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Research review paper

Tailored functionalization of iron oxide nanoparticles for MRI, drug delivery, magnetic separation and immobilization of biosubstances

Katerina Hola, Zdenka Markova, Giorgio Zoppellaro, Jiri Tucek, Radek Zboril *

Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry, Faculty of Science, Palacky University, 17. listopadu 1192/12, 771 46 Olomouc, Czech Republic

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ABSTRACT

In this critical review, we outline various covalent and non-covalent approaches for the functionalization of iron oxide nanoparticles (IONPs). Tuning the surface chemistry and design of magnetic nanoparticles are described in relation to their applicability in advanced medical technologies and biotechnologies including magnetic resonance imaging (MRI) contrast agents, targeted drug delivery, magnetic separations and immobilizations of proteins, enzymes, antibodies, targeting agents and other biosubstances. We review synthetic strategies for the controlled preparation of IONPs modified with frequently used functional groups including amine, carboxyl and hydroxyl groups as well as the preparation of IONPs functionalized with other species, e.g., epoxy, thiol, alkane, azide, and alkyne groups. Three main coupling strategies for linking IONPs with active agents are presented: (i) chemical modification of amine groups on the surface of IONPs, (ii) chemical modification of bioactive substances (e.g. with fluorescent dyes), and (iii) the activation of carboxyl groups mainly for enzyme immobilization. Applications for drug delivery using click chemistry linking or biodegradable bonds are compared to non-covalent methods based on polymer modified condensed magnetic nanoclusters. Among many challenges, we highlight the specific surface engineering allowing both therapeutic and diagnostic applications (theranostics) of IONPs and magnetic/metallic hybrid nanostructures possessing a huge potential in biocatalysis, green chemistry, magnetic bioseparations and bioimaging.

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* Corresponding author. Tel.: +420 585634337; fax: +420 585634167.
E-mail address: radek.zboril@upol.cz (R. Zboril).

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1. Introduction — basic properties and synthetic strategies towards IONPs

The properties of iron oxide nanoparticles (IONPs) – a flexible and modular design combined with low toxicity – lend themselves to numerous technological applications in applied nanoscience (Gupta and Gupta, 2005a). The bottom-up engineering of IONPs generates excellent platforms in biomedical research for magnetically controllable drug delivery systems (Sun et al., 2008), biosensors and contrast agents for medical diagnostics (Xie et al., 2011). Research in biotechnology, the environmental sciences and green (environmentally friendly) chemistry has shown that IONPs' high magnetic response under an external magnetic field allows them to act as efficient carriers in the magnetic separation of various biosubstances or environmentally important species (e.g., proteins, low molecular weight agents, ions, inorganic or organic pollutants etc.), be tailored to work as separable and renewable nanocarriers for catalysis (Gawande et al., 2013) and be used as magnetically drivable platforms for enzyme immobilization (Ansari and Husain, 2012).

The six non-hydrated crystalline iron oxide phases identified so far (Cornell and Schwertmann, 2003) are most frequently classified according to the valence state of iron in their crystal structure. FeO, known mineralogically as wüstite, has only Fe^{2+} ions in its cubic crystal structure, and is thermodynamically unstable and paramagnetic at room temperature. On the other hand, Fe_2O_3, or ferric oxide, contains only Fe^{3+} ions and displays polymorphism, that is, the existence of further isochemical phases with different physical properties revealed by their different crystal structures. So far, four Fe_2O_3 polymorphs have been described (Machala et al., 2007, 2011; Tucek et al., 2010; Zboril et al., 2002): (i) α-Fe_2O_3, known mineralogically as hematite, with a rhombohedrally centered hexagonal crystal structure (space group $R\bar{3}c$); (ii) β-Fe_2O_3 with a cubic-body-centered crystal structure of a bixbyite type (space group $Ia\bar{3}$); (iii) γ-Fe_2O_3 with a cubic crystal structure of inverse spinel type (space group $Fd3m$); and (iv) ε-Fe_2O_3 with an orthorhombic crystal structure (space group $Pna2_1$). The last non-hydrated iron oxide phase, Fe_3O_4 (known mineralogically as magnetite) has both Fe^{2+} and Fe^{3+} ions distributed over a cubic inverse spinel crystal structure. However, only Fe_3O_4 and γ-Fe_2O_3 have been found to be functional and promising candidates in biomedical and biotechnological applications due to their favorable magnetic properties (Gupta and Gupta, 2005b; Laurent et al., 2008; Pankhurst et al., 2003; Tucek et al., 2014). They are both strong ferrimagnetic materials with two magnetic sublattices mirroring tetrahedral (T) and octahedral (O) sites, the two non-equivalent cation positions in their crystal lattice (Machala et al., 2011; Zboril et al., 2002). In stoichiometric Fe_3O_4, Fe^{3+} ions are distributed over all T-sites and half the O-sites whereas Fe^{2+} ions occupy the remaining O-sites leaving no vacant places. On the other hand, in stoichiometric γ-Fe_2O_3, Fe^{3+} ions are located at all the T-sites and 5/3 of O-sites; the remaining 1/3 of O-sites are left vacant (with virtual negative charge) to establish the neural charge in the γ-Fe_2O_3 crystal lattice. O-site vacancies can appear in a random manner, or be partially or fully ordered changing the crystal symmetry from high (cubic) to low (tetragonal). Replacing iron cations with foreign (non-iron) cations in the γ-Fe_2O_3 or Fe_3O_4 crystal structure alters the γ-Fe_2O_3 or Fe_3O_4 hysteresis parameters (magnetic hardness), thus modifying their magnetic properties and requirements for a given application. More importantly, if the size of γ-Fe_2O_3 or Fe_3O_4 nanoparticles falls below a certain threshold (usually below ~15 nm), they display superparamagnetism, a relaxation phenomenon associated with thermally activated spontaneous oscillations of nanoparticle superspin among the orientations along easy axes of magnetization energetically favored by nanoparticle magnetic anisotropy (Tucek et al., 2006). In a superparamagnetic regime (i.e., above a size-determined temperature), γ-Fe_2O_3 and Fe_3O_4 nanoparticles exhibit a remarkably high MRI contrast effect, are easily controlled by a magnetic field gradient, and display a strong magnetic response in a relatively low applied magnetic field (less than 1 T). Like all other iron oxide phases, γ-Fe_2O_3 and Fe_3O_4 are non-toxic (at low dosage), biodegradable and biocompatible, significantly raising their application potential in bio-related fields (Gupta and Gupta, 2005b).

For biomedical and biotechnological applications, the γ-Fe_2O_3 and Fe_3O_4 nanoparticle surface must be covered by a suitable biocompatible compound to (i) prevent the degradation of iron oxide nanoparticles in an unfriendly and aggressive environment (e.g., blood), (ii) suppress the aggregation of iron oxide nanoparticles by suppressing their magnetic interactions and (iii) provide functional groups for bioactive compound (e.g., drug) attachment (Gupta and Gupta, 2005b). The resulting inorganic/organic complex has a core/shell nanoarchitecture, which may be functionalized by adding various compounds. Added functions include prolonging the recognition time by the immune system or treating unhealthy cells and tissues. Such a functional complex has theranostic properties in that it can be used in both diagnostic and therapeutic procedures (e.g., MRI imaging, targeted drug delivery, cell labeling and separation, or magnetically-assisted hyperthermia) (Gupta and Gupta, 2005b).

The chemistry of a core-shell IONP's magnetic core and surface dictates its function and working environments (Veiseh et al., 2010). A functionalization of the surface enables a fine modulation of IONP behavior in solution, such as colloidal stability, pH response, overall toxicity and proclivity to bind and transport active substances. The design of the magnetic core (chemical nature and crystal lattice), on the other hand, allows the nanoparticle's magnetic response (ferrimagnetic/superparamagnetic, magnetic hardness) to be adjusted by modifying the core size and chemical composition (e.g., upon using admixture of other metals diverse from Fe). It is not a surprise that in the last 20 years, a tremendous research effort has been devoted towards the exploration and the development of different routes for controlling the chemical and physical properties of IONPs.

Thus, many synthetic routes for the preparation of IONPs of various shapes and morphologies have been described. These synthetic approaches can be shortly classified as (i) synthesis in constrained-environments, (ii) hydrothermal synthesis, (iii) sol–gel reactions, (iv) flow injections, (v) microwave assisted processes, and (vi) co-precipitation processes. In addition to the solvent or thermal degradation-based methods, IONPs can be also obtained via biomineralization in living organisms from magnetotactic bacteria (Blakemore, 1975; Markova et al., 2012a). However, the most commonly used synthetic procedures rely on the co-precipitation processes of IONPs from suitable admixtures of metal salts, a method first reported by Massart (1981), the thermal decomposition of iron precursors in organic solvents (Fang et al., 2009; Gautier et al., 2013), and solid-state thermal decompositions of iron precursors under controlled conditions (Machala et al., 2011; Zboril et al., 2004).

