magnetic fields to align the nuclear magnetization of the body’s hydrogen atoms to which a radio frequency (RF) pulse is applied that changes the alignment of these nuclei. When the RF pulse is removed, the nuclei “relax” back to their original state. This process can be measured by either its longitudinal relaxation (T1) or transverse relaxation (T2), each of which can be used to generate an MR image. Variation in relaxation rates corresponds to image contrast, allowing for discrimination between tissue types. MR imaging has the benefit of a high three dimensional spatial resolution and high contrast differentiation between soft tissues, which enables simultaneous extraction of physiological, molecular and anatomical information.

Contrast agents developed for MR imaging include MNPs and paramagnetic chelates (e.g. Gd atoms chelated by DTPA, DTPA-BMA, and DTPA-BMEA). The localized interaction of these agents with protons of water molecules creates contrast by reducing T1 or T2 decay relaxation times. SPIONs are typically more effective at shortening T2 rather than T1 relaxation times [22].

Currently, with more advanced MR imaging systems and contrast agents this technique can offer a spatial resolution of 10–100 μm , with no imaging depth limitations (unlike other imaging systems) [233]. At the same time, MR imaging requires relatively long acquisition times (minutes to hours), that patients be placed in the confines of the MR imaging machine, and shows high sensitivity to motion artifacts.

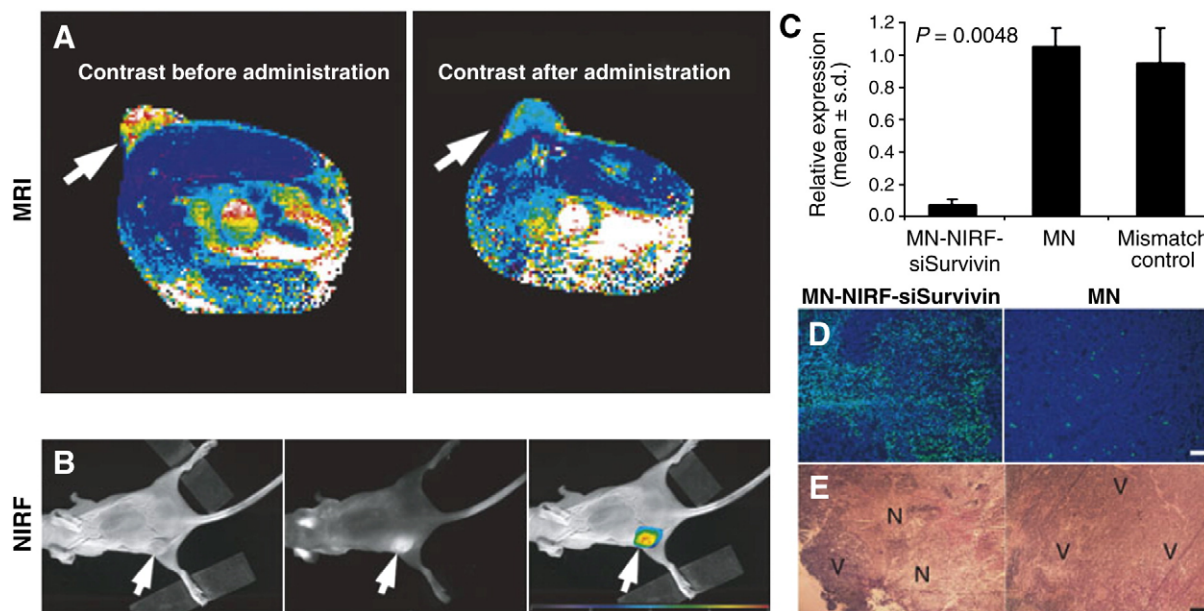


Fig. 7. (A) *In vivo* MRI of mice bearing subcutaneous LS174T human colorectal adenocarcinoma (arrows). There was a significant drop in T2 relaxivity in images acquired after administration of the contrast agent ($P > 0.003$), indicating probe delivery. (B) A high-intensity NIRF signal on *in vivo* optical images associated with the tumor following injection of MNP-NIRF-siSurvivin confirmed the delivery of the probe to this tissue (left, white light; middle, NIRF; right, color-coded overlay). (C) Quantitative RT-PCR analysis of survivin expression in LS174T tumors after injection with either MNP-NIRF-siSurvivin, a mismatch control or the parental magnetic nanoparticle (MNP). Data are representative of three separate experiments. (D) Note distinct areas with a high density of apoptotic nuclei (green) in tumors treated with MNP-NIRF-siSurvivin (left). Such areas were not identified in tumors treated with the control MNPs (right). Sections were counter-stained with 6-diamidino-2-phenylindole (DAPI, blue). (E) H&E staining of frozen tumor sections revealed considerable eosinophilic areas of tumor necrosis (N) in tumors treated with MNP-NIRF-siSurvivin (left). Tumors treated with MNPs were devoid of necrotic tissue (right). Purple hematoxyphilic regions (V) indicate viable tumor tissues. Scale bar, 50 μm . Reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine* [201], copyright 2007.

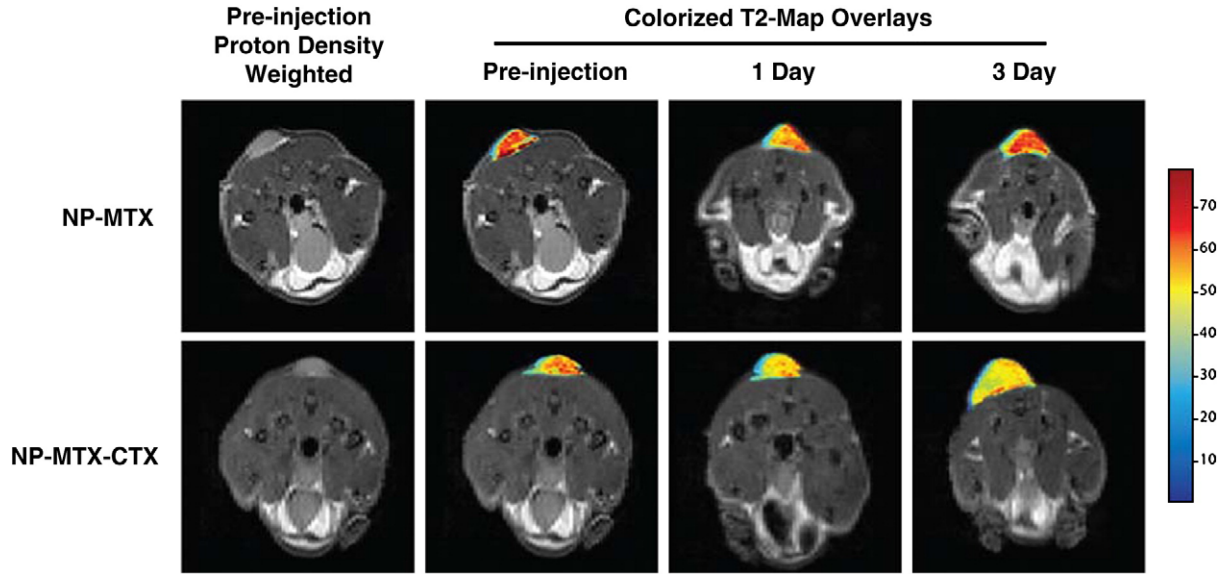


Fig. 8. Axial cross sections displaying 9L tumors of mice before injection of nanoparticle conjugates and 1 and 3 days post-injection. T2 map overlays of the tumor region show decreased T2 for both NP-MTX and NP-MTX-CTX nanoprobe conjugates 1 day after administration. However, the reduction is more significant and uniform in tumor of mouse receiving NP-MTX-CTX. A total of 3 days post-injection, the tumor T2 values of the mouse receiving NP-MTX-CTX remained at the decreased level, while those of mouse receiving NP-MTX returned to the post-injection level suggesting clearance of NP-MTX from tumor tissue. (CTX > Chlorotoxin, MTX > Methotrexate, NP > Nanoparticle). Reproduced with permission from Future Medicine Ltd: Nanomedicine [156] Copyright 2008.

5.1.2. Optical imaging

Optical imaging *in vivo* typically relies on the monitoring of photons emitted from fluorescent agents in the near infrared range (NIR; ~650–

900 nm wavelengths), because of their ability to efficiently pass through biological tissue [22,235]. These fluorescent molecules absorb light of a particular wavelength, and emit light back at a longer wavelength [233].