A graphical overview that collects several types of IONP architectures with different core/shell arrangements is depicted in Fig. 1, examples that help to illustrate the tremendous creativity-driven-design beneath this field of research. The size of IONPs can vary from a few nm up to several hundred nm in some condensed IONP assemblies and they also exhibit diverse structural shapes. The ultrasmall superparamagnetic iron oxide nanoparticles, called USPIOS, usually exhibit a spherical shape and small size, from 4 to 13 nm (Park et al., 2005), whereas nanoclusters obtained from condensed IONPs (Bakandritsos et al., 2010; Kim et al., 2008; Zoppellaro et al., 2014) are much larger (>50 nm), but can also adopt a nearly spherical morphology. Apart from the spherical motif, other IONP shapes have been described such as iron oxide nanocubes (Guardia et al., 2012; Lee et al., 2011), tetrahedral nanoparticles (Arndt et al., 2014), magnetic nanostars (Li et al., 2014), and nanorods (Xiao et al., 2012). The chemical behavior of IONPs is determined and driven not only by their shape and size but also by their surface passivation, which regulates the hydrodynamic diameter, solvent dispersability, surface charge, colloidal

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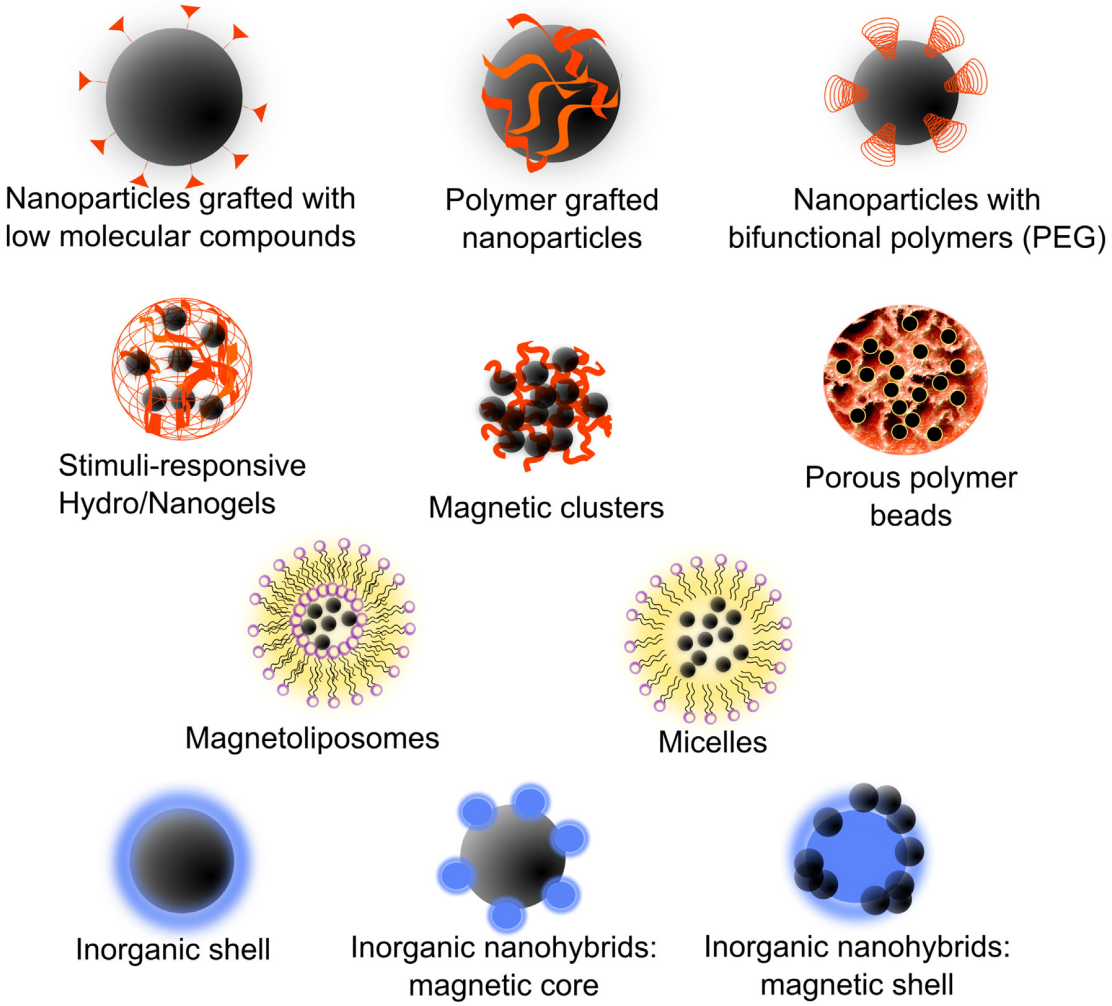

Fig. 1. General types of IONP arrangements with polymers, molecules and inorganic nanoparticles. Nanoparticles/hybrids are not depicted to scale.

stability or the speed of response to an external magnetic field. Further IONP characteristics and various surface shells are summarized and discussed in the next section.

Herein, we provide an overview of the physical and morphological IONP characteristics that dictate their applications in biological environments, soil or water. We illustrate the interplay of IONP design (size, shape, composition, and surface chemistry) that allows the assembly of versatile and truly multifunctional magnetic platforms. Most importantly, we critically discuss the principal requirements of IONP properties, their surface chemistry, and conjugation methods used in selected bioapplications, namely MRI contrast agents, drug delivery, magnetic separation and immobilization of enzymes and other biosubstances. The conjugation routes and applications potential of IONPs/metal hybrids are also highlighted.

2. Principles and specific requirements for applications of IONPs in biomedicine and biotechnologies

IONPs have found their application in various fields of biomedicine and biotechnology. Owing to their small size, they are an ideal platform for use as nanocarriers in drug delivery. A further advantage of IONPs is that due to their magnetic properties, mainly superparamagnetism, they can be used as contrast agents for nuclear magnetic resonance imaging (MRI). Their magnetic response on the external magnetic field can be even used for local overheating of cancer tissues known as hyperthermia therapy. Among these biomedical applications, magnetic properties of IONPs can be also fully explored in separation techniques of various (bio)substances or pollutants. Magnetic separation attracts a big scientific interest mainly because of its speed and effectiveness. This is also a reason why IONPs have been employed as a support for enzyme immobilization. The renewable magnetic-enzyme platform is very promising for various industrial applications and even proteomics. The overview of utilization of IONPs is depicted in Fig. 2. In the following subchapters, all of the mentioned areas of IONPs use will be addressed in greater details.

2.1. Contrast agents in MRI

MRI is a powerful and non-invasive technique used in diagnostics, characterized by ultrahigh spatial resolution based on the detection of proton relaxation in an external magnetic field. The relaxation and the enhancement of the signal/contrast can be increased with the aid of contrast agent material. Those based on superparamagnetic iron oxides (SPIOs) are able to substantially alter the spin–spin relaxation of water molecules (T2 relaxation) near the magnetic nanoparticle and to enhance the negative contrast of the image (Wang et al., 2001). The contrast properties can also be influenced by the size and hydrophilicity of the nanoparticles (Duan et al., 2008) or by the thickness of the surface shell (LaConte et al., 2007; Veiseh et al., 2010). The water relaxivity can also be enhanced when low molecular-weight compounds used in the

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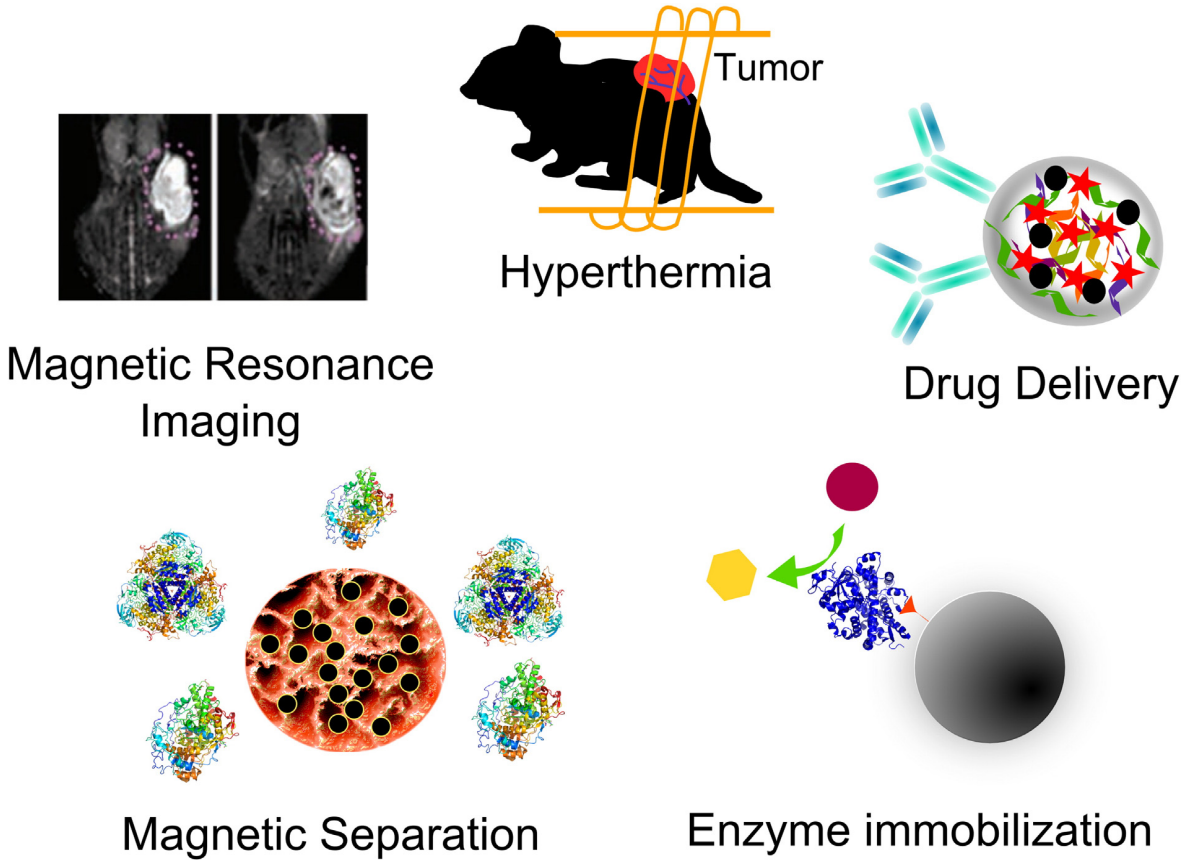

Fig. 2. Schematic representation of biomedical and biotechnological applications of IONPs. The image for magnetic resonance imaging was reprinted with permission from Yang et al. (2009).

IONP shell encode π-conjugation paths that allow an effective spin transfer from the magnetic core to the surrounding water (Maity et al., 2012).

Two general types of negative contrast agents (CAs) based on IONPs can be found in literature; the peroral CAs for imaging of digestive system (Kluchova et al., 2009) and CAs used through intravenous administrations. The key characteristics of peroral CAs are their low toxicity combined with the good stability to withstand the acidic environment of the stomach (Kluchova et al., 2009). The precise design of the surface coating materials is not so essential for peroral CA preparations. In contrast, IONPs used for intravenous administration are much more challenging to design.

The basic requirements for the application of IONPs as intravenous CA in MRI are: (i) low toxicity, (ii) colloidal stability at physiological conditions (37 °C, PBS buffer, pH = 7.4, 0.154 M ionic strength) (Qiao et al., 2009; Veiseh et al., 2010), (iii) low and stable hydrodynamic diameter in plasma, (iv) little proclivity towards aggregation, (v) well controlled surface charges (Chapman et al., 2013), and (vi) low nonspecific protein adsorption (Nie, 2010). All these variables determine the nanoparticle's biodistribution and its ability to overcome biological barriers in vivo (Chapman et al., 2013; Veiseh et al., 2010). In general, nanoparticles with hydrodynamic sizes of up to 5–8 nm (similar to the size of 30–50 kDa serum proteins) and low molecular weight contrast agents can undergo renal excretion (Choi et al., 2007; Liu et al., 2013; Longmire et al., 2012; Nie, 2010). IONPs below 5 nm are quite unusual (Wang et al., 2014) and they are much more prone to extravasation processes, thus are cleared very fast. The majority of functional IONPs have larger hydrodynamic diameters and are captured by the reticuloendothelial system (RES, e.g., in liver and spleen) (Weissleder et al., 1989). This is also the reason why IONPs were originally used for imaging and detection of hepatic (Weissleder et al., 1987) and splenic tumors (Weissleder et al., 1988). Nanoparticles from 20/30 nm up to 100/150 nm (Elsabahy and Wooley, 2012; Veiseh et al., 2010) can also accumulate in the stomach (Banerjee et al., 2002), bones and kidneys (Lee et al., 2010). However, these medium size nanoparticles have a high potential for cancer imaging as well as for drug delivery, due to their prolonged circulation time in blood vessels and their tendency to passively target tumors due to the Enhanced Permeation and Retention (EPR) effect of solid tumors (Elsabahy and Wooley, 2012; Tanaka et al., 2004). These medium-sized particles can escape the renal clearance and are not so big as to be immediately opsonized (coated with serum proteins) and captured by the RES. Another important parameter for avoiding a fast opsonization of the IONPs is the surface charge of the nanoparticle. Neutral nanoparticles with coats containing hydrophilic polymers, for example polyethylene glycol or dextran (Hong et al., 2008), do not strongly interact with opsonin proteins such as albumins, immunoglobulins, fibronectins, and complement proteins (Nie, 2010; Roser et al., 1998). The IONP's surface charge can be reduced by assembling complex surface architectures combining two types of polymeric coating (e.g., polyethylene glycol branched chitosan (Veiseh et al., 2009) or polyacrylic acid with polyethylene glycol (Pothayee et al., 2013)). In contrast, positively or negatively charged IONPs strongly interact with plasma proteins and are rapidly removed from the bloodstream by macrophages, a process activated by the enhanced stimulation of the immune system (Dobrovolskaia and McNeil, 2007).

The advantage of using IONPs as contrast agents over other types of metal oxide is their excellent biocompatibility, even if they end up localized in the spleen or liver and are metabolically degraded (Jain et al., 2008). In vitro studies show that IONP surface coverage strongly affects its cytotoxicity, cellular uptake, and mechanism of endocytosis (Gupta

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and Gupta, 2005a). Coated IONPs exhibit much lower cytotoxicity and are almost nontoxic in comparison with the particles without surface coverage. Nevertheless, it has been shown that the IC50 of IONPs without surface coverage was around 0.5 mg/mL, which is still better than the values reported for other nanomaterials used for biomedical applications (Lewinski et al., 2008). The particles without surface coverage can, e.g., determine the organization of the cytoskeleton, ability of cells to adhesion (Gupta and Gupta, 2005a) or the kinetics of their biological degradation (Lartigue et al., 2013). The cytotoxicity of IONP degradation in a biological environment also needs to be taken into account. IONP degradation follows, in general, the path of lysosomal digestion (Lartigue et al., 2013). Lysosomal enzymes degrade nanoparticles with a simultaneous release of iron cations. The kinetics of degradation is strongly determined by the "active" surface of IONPs in terms of enzyme recognition, the surface polymer's hydrophilicity or the ability of particles not to aggregate in the lysosomes (Lartigue et al., 2013). The release of iron cations can potentially promote severe oxidative reactions in cells. However, iron homeostasis is highly regulated and excess iron is quickly stored in ferritin proteins (Lartigue et al., 2013), one of the enzymatic systems that help to control the iron level in the body (Jain et al., 2008).

The effective application of IONPs for MRI imaging and preventive medicine is also associated to their ability of an early detection of cancers in various organs, following their preferential localization in such areas due to the EPR effect. While the passive targeting has proven to be an effective tool for the early diagnostic, the active tumor targeting approach became in recent years one of the leading strategies that directed the IONP chemical design. In this context, the surface engineering of functional IONPs can be augmented by specific cancer markers, such as antibodies and glycoproteins (Tanaka et al., 2004). These bioactive compounds are usually immobilized onto IONPs in a late synthetic step, via coupling reactions following an initial coating of the nanoparticle surface with polymeric materials that contain functional groups responsive to the coupling. An overview of polymers/molecules for coating IONP systems suitable for MRI is presented in Fig. 3. Coupling methods for immobilizing targeting compounds are discussed in the third part of this review.

2.2. Targeted drug delivery

Drug delivery refers to pharmaceutical approaches to transporting drugs to a desired place in the body. Nanotechnology is used mostly for intravenous delivery, in particular, to cancer tissues. The goals of targeted drug delivery are to reduce the toxic dose for non-cancerous tissues and increase the therapeutic effect of the drug in the desirable places. Nanoparticles are the ideal candidates for drug delivery because they do not only act as a nanocarrier for passive tumor targeting but can be coated for active tumor targeting and, in the case of IONPs, can be delivered to cancer tissues by magnetic targeting (Chertok et al., 2008).

IONP systems have the potential to act as magnetic nanocarriers because IONP surfaces may be coated with diverse organic polymers (see Fig. 3) that, in turn, are capable of interacting with or binding pharmaceutically active molecules. Such properties create biomedical applications for IONPs, especially in cancer therapies.

Morphological differences in the physical surroundings of healthy and cancer tissues gives rise to a selective penetration by IONPs of

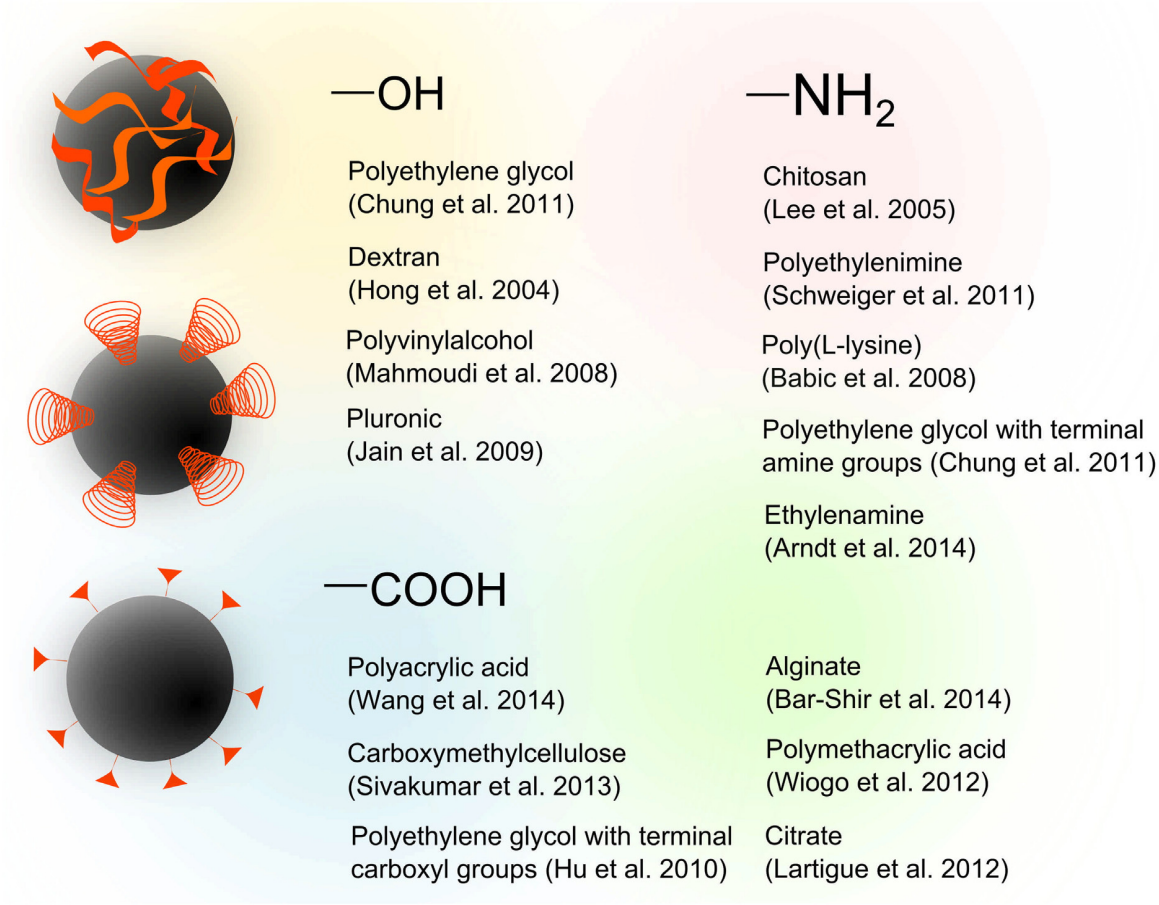

Fig. 3. Polymers and organic molecules (sorted by functional group) typically used for primary coating and stabilization of IONPs for MRI. The appropriate coating/functionalization method can be found in given literature (Arndt et al., 2014; Babic et al., 2008; Bar-Shir et al., 2014; Chung et al., 2011; Hong et al., 2004; Hu et al., 2010; Jain et al., 2009; Lartigue et al., 2012; Lee et al., 2005; Mahmoudi et al., 2008; Schweiger et al., 2011; Sivakumar et al., 2013; Wang et al., 2014; Wiogo et al., 2012).