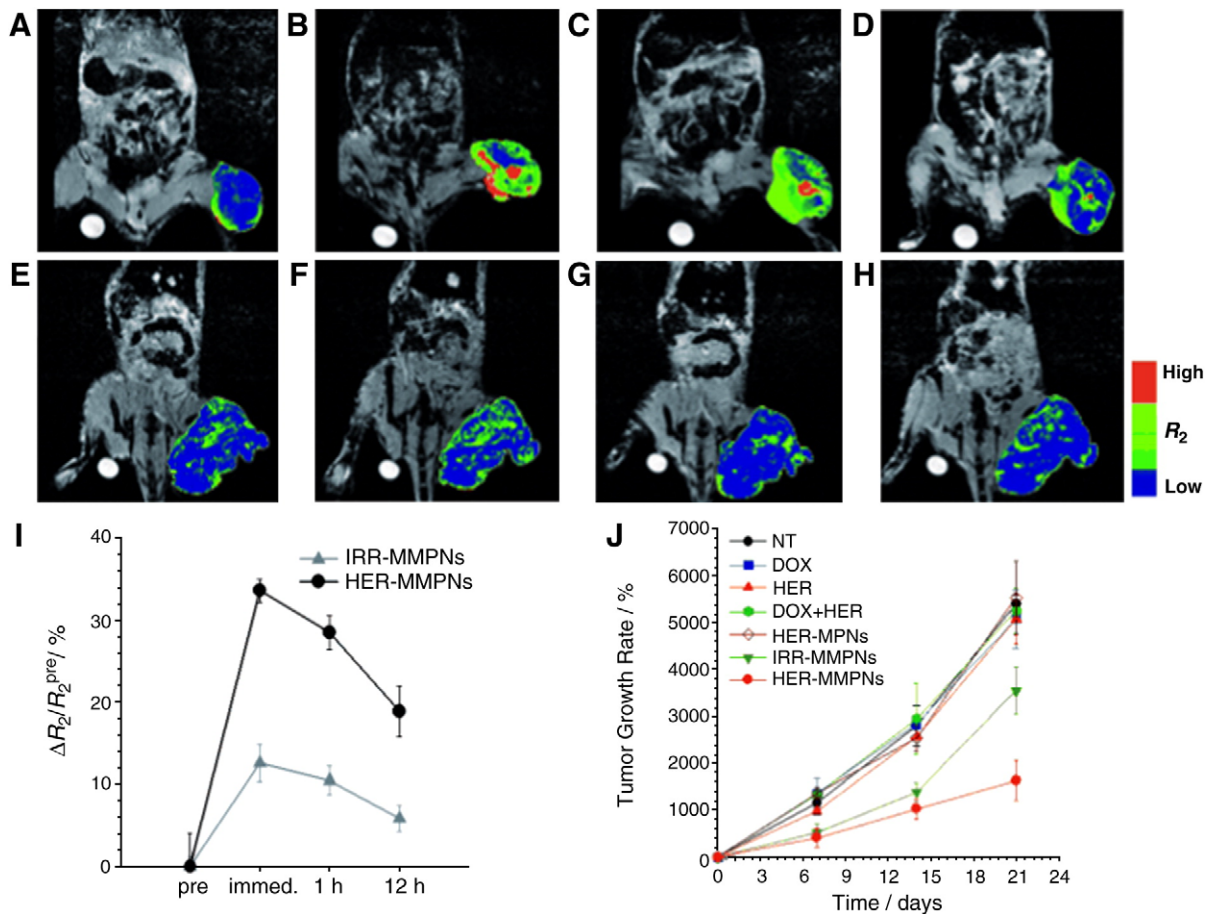


Fig. 9. A) MR images and their color maps (tumor region) of cancer-targeting events of HER-MMPNs (A–D) and IRR-MMPNs (E–H) in NIH3T6.7 cells implanted in mice at various time intervals: A,E) preinjection; B,F) immediately; C,G) 1 h; D,H) 12 h after injection of the MMPNs. I) $\Delta R_2/R_2^{\text{pre}}$ graph versus time before and after injection of MMPNs. J) Comparative therapeutic-efficacy study in an *in vivo* model. Reproduced with permission from Wiley-VCH Verlag GmbH & Co. KGa: Angew. Chem. Int. Ed. [241].

Fluorescent contrast agents are most often used in fluorescence reflectance imaging and fluorescence-mediated topography systems. These techniques are highly useful in molecular screening of surface-based diseases or facilitation of disease resection. State of the art systems provide resolutions on the order of millimeters, and imaging depths up to 10 cm from the surface [233]. Significantly, these techniques offer continuous imaging capabilities (imaging is on the order of seconds). Conversely, optical imaging suffers from poor resolution and limited penetration of light.

5.1.3. Positron Emission Tomography (PET) Imaging

Positron emission tomography (PET) is a nuclear medicine imaging technique that relies on using pairs of high-energy gamma rays produced indirectly by decay of positron emitting radio nucleotides (tracers) introduced into the body. Through this technique, quantitative, tracer concentration dependent, three-dimensional images can be constructed illuminating functional biological processes. Independently this technique does not provide anatomical information. Currently available PET systems offer a relatively poor effective spatial resolution of approximately 1 mm [236]. However, in comparison to other imaging modalities PET offers extremely high sensitivity allowing for detection of as few as 10^{-11} moles of radionuclides deposited in the living subjects.

Radionuclides used for PET imaging include isotopes with short half-lives. Some examples include carbon- 11 (~20 min), nitrogen- 13 (~10 min), oxygen- 15 (~2 min), and fluorine- 18 (~110 min). For imaging applications these agents can either be incorporated into metabolites (e.g. sugar, water, oxygen), or linked to targeting ligands or nanoparticles including MNPs [236].

5.1.4. Conventional imaging compounds vs. MNPs

The purpose of a contrast-imaging agent is to report its location, thus providing physiological or biochemical information about the tissue around it. The success of the agent is dependent on both the concentration at which it accumulates at target site and the strength of the signal it emits. For example, radionuclides can produce highly energetic signals where as few as 10^{-11} moles of radionuclides can be detected. At this signal to noise ratio a single targeting ligand carrying a radionuclide can be detected in a living organism [236]. A fluorescent contrast agent has a detection sensitivity of $\sim 10^{-8}$ moles, one thousand times weaker. This means that to achieve the same sensitivity as PET, an optical imaging agent would have to either accommodate 1000 fluorophores or the target site would have to bind 1000 targeting ligand-fluorophore conjugates. Neither scenario could be practically reached. Gadolinium chelates in water emit signals detectable $\sim 10^{-4}$ mole concentrations roughly 10,000 times even weaker than fluorophores making it much more difficult to image using this agent.

A NP platform can be used to overcome these limitations. The number of imaging reporter and targeting ligands can be tuned to achieve desired signal to noise sensitivity. MNPs are ideally suited for use in these imaging platforms because of their intrinsic magnetic properties. Their detectability is dependent on the size, crystallinity, and coating of the material. However formulations can be created with a detection sensitivity of 10^{-12} M [237]. In practice the magnetic sensitivity depends on parameters such as magnetic field strength, gradient characteristics, and acquisition time [236].

MNPs can be additionally modified with other reporters to create multimodal imaging agents. For example NIR fluorophores have been attached to MNPs to create multimodal contrast agents that offer both the high spatial and temporal resolution and deep tissue penetration of MR imaging and rapid response and sensitivity of optical imaging [22]. Applications of these constructs include cell death monitoring, intra-operative imaging, and epithelial lesion detection [233]. Several studies highlighted the use of these constructs for preoperative diagnosis, and intra-operative resection of brain tumors [85,238,239].

SPIONs have similarly been labeled with ^{64}Cu radionuclides and arginine-glycine-aspartic (RGD) peptides to create a multifunctional PET/MRI contrast agent for imaging of integrin expressing tumors [240]. The multimodal imaging approach can facilitate verification of the accuracy in tumor detection and provide additional information regarding the pathology of the tumor.