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tumor regions. Solid tumors are usually characterized by a compromised leaky vasculature that promotes extravasation of the IONPs from the blood and their subsequent retention in the tumor interstitium. In contrast, the densely packed endothelial cells in healthy-tissue vessels form effective barriers that prevent IONP extravasation. Selective penetration can be combined with magnetic gated-delivery of IONPs (Cole et al., 2011).

The therapeutic effect can then be triggered by local overheating of the cancer cells via hyperthermia (Guardia et al., 2012) and/or upon the IONP's gated-delivery of cancer drugs. The hyperthermia effect, namely, the ability of magnetic nanoparticles to adsorb alternating current (AC) energy and to convert it into heat, although very promising as a therapeutical approach, may suffer from difficulties in controlling the effect of overheating effects in both the tumor region and its healthy surroundings. If local overheating by hyperthermia is in the temperature range from 41 to 46 °C, the cancer cells undergo apoptosis, programmed cell death caused by thermal stress (Deatsch and Evans, 2014; Kumar and Mohammad, 2011). Higher temperatures can cause dangerous necrosis or coagulation of tissues often associated with inflammation (Gnant et al., 2000). Against this background, IONPs used for cancer drug delivery may be safer, although a combination of drug-delivery and hyperthermia may provide the ultimate solution for therapy.

For this reason, various synthetic strategies have been explored to obtain drug–IONP conjugates where the triggered release can benefit from the heating of IONPs. Various drugs have been successfully incorporated into IONPs to achieve these properties, such as paclitaxel, doxorubicin and methotrexate, photoactivated agents and even radiochemicals.

A variety of methods are used to attach a drug to the IONPs. The drug molecule can be loaded in the polymer interspace of magnetic nanoclusters (Bakandritsos et al., 2012), encapsulated in stimuli responsive hydrogel/polymer frameworks (Jaiswal et al., 2014), linked to the activated surface of IONPs (Magro et al., 2014) or trapped in magnetoliposomes (Tai et al., 2009). In addition, drugs can be connected to the IONP's organic canopy by covalent linkages that are susceptible to degradation (e.g., by hydrolysis) as part of the cell's metabolism (see Fig. 4).

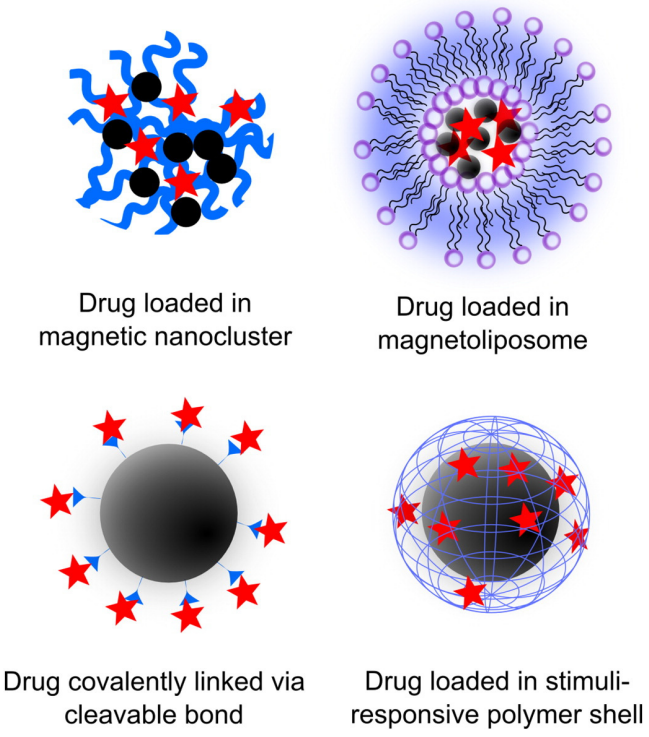

Fig. 4. Schematic representation of various IONP–drug nanosystems for targeted magnetic drug delivery.

The in vivo kinetics of drug release is a key factor that can be tailored by successful drug–IONP conjugate design. Drug release can be induced not only by external stimuli such as a local temperature increase but also by low pH, typical for cancer tissues. Tai et al. (2009) reported the preparation of thermosensitive magnetoliposomes that were able to release the loaded compounds after exposure to alternating magnetic field and following a local temperature increase. Another example of controlled drug (doxorubicin) release by magnetic IONPs has been described in condensed IONP nanoclusters containing alginate chains as polymer-coronas (Zoppellaro et al., 2014). These clusters exhibited very high loading capacity of anticancer drug (doxorubicin, 26% in weight) and were able to release almost 70% of the loaded drug within 5 min after exposure to an alternating magnetic field. A similar study was performed by Deka et al. (2011), using IONPs in the form of nanobeads that were surface-functionalized with thermoresponsive N-isopropylacrylamide (PNIPAM). Such systems were capable of loading doxorubicin molecules up to 45% in weight. In this case, the drug release topped 85% after 110 h at 37 °C. It is important to note that a slower sustained release of the drug can sometimes be considered a therapeutical advantage, since it may mitigate several of the side-effects typically observed in the patient undergoing the chemotherapy; in fact, Ponta and colleagues recently reported that a more effective cancer treatment can be obtained using a slower release of the active drug from micelles (Ponta and Bae, 2014). Another effective IONP drug-delivery therapy was reported by Lim and co-authors. In their study, the magnetic nanoparticles were encapsulated into a pH-responsive nanoparticle, conjugated with HER2/neu antibody (HER = Herceptin) and loaded with doxorubicin (Lim et al., 2011). The system exhibited an excellent selectivity for targeted tumor tissues. A nearly 100% release of the drug doxorubicin was observed in the tumor regions characterized by an acidic environment (pH = 5.5) over 5 days. In contrast, just 50% release of the drug was observed in healthy tissues where pH is nearly neutral (~7.4). As mentioned earlier, the drug can also be attached to the surface of IONPs by pH degradable bonds. Banerjee and colleagues reported the successful linkage of doxorubicin to IONPs coated with arabic gum via pH-sensitive hydrazone bonds (Banerjee and Chen, 2008). The loaded doxorubicin (80%) was found to be released fast, within 4 h, when the environmental pH was made acidic enough (pH = 5.0). From these examples, the research on drug delivery is well on its way to generate highly tailored IONPs; as such, they will provide in the close future better plasma pharmacokinetics, tumor selectivity and, in general, a truly personalized platform for medical therapies.

2.3. Magnetic separation

Another highly effective technological application of IONPs is their use in the magnetic separation processes of various substances. Suitably modified IONPs efficiently separate ions (Liu et al., 2009), pollutants (Ambashta and Sillanpää, 2010), cells (Borlido et al., 2013), proteins/antibodies (Heebøll-Nielsen et al., 2004; Safarik and Safarikova, 2004), and various bioactive substances (Müller-Schulte and Brunner, 1995).

The advantages of using magnetic separation techniques in comparison to more conventional procedures such as chromatography, are their (i) high versatility, (ii) the possibility to work without pre-treatment of the active material even in heterogeneous crude media, (iii) fast, highly cost-effective and straightforward methodology, and (iv) the reusability of sorbents after the magnetic separation. Today, several ready-to-use magnetic sorbents are available commercially, some containing an activation reagent for the immobilization of various substances, such as magnetic Sepharose®, Dynabeads® and Chemicell®. Two types of magnetic sorbents have been reported, (i) IONPs coated with specific functional groups and

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(ii) IONPs encapsulated in the porous matrix of a polymer bead (polymer microsphere). The separation of different substances can be finely tailored by the synthetic design for use in processes such as ion-exchange, hydrophobic interactions, affinity adsorptions, and even specific interaction through antibody–antigen interactions. Fig. 5 shows these two types of architectures in IONPs used in magnetic separation processes.

From the more general perspective, the key requirement for magnetic sorbents is a large amount of functional groups per mass of magnetic material in combination with an appreciable saturation magnetization value (Franzreb et al., 2006). These properties are pivotal for obtaining both high binding proclivity for the target substances and a fast magnetic response of the composite. Magnetic microspheres (Fig. 5a) fulfill these requirements by caging a high content of magnetic material and having a porous polymer framework. Similarly, core-shell magnetic nanoparticles (Fig. 5b) have both a high surface area and the ability to incorporate a large amount of functional groups in the polymeric or inorganic shell.

Magnetic microspheres are usually prepared by adding IONPs to the polymer solution during formation (spraying or microemulsion method) of the polymer bead (Yang et al., 2008). IONPs can also be synthesized within the pores of the polymer microspheres in situ (Luo et al., 2009). The choice of polymer coating material is dictated by the type of compound that needs to be separated. An overview of the synthesis techniques used for surface modification of IONPs is given in the third section of this review. Krizova and colleagues prepared magnetic microspheres from methacrylates with low porosity and a carboxylic acid functional group for the separation of genomic DNA (Krizova et al., 2005). In the case of proteins, separation can be achieved by using highly porous microspheres coated with hydrophilic polymers such as cellulose (Safarik et al., 2007), agarose (Zhang et al., 1999) or poly(vinylalcohol) (Yang et al., 2008). The hydrophilicity of these polymers prevents any denaturation of the proteins. Other types of magnetic microsphere include the ion-exchange resins. These composites are suitable for separation of ions and low molecular organic substances and are usually composed of microspheres prepared by the polymerization of styrene in the presence of IONPs (Lee et al., 2003).

In addition to the separation methods based on specific interactions, IONPs can also be used for non-specific sorption such as the sorption of heavy metals (Hua et al., 2012). This separation is partly based on electrostatic interaction but, more importantly, uses the large surface of IONPs (Xu et al., 2012). Hu and colleagues used IONPs for the removal of hexavalent chromium. The particles had a surface area of 178 $m^2 g^{-1}$ and a very high sorption capacity for chromium amounting to 19.2 mg g^{-1} (Hu et al., 2005). Similarly, superparamagnetic IONPs are highly efficient for arsenate removal from an aqueous environment (Kilianova et al., 2013). Apparently, even IONPs without specific surface coverage can be used to separate or remove pollutants in waste water through non-specific interactions.

2.4. Application of IONPs conjugated with enzymes

Magnetic nanosystems combining enzymes and IONPs via biopolymer immobilization are another fast expanding area of biotechnology research. Such nanocomposites are potentially useful to the food industry, in the form of food processing units. As renewable "bio-catalysts", they can also be implemented for the greener generation of biofuels (Ansari and Husain, 2012). There are several technological advantages to immobilize enzymes onto IONPs. Above all, the magnetic recovery of the active material decreases the cost of production. Furthermore, immobilized enzymes often exhibit better temperature stability, pH stability and/or higher activity compared to unsupported proteins (Khoshnevisan et al., 2011; Pecova et al., 2013).

An example of a highly cost-effective industrial implementation of enzyme–IONP conjugates can be found in the immobilization of the enzyme lactase onto IONPs. The conjugate is used to deplete milk of lactose for use by lactose intolerant individuals (Talbert and Goddard, 2013). Another potential application of enzyme–IONP conjugates is the green synthesis of cellulosic ethanol. The degradation of cellulose into glucose by cellulase is, however, not yet as cost-effective as the production of ethanol from glucose in yeast, but such a platform may enable a further development of the process (Jordan et al., 2011).

The conjugation of enzymes to IONPs can be achieved by various synthetic approaches and an overview of strategies is provided in Section 3. In general, the enzyme can be attached to the magnetic polymer microspheres or simply to the magnetic nanoparticles that contain suitable surface functionalities (e.g., amino groups, thiol moieties, carboxylate residues). Compared to antibody–IONPs for drug delivery, there is no strict requirement for colloidal stability of the enzyme–IONP system because of the needed faster response of IONP aggregates to an external magnetic field. Importantly, the enzymatic activity in the supported nanocomposite must be carefully preserved.

Enzyme–IONP conjugates can also be used in other applications such as biosensing or proteomics. For example, trypsin conjugated IONPs are regarded as a promising platform for proteomic analysis (Lin et al., 2008). In this case, immobilization of trypsin onto magnetic nanoparticles decreases autolysis of the enzyme and increases its re-usability (Lin et al., 2008; Pecova et al., 2013). Trypsin's conjugation with IONPs even

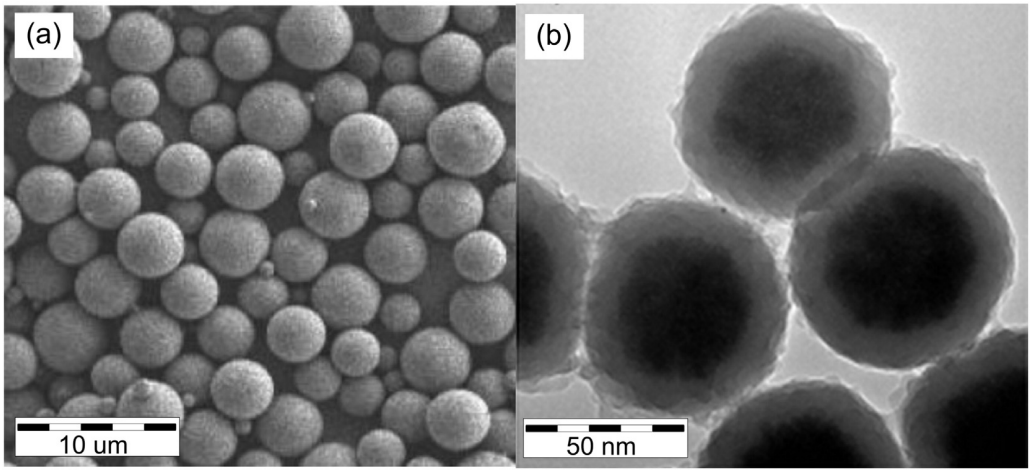

Fig. 5. Examples of sorbents for magnetic separation. (a) Magnetic microspheres and (b) magnetic nanoparticles with surface coating; (a) scanning electron microscope image of porous microspheres from poly(vinylalcohol) with IONPs encapsulated in the matrix (reprinted with permission from (Yang et al., 2008)). (b) Transmission electron microscope micrograph of superparamagnetic IONPs with polymer-silica shell (reprinted with permission from (Fang et al., 2010b)).

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improves its thermal stability such that trypsin-induced digestion of various proteins can take place at a higher temperature. Enzyme–IONP conjugates have also been used for biosensing, enabling the detection of various analytes (gases, glucose, drugs, cells, metabolic products, contaminants, etc.). The various biosensing systems based on IONPs have been excellently reviewed elsewhere (Xu and Wang, 2012).

2.5. Applications of magnetic/metallic hybrid nanostructures

Another class of magnetic nanostructure is the IONP/metal hybrid nanostructure, which can be obtained by merging two or more nanometer-scale components, allowing complex technological features to exist in a single unit. Such materials can be multifunctional (magnetic, electronic, optical), since the functionality of each component can be fully integrated into the merged unit. The different components' material and size parameters can be independently optimized in order to fine-tune their function or to induce synergistic phenomena (Zeng and Sun, 2008).

The IONP/metal nanocomposites have demonstrated the huge application potential and real possibility to obtain a large variety of catalytic, magnetic, electric, optical, and plasmonic properties. Of all the IONP/metal nanohybrids, those combining IONPs with noble metals (mainly silver, gold, and palladium) represent the most promising superstructures with a versatile portfolio of applications.

IONP/Ag nanocomposites are successfully used as hydrogen peroxide sensors in electrochemistry. They are also used as substrates in magnetically assisted surface enhanced Raman scattering (SERS) for the chemical analysis of organic species, bioactive substances or toxic molecules with record detection limits (Choi et al., 2010; Han et al., 2013; Ranc et al., 2014). Moreover, IONP/Ag hybrid nanostructures also exhibit antibacterial, antifungal and even antiviral properties and they are utilized in antimicrobial targeting (Chudasama et al., 2011; Nangmenyi et al., 2011; Prucek et al., 2011).

The IONP/Au nanohybrids can serve as multimodal contrast agents for MRI and CT bioimaging (Narayanan et al., 2012). Furthermore, they can be used in bioseparation and immunoassays based on surface-enhanced Raman spectroscopy at favorably low detection levels (Bao et al., 2009; Ranc et al., 2014). The magnetic-gold hybrids possess a highly tunable plasmon resonance, a property that may be exploited in photothermal therapy for selective destruction of target carcinoma cells (Salgueirino-Maceira et al., 2006).

IONP/Au hybrid nanostructures are also being used as magnetically separable catalysts. Gawande and colleagues demonstrated that highly tuneable catalytic processes can be obtained from magnetite–gold nanoparticles in oxidative esterification and hydrogen transfer reactions (Gawande et al., 2013, 2014). Nanocatalysts based on magnetite and palladium nanoparticles were successfully applied in the Buchwald–Hartwing reactions for arylation of amines and amides (Sa et al., 2014). Similarly, palladium-supported magnetic materials have successfully catalyzed the hydrodechlorination of the chlorohydrocarbons trichloroethene or chlorobenzene, as described by Hildebrand et al. (2009).

Several other mixed metal composites have been reported, such as magnetite–nickel (Costa et al., 2014), magnetite–platinum (Wu et al., 2012) or magnetite–ruthenium (Baig and Varma, 2014) nanocatalysts. These are but a few applications of magnetic/metallic hybrid nanostructures for catalysis, a popular research topic for material chemists in recent years. The wide range of applications for IONP/noble metal hybrids is illustrated in Fig. 6.

3. Surface design of magnetic nanoparticles

The manner in which active substances/groups/nanomaterials are immobilized onto the surface of IONPs is pivotal to their function. Primary functional groups prevent nanoparticle aggregation and provide suitable binding sites for further conjugation with biosubstances. Usually, primary functional groups can be attached to IONPs via adsorbed polymer/small molecules or covalently linked to the particle via polymerized silane structures with an attached functional group (e.g., amine group via APTES 3-aminopropyltriethoxysilane). Typical functional groups used for primary functionalization are hydroxyl groups (e.g., polysaccharides, PEG), amine groups (e.g., chitosan, polyethylenimine), and carboxylic groups (e.g., polyacrylic acid, alginate, citrate). Other types of functional groups such as epoxy, sulfhydryl (thiol), alkyne, azide, and carbonyl are very often used for specific conjugation methods such as "click" chemistry. The synthetic strategies used for functionalization of IONPs with various types of functional groups are depicted in Fig. 7.

3.1. Immobilization of proteins, enzymes, antibodies and targeting agents

The suitable synthetic strategy for immobilizing active substances ("vectorization") is crucial in IONP design. Many conjugation techniques exist, each with specific characteristics and requirements. Immobilization should neither affect the active agent's recognition site nor the required IONP characteristics (e.g., colloidal stability for drug delivery). Proteins, enzymes and even antibodies contain multiple functional groups such as amine, carboxyl, thiol (sulfhydryl) or hydroxyl groups, in the case of glycoproteins. In general, any of these functional groups can be used for attachment to the IONP surface. The optimal conjugation strategy must also take the number of particular groups into account.

There are three approaches for covalently conjugating IONPs with bioactive substances (antibodies, peptides, fluorescent dyes, molecules expressed by cancer cells). The first involves a chemical modification of the functional group on the IONP's surface followed by its attachment to the specified bioactive substance's functional group. The second approach uses an activation of the IONP's functional groups and the third strategy relies on a chemical modification of the bioactive substance.