5.2. Dual imaging and drug delivery applications

MNPs engineered as drug delivery devices retain the ability to track their movement through the body. This is significant because it allow clinicians to monitor the effectivity of injected therapeutics to reach their target sites. There remains significant flexibility in the contrast agents implemented in these constructs and the manner in which drugs are delivered. Here we profile two notable examples of what this technology can offer.

Medarova et al recently developed a CLIO modified with a NIR fluorophore, therapeutic siRNA sequences, and a cell penetrating peptide [201]. The MNP used passive targeting by the EPR effect to direct tumor localization. *In vivo*, these MNPs demonstrated therapeutic efficacy against target tissue, as determined by real time PCR and histological evaluation, while simultaneously demonstrating image contrast in both MR and optical imaging (Fig. 7).

In a study by Sun et al, active cell targeting was shown by PEG-coated SPIONs to which the chemotherapeutic, MTX, and targeting molecule, CTX, were attached [155]. Shown in Fig. 8 are MR images obtained from mice with flank 9L brain tumors injected with NP-MTX or NP-MTX-CTX. The selective contrast enhancement of the 9L brain tumor by these SPIONs indicates preferential accumulation compared with the same SPION construct without the CTX peptide in a 3-day study.

In another recent study by Yang et al. simultaneous targeted drug delivery and MR imaging of breast cancer tumors were demonstrated through the use multifunctional magneto-polymeric nanohybrids (MMPNs) composed of magnetic nanocrystals and doxorubicin (chemotherapeutic agent) which were simultaneously encapsulated within an amphiphilic block copolymer shell [241]. The surfaces of these micelles were additionally functionalized with the breast cancer targeting/therapeutic ligand, anti-Herceptin antibody. *In vivo* evaluations of this nanoparticle system were performed in nude mice bearing NIH3T6.7 breast cancer tumors. Shown in Fig. 9 are MR images acquired from tumor bearing mice at various time points prior to and post injection with either HER-MMPNs (a-d), or a control irrelevant IgG human antibody (IRR) IRR-MMPNs (e-h). The quantitative evaluation of MR images revealed preferential accumulation of the targeted MNPs compared to the control MNPs (i). The therapeutic functionality of the MNPs developed in this study were additionally evaluated and it was determined that the HER-MMPNs which were decorated with targeting ligands and loaded with doxorubicin were most effective in inhibiting tumor growth (j). Combined, these findings illustrate the functionality and efficacy of targeted multifunctional MNPs for simultaneous MR imaging and drug delivery.

6. Conclusions

Advancements in our ability to fabricate MNPs with greater control over physicochemical and bioactive properties have led to new NP candidates for imaging and therapeutic use. These formulations offer (1) disease diagnosis at their earliest stages and improved pre-operative staging, (2) delivery of therapeutics specifically to diseased tissue, limiting unwanted side effects, and (3) non-invasive monitoring capabilities of new therapeutics. To take advantage of these imaging and therapeutic opportunities, it is critical to be aware of the design parameters discussed, especially when more elaborate constructs are prepared.

Importantly, MNP success ultimately depends upon its ability to bypass *in vivo* barriers. This is highly influenced by the physicochemical

properties of MNPs. Size, shape, and surface chemistry dictate *in vivo* behavior, including biodistribution, biocompatibility, and pharmacokinetics. As such, these parameters can be tuned to achieve enhanced targeting via passive, active, and magnetic targeting mechanisms. Active targeting, in particular, offers high sensitivity due to the ability to direct MNP localization, but requires added attention be paid to the targeting agent used, and the method of MNP attachment employed. A number of bioconjugation strategies including physical methods, covalent strategies, and click chemistries are available, each having distinct advantages. In addition to assisting in disease imaging, cell targeting by MNPs can also assist in disease treatment if a therapeutic payload is integrated into the MNP. This requires additional design considerations, though, including type of therapeutic, method of release, and intracellular activity.

As we move forward, better characterization tools are needed to both evaluate new MNPs and better understand their behavior in the body. Controlled studies of individual physicochemical parameters will offer NP engineers the basic understanding to successfully build more elaborate and functional MNPs. At the same time, questions about MNP elimination and long term toxicity remain barriers to clinical entry. Once these concerns are addressed, MNPs will move closer to clinical application, improving the diagnosis, treatment, and monitoring of our most unmanageable diseases.

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