The modification of suitable IONP functional groups (mainly the amine group) is a very efficient strategy for immobilization of proteins, enzymes and antibodies with thiol functional groups (Laurent et al., 2008; Veiseh et al., 2010). The advantage of this strategy is the selectivity of the covalent bonds and the reduced probability of aggregation for proteins with few thiol groups. Several examples of the chemical modification of IONPs (with maleimide, pyridyl disulfide, iodoacetate and hydrazine) for the attachment of thiol-active compounds are given in Fig. 8.

Another way of chemically modifying IONPs is to attach bifunctional aldehydes (e.g., glutaraldehyde) to their amine groups (Kluchova et al., 2009). Thus, aldehydes are used to immobilize another amine containing compound. However, the formed condensed Schiff-base is not stable in acidic pH and should be stabilized via a reducing agent such as cyanoborohydride. This conjugation technique is not very selective due to the high amount of amine groups in proteins and is suitable mainly for enzyme immobilization where colloidal stability is not necessary. However, the slightly modified approach based on condensing a Schiff-base can be used to immobilize antibodies for drug delivery. Pereira and colleagues treated the antibody with sodium periodate, which oxidized the minor sugar units of the antibody into the aldehyde functional group (Pereira and Lai, 2008). The aldehyde functional groups were next coupled with a hydrazine bond that was covalently attached to particles via adipic acid dihydrazide (see Fig. 8d) (Pereira and Lai, 2008).

Bioactive substances may also be attached by activating an IONP's functional group. This strategy is used mainly for immobilizing small molecules onto carboxylic group-containing IONPs. The carboxylic group is activated to be more reactive for the covalent attachment of various substances. Compared to the chemical modification described above, the activation agent does not become part of the final conjugate. Carboxylic acid can be activated via a reaction with thionyl chloride followed by coupling with a hydroxyl group (McCarthy and Weissleder, 2008). However, this strategy is not suitable for

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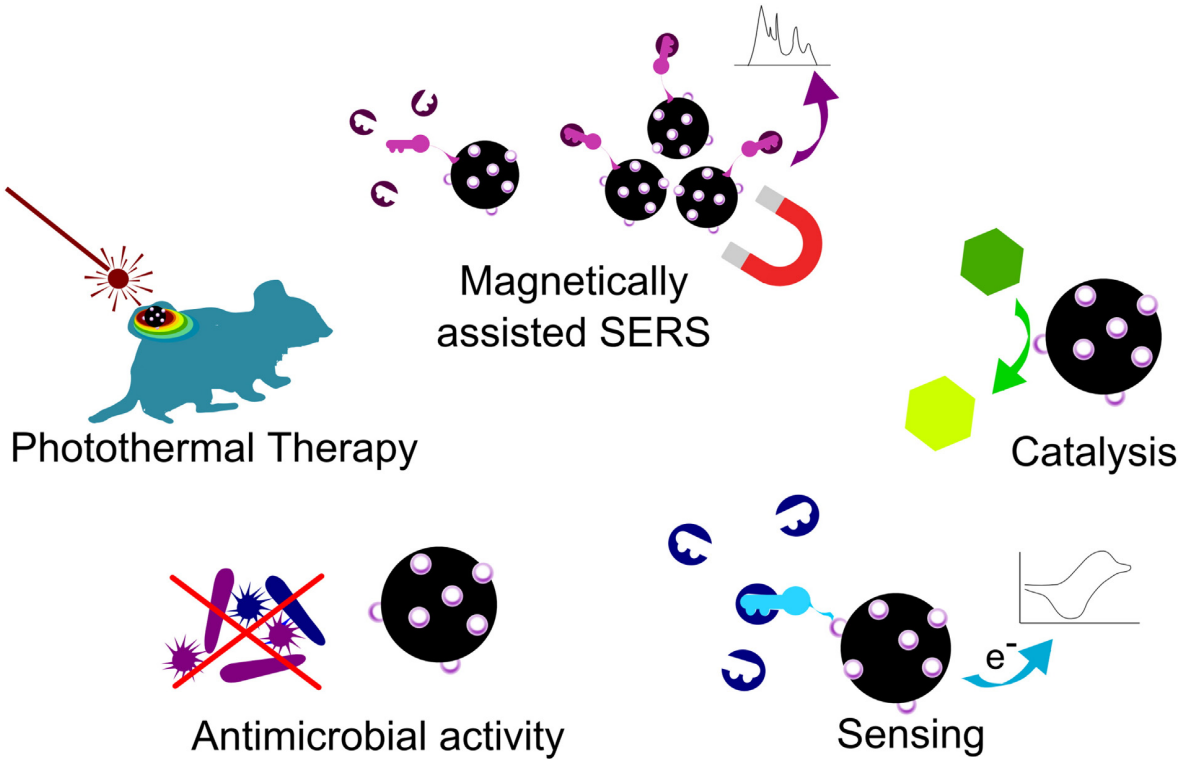

Fig. 6. Schematic illustration of various applications of IONP/noble metal hybrids.

immobilizing antibodies or enzymes because the reaction has to be performed under anhydrous conditions, e.g., in DMSO (Aksoy et al., 1998; McCarthy and Weissleder, 2008). Another example of this conjugation strategy is the activation of the carboxyl group via a reaction with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxosuccinimide) (Veiseh et al., 2010). This activation is suitable mainly for small molecules containing one amine group or for enzyme immobilization because of the possible aggregation of IONPs via crosslinking of the particles (not suitable for immobilization of proteins/antibodies to IONPs for biomedical applications).

The direct modification of bioactive substances is another method used for their conjugation with IONPs (see Fig. 8). Some compounds such as fluorescent dyes are commercially available in the activated form, e.g., fluorescein isothiocyanate or rhodamine B isothiocyanate. Such compounds may be used for reactions with amine groups, aldehydes or even polysaccharides modifying the surface of IONPs. The main conjugation possibilities are depicted in Fig. 8.

"Click" chemistry, as shown in Fig. 9, is a special conjugation strategy. The alkyne/azide and thiol/alkene groups can be attached to bioactive substances using the same strategies summarized in Fig. 7. The key advantages of this conjugation strategy include high yield and selectivity, and also good physiological stability. The possibility of perfectly controlling the conjugation route makes "click" chemistry one of the most powerful tools for surface nanoengineering (Nandivada et al., 2007). Finally, the above mentioned linking techniques represent an overview of the basic principles of conjugation chemistry and many other conjugation alternatives exist (Biju, 2014).

3.2. Biodegradable bonds for targeted drug delivery

One of the challenges in drug delivery is to design nanocarriers that release the attached drug in the desired place. In addition to the non-covalent techniques discussed in Section 2.2, the most promising nanosystems for this purpose consist of nanoparticles with a drug covalently attached via biologically degradable or cleavable bonds.

Several types of biologically cleavable bonds exist: the hydrazone bond that is cleavable at low pH (cancer tissue, lysosomal environment), the S–S bond that is enzymatically degradable by thiol reductase in the lysosomes (Arunachalam et al., 2000), the peptide sequence Gly–Phe–Leu–Gly cleavable by lysosomal cathepsin (proteinase) (Hanessian et al., 2008) or the biologically cleavable ester bond that has been used for linking drugs to carbon nanotubes (Liu et al., 2008) or polymers (Kopecek and Kopeckova, 2011). An overview of these bonds is given in Fig. 9.

Although enzymatically cleavable bonds are highly promising they have not yet been fully explored for use with IONPs. The main advantage lies in their selectivity for drug release. They can be degraded (with the drug being released) just after internalization into the cell. By comparison, pH-triggered drug release is less selective because the pH differences between the bloodstream and the lysosomal environment are not so dramatic and can cause unwanted slow drug release in the bloodstream or a slow hydrolysis in the lysosomes (Kopecek and Kopeckova, 2011).

3.3. Functionalization for magnetic separation

Magnetic separations based on IONPs offer enormous variability in applications and even in preparation techniques of magnetic sorbent. IONPs can be used to separate cells via a conjugated antibody (Borlido et al., 2013) as well as separate ions via electrostatic interaction. The antibodies are attached to the magnetic sorbent using methods similar to those mentioned in the previous section. Magnetic separation is also used to isolate antibodies from crude media. For example, boronic acid can capture antibodies via the formation of a bond with the antibodies' cis-diol (glycol) units (Borlido et al., 2011). The conjugation of boronic acid to IONPs can be performed, for example, by thiol-en click chemistry (Zhang et al., 2014a, 2014b).

The functionalization of magnetic microspheres with typical ion-exchange separation groups such as diethylaminoethyl (DEAE), carboxymethyl (CM), sulfopropyl (SP) and hydrophobic groups (pentyl, octyl) can be performed in a manner similar to the typical

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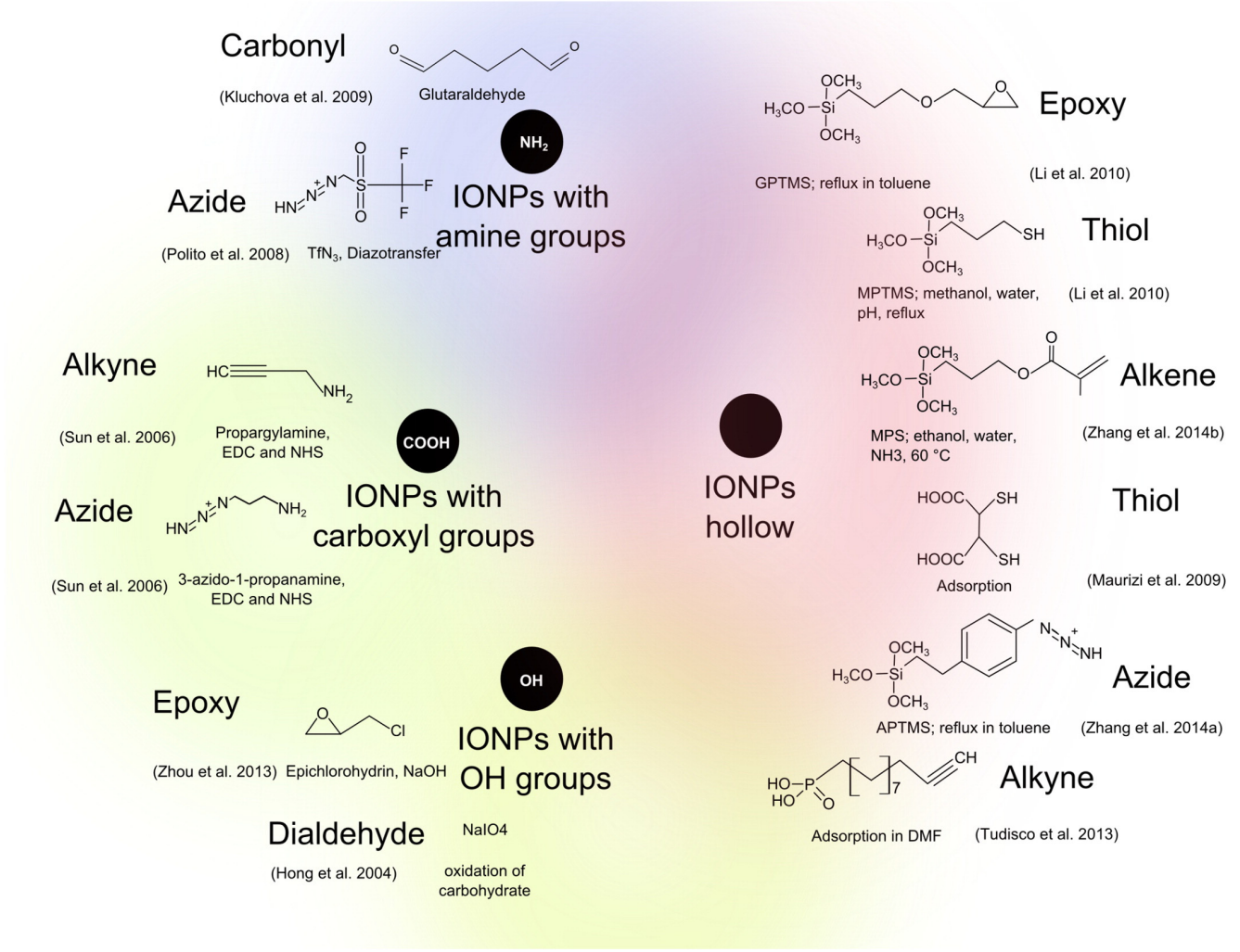

Fig. 7. Strategies for functionalization of IONPs with specific functional groups. GPTMS: 3-(glycidoxy)propyltrimethoxylsilane, MPTMS: 3-(mercapto) propyltrimethoxysilane, MPS: 3-(methacryloyloxy)propyltrimethoxylsilane, NHS: (N-hydroxosuccinimide), APTMS: 4-(azidomethyl)phenethyltrimethoxysilane, EDC: (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). The appropriate coating/functionalization method can be found in given literature (Hong et al., 2004; Kluchova et al., 2009; Li et al., 2010; Maurizi et al., 2009; Polito et al., 2008; Sun et al., 2006; Tudisco et al. 2013; Zhou et al., 2013; Zhang et al., 2014a; Zhang et al., 2014b).

functionalization of agarose/cellulose microspheres. These synthetic strategies are based on the activation of the polysaccharide in sodium hydroxide followed by the addition of a compound with a Cl–C bond such as chloroacetic acid or 2-chlorethyl-diethylamine (Boeden et al., 1991; Peška et al., 1976). The hydrophobic group can be there attached via n-alkylamines/carbonyl Schiff base coupling (Boeden et al., 1991).

3.4. IONP/metal hybrid synthesis

There are several approaches for preparing multicomponent nanomaterials containing IONPs and metallic nanoparticles of various arrangements (see Fig. 10), namely, the raspberry like structure, dumbbell like structure or core-shell arrangement. The first and most widely used synthesis involves the heterogeneous nucleation and growth of a metallic component onto seed nanoparticles. The seed nanoparticles are usually magnetite or maghemite cores. They can be pristine (Sa et al., 2014) or surface functionalized by amines (APTES (Liu et al., 2010)), dopamine (Chin et al., 2009), or chitosan (Markova et al., 2012b), carboxylic groups/polyacrylate acid (Prucek et al., 2011), thiol (APTMS (Han et al., 2013; Odio et al., 2014)), or hydroxide groups (TEOS (Choi et al., 2010)). The reduction of metals on a magnetic seed surface is mediated by common reducing agents such as $NaBH_4$ (Tamer et al., 2010), reducing sugars (Iglesias-Silva et al., 2007; Mandal et al., 2005), butylamine (Choi et al., 2010), amine groups on the surface of IONPS in a basic environment (Gawande et al., 2014; Sa et al., 2014) and others. This type of reaction usually results in raspberry like structures of nanocomposites.

The second type of preparation is based on a physico-chemical approach. Gu and colleagues described heterodimeric magnetite–Ag nanoparticles prepared at a liquid–liquid interface of "colloidosomes" provided by ultrasonication (Gu et al., 2005). A dumbbell-like structure is formed due to the limited number of catalytic centers available for the reduction of Ag^+ onto the magnetite surface. The solution-phase route supported by heating to prepare magnetite–Ag colloidal dimer nanoparticles with a dumbbell-like shape was also described by Lopes et al. (2010). Furthermore, sonochemical irradiation of iron(II) acetate solution in the presence of silver nanopowder can result in the deposition of magnetic nanoparticles on metal nanocrystals and the formation of metal@magnetite raspberry like structures, as described by Perkas et al. (2009).

The last category of IONP/metal hybrid synthesis involves non-covalent electrostatic or Van der Waals interactions between separately prepared magnetic and metal nanoparticles. An electrostatic interaction causes the formation of hydrides in syntheses described by Liu et al. (2010) where mixing of aminated magnetic nanoparticles and citrate

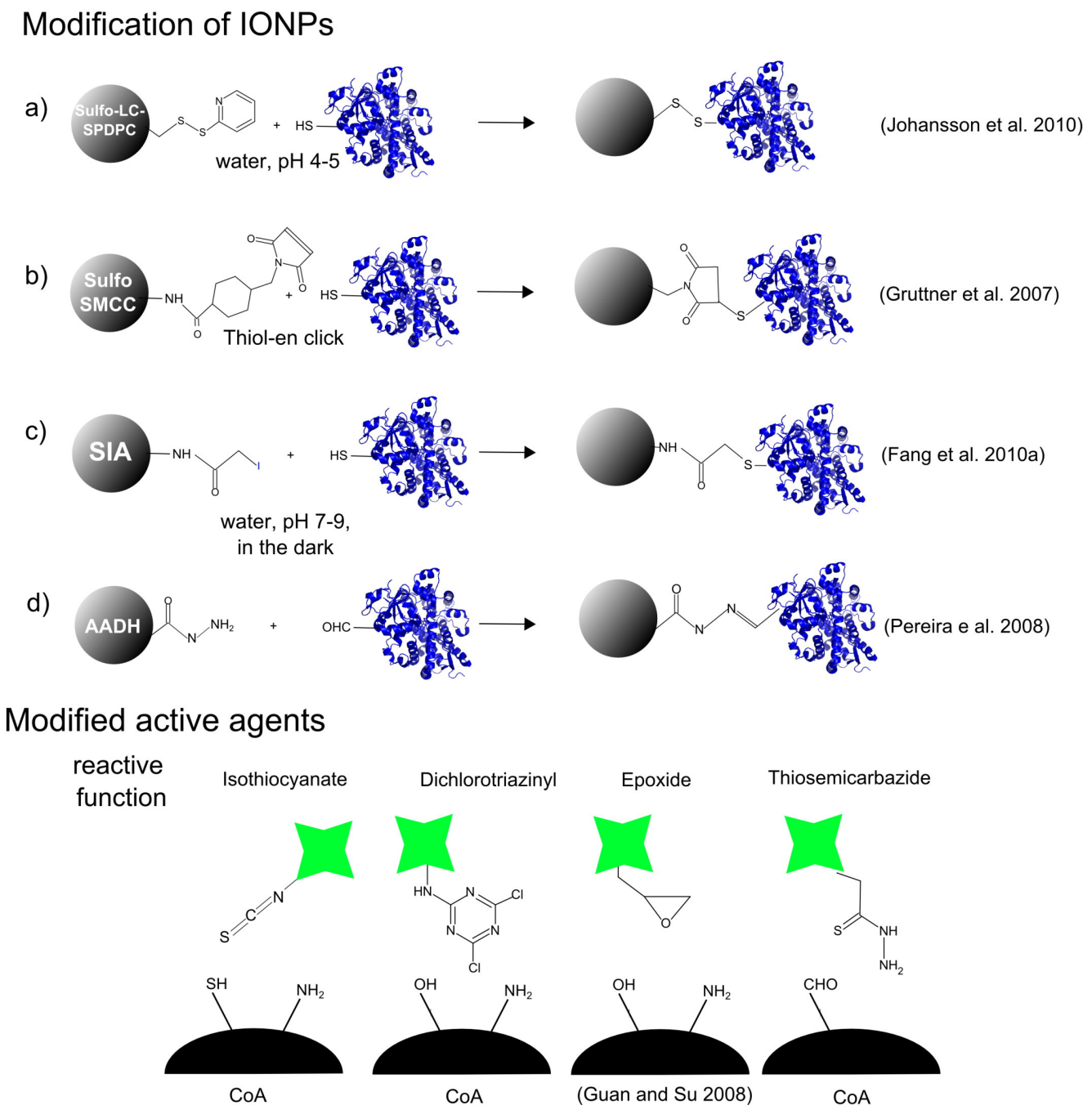

Fig. 8. Coupling strategies for linking IONPs with bioactive substances. Modification of the IONP's surface for immobilization of active compounds (a) via Sulfo-LC-SPDP: sulfosuccinimidyl 6-[3´(2-pyridyldithio)-propionamido] hexanoate, (b) Sulfo-SMCC: sulfosuccinimidyl-4-N-(maleimidomethyl)cyclohexyne-1-carboxylate, (c) SIA: succinimidyl iodoacetate. (d) Hydrazine strategy (AASH: adipic acid dihydrazide) for immobilization of antibodies. All of the reagents are commercially available. Modified active agents: commercially available activated agents (mainly fluorescent dyes); CoA: commercially available. The appropriate details for these coupling strategies can be found in given literature (Fang et al., 2010a; Grüttner et al., 2007; Guan and Su, 2008; Johansson et al., 2010; Pereira and Lai, 2008).

stabilized Ag nanoparticles led to magnetite–Ag submicrospheres. The Van der Waals interactions play a crucial role in impregnating the magnetite surface with Ni and NiPd nanoparticles, as described by Costa et al. (2014) or in the simple adsorption of Au seed on silica coated magnetite nanoparticles prepared in the study by Maciera (Salgueirino-Maceira et al., 2006). However, the covalent interaction can also be used to attach metallic nanoparticles to thiol-capped magnetite nanoparticles as described by Odio (Odio et al., 2014) or Han (Han et al., 2013). The deposited metal seeds on magnetic core can form a template for growing the shell. Salgueirino-Maceira et al. (2006) developed a shell of gold on magnetic silica spheres decorated with Au nanoparticulate seeds in a step-by-step reduction of $HAuCl_4$ aliquots with ascorbic acid. Similarly, a silver shell is formed by the addition of $AgNO_3$ solution instead of gold salt, as described by Chin (Chin et al., 2009) or Liu (Liu et al., 2010).

4. Concluding remarks

Much research has been devoted to the surface modification of magnetic IONPs. The biocompatibility, biodegradability, colloidal

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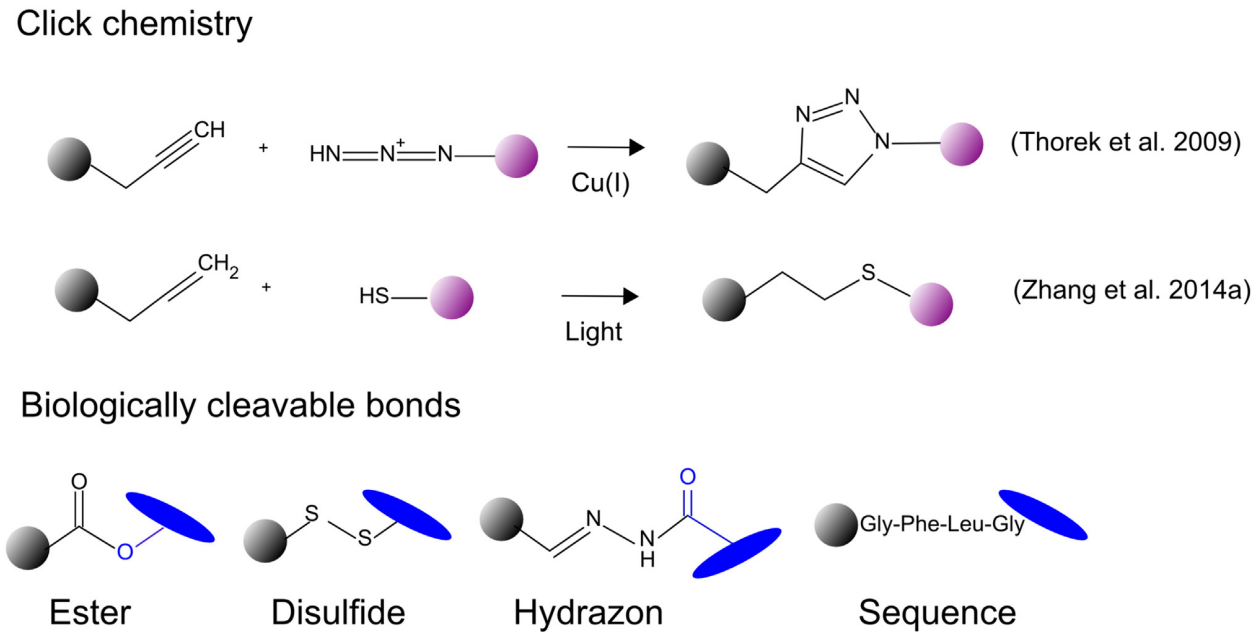

Fig. 9. Click chemistry linking approaches and biodegradable bonds for drug delivery. The appropriate details for these click chemistry coupling strategies can be found in given literature (Thorek et al., 2009; Zhang et al., 2014a).

stability, magnetic separability, nanometer scale and contrast enhancing in MRI make IONPs one of the most promising tools in various fields of biomedicine and biotechnology. In this short critical review, we summarize and critically discuss the principles, functionalization procedures, and conjugation strategies for applications of IONPs in magnetic resonance imaging, drug delivery, magnetic separation, and immobilization of various biosubstances (e.g., proteins, enzymes, drugs, antibodies). This review demonstrates that despite many similarities, each nanosystem application requires specific core-shell properties, and as a result, tailored functionalization and conjugation procedures. Because of this, a challenge in this field is to develop universal systems and functionalization procedures that allow simultaneous diagnostic (MRI) and therapeutic (drug delivery, hyperthermia) applications of IONPs. In addition to these so called theranostic agents,

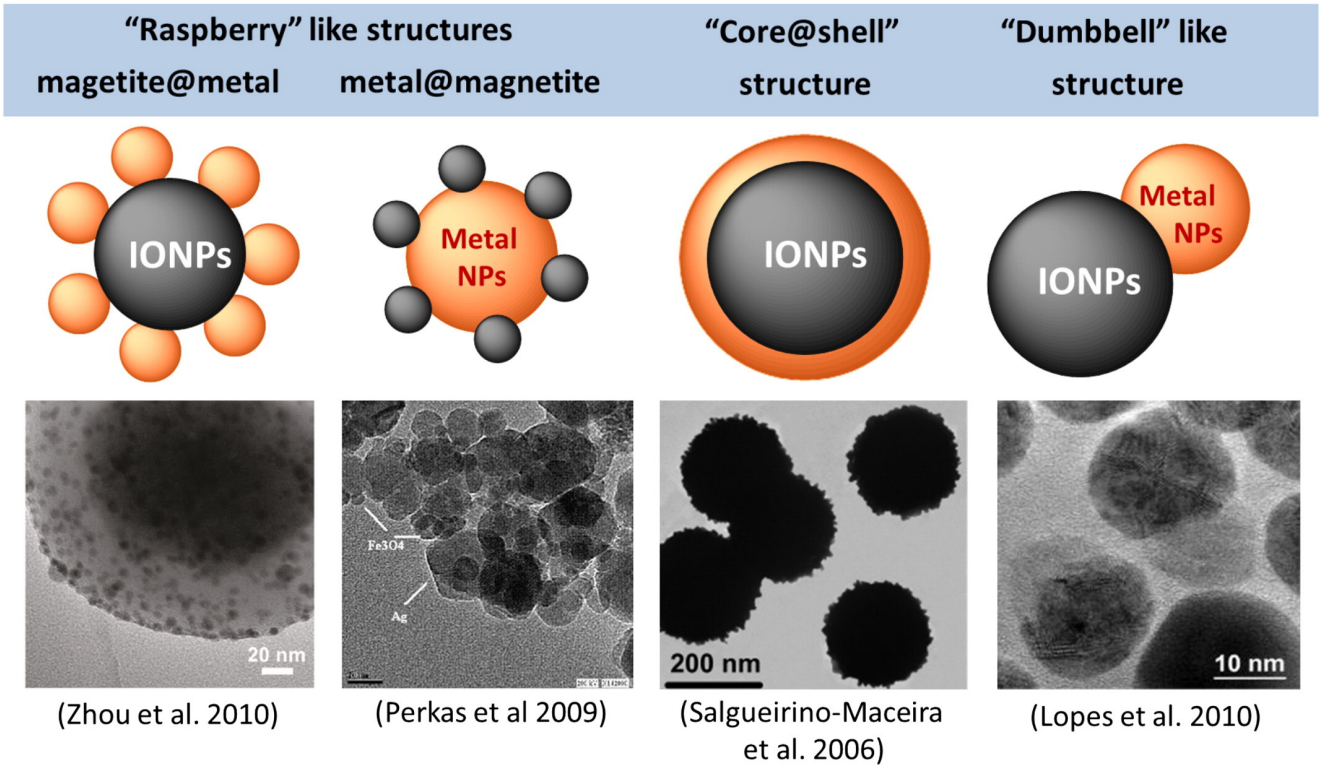

Fig. 10. Schematic illustration of IONPs/metal NP (nanoparticle) assemblies.
Reprinted with permissions from Lopes et al. (2010), Perkas et al. (2009), Salgueirino-Maceira et al. (2006), and Zhou et al. (2010).

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multicomponent hybrids combining the properties of magnetic IONPs and metallic nanoparticles represent another type of biomaterial with a huge potential in biocatalysis, biosensing, analytical chemistry, bioimaging, and environmental and antibacterial technologies.

In the field of biomedical application of IONPs, huge progress has been made so far. We now understand the in vivo toxicity of the particles, their bioprocesses, how to avoid opsonization and RES-uptake and how to target the particles to desired places by surface functionalization. However, to fully harness the potential of IONPs for targeted healthcare, the pharmacokinetics of drug release from the particles must be explored for specific diseases. The combination of targeted delivery, controlled drug release/therapy and parallel MRI imaging makes IONPs an excellent platform for personalized medicine. Challenges are also found in the areas of magnetic separations or enzyme immobilization. Much attention is focused on upscaling systems in an economically viable manner. Research is also focused on the new and emerging applications of magnetic/metallic hybrid nanostructures, for example, in heterogeneous catalysis or in new technologies for early detection of disease based on surface-enhanced Raman spectroscopy. These highly sophisticated nanosystems and their applications can expect enormous development in the future.

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