J. Iran. Chem. Soc., Vol. 7, Suppl., July 2010, pp. S1-S27.

JOURNAL OF THE
Iranian
Chemical Society

Recent Advances in Surface Engineering of Superparamagnetic Iron Oxide Nanoparticles for Biomedical Applications

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(Received 25 December 2009, Accepted 7 February 2010)

Superparamagnetic iron oxide nanoparticles (SPIONs) are promising materials for various biomedical applications including targeted drug delivery and imaging, hyperthermia, magneto-transfections, gene therapy, stem cell tracking, molecular/cellular tracking, magnetic separation technologies (e.g. rapid DNA sequencing), and detection of liver and lymph node metastases. The most recent applications for SPIONs for early detection of inflammatory, cancer, diabetes and atherosclerosis have also increased their popularity in academia. In order to increase the efficacy of SPIONs in the desired applications, especial surface coating/characteristics are required. The aim of this article is to review the surface properties of magnetic nanoparticles upon synthesis and the surface engineering by different coatings. The biological aspects, cytotoxicity, and health risks are addressed. Special emphasis is given to organic and inorganic-based coatings due to their determinant role in biocompatibility or toxicity of the final particles.

Keywords: Magnetic iron oxide nanoparticles, Coatings, Cytotoxicity, Biocompatibility, Biomedical applications

INTRODUCTION

The wide applications of superparamagnetic iron oxide nanoparticles (SPIONs) in magnetic fluids [1-5], data storage [6], catalysis [7-10], bioengineering and biosensors [11-16], environmental remediation [17-21], and magnetic inks for jet printing [22, 23] have attarcted significatont attention worldwide. Recently, the potential usage of SPIONs have significantly increased due to their new applications in biotechnology and medicine [24]. More specifically, in the human clinical usage, the SPIONs are being utilized as delivery systems for drugs [25], genes [26], bio-molecules [27] and radio-drugs [28], stem cell tracking [29], diabetes [30, 31] and cancer detection [32]. There are also numerous *in vitro* applications for SPIONs in medical diagnostics, such as immune magnetic separation of cells, proteins, DNA/RNA, bacteria, viruses and other biomolecules [33-35]. SPIONs can be used for these applications in different architectures including magnetosomes [36] (liposomes containing SPIONs), magnetic beads [37] (random dispersion of SPIONs inside the polymeric matrix), CNCs [38] (magnetite colloidal nanocrystal clusters), and core-shell [39] (single coated nanoparticles). Regardless of the type of the biomedical applications, the superparamagnetism is an essential property because once the external magnetic field is removed, the magnetization disappears that eventually avoid the possible embolization of

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the capillary beds [40]. In spite of the vast variety of the envisaged applications for SPIONs in biomedicine, inadequate characterization techniques, inhomogeneous properties, severe agglomeration in biological environment and fast recognition by the body immune system have limited the usage of these promising nanoparticles (NPs) in clinical applications. Recently, researches have focused on developing innovative technologies for controlled synthesis and analysis of SPIONs coated with novel organic or inorganic coatings in order to overcome the above barriers, making the application of SPIONs in diagnostics and therapeutics purposes feasible [41, 42].

In this review, the methods used for the synthesis of SPIONs with controlled size distribution are briefly addressed. Then, recent developments and achievements in surface engineering of SPIONs to make them applicable for biomedical applications are presented. Finally, the biological response to the functionalized nanoparticles is discussed.

Synthesis of the superparamagnetic nanoparticles

The shape, particle size characteristics, and surface properties of SPIONs are crucial factors affecting their specific applications. Evidently, these factors are controlled by the method of synthesis and the processing conditions utilized. On the other hand, the process yield and its reproducibility is very important in scale production of SPIONs [22, 43-46]. All these factors affect the colloidal stability of the final product that is an essential prerequisite *per se* in biomedical applicability of SPIONs [47, 48]. For instance, to precisely control the produced heat in a therapeutic hyperthermia procedure using magnetic NPs, size characteristics of SPIONs is crucial in order to achieve single- or multi-domain [49-51] with a very narrow size distribution to imply a homogeneous properties [52]. There are a plenty of techniques available to facilitate production of SPIONs with specific physical and chemical properties. Co-precipitation of iron salts [53], microemulsion formation [54], thermal decomposition of iron precursors [55], hydrothermal methods [56], sol-gel transition [57], chemical vapor deposition (CVD) [58], flow injection [59], electrochemical [60] techniques under oxidizing conditions, and laser-induced pyrolysis of pentacarbonyl iron vapors have been used. The first three procedures are the most common routes which have been utilized by many researchers. The other methods are of less importance and interested reader are referred to the comprehensive review published by *Laurent et al*.[3]

SPIONs are commonly produced by reduction of iron salts under alkaline condition, a procedure which is fast and simple and is universally termed as co-precipitation method. *Massart et al.* (1981) reported the first well-controlled co-precipitation method to prepare SPIONs in both alkaline and acidic media without addition of any stabilizing molecule [61]. Since then, enormous investigations on co-precipitation method have been reported and continue to be reported to optimize the synthesis parameters. The distinguished features of the co-precipitation method in comparison to the other available procedures are related to its convenience, efficiency, and possibility to easily synthesize the major types of iron oxide nanoparticles, *i.e,*. magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) [22, 62]. Literature reports indicate that the size characteristics of the particles, shape and morphology of the individual grains, the magnetic saturation, and the composition of particles vary with the synthesis condition. Parameters such as iron charge (Fe^{2+}/Fe^{3+}) [63] and their concentration in the primary media [64], acidity and ionic strength of the final media [65, 66], reaction temperature [39, 67], injection flux rates [63], homogenization rates [40, 68], bubbling inert gas thorough the solution [40, 69, 70] are effective. In spite of the main advantage of the co-precipitation method in easily synthesis of SPIONs with a high process yield, size polydispersity [71, 72] of the product is distractive because the polydispersity in particle size could result in non-suitable magnetic properties as the blocking temperature and saturation magnetization depend on the particle size. Many studies have been performed to optimize and control the process in order to reduce the size polydispersity, for example, by tuning of the homogenization rate and base molarities [40].

Microemulsion is an alternative method to synthesize SPIONs with a narrow-size distribution. A microemulsion is a thermodynamically stable isotropic dispersion of two immiscible liquids, where the micro-domain of either or both liquids is stabilized by a densely packed interfacial film of surface-active molecules composed of a surfactant and a cosurfactant *i.e,*. a medium chain alcohol [73, 74]. In order to prepare NPs with uniform size, inverse micelle (water-in-oil or W/O) has been extensively used [75-78]. Herein, the aqueous

phase is dispersed as tiny droplets (typically between 10-100 nm in diameter) encircled by a monolayer of amphiphilic molecule (10-100 nm) in continuous hydrocarbon phase [79]. Microemulsions are inherently stable emulsions (long shelf life) and transparent which formed easily due to a small interfacial tension and have a low viscosity with Newtonian behavior. The particle size of the product is directly determined by the droplet size in the reverse micelles and thus by the type and concentration of surfactants. Anionic [80-82] (*e.g.,* sodium bis(2-ethylhexylsulfosuccinate) (AOT) and sodium dodecylsulfate (SDS)), non-anionic [83, 84] (*e.g.,* polyethoxylates and Lutensol AT50) and cationic [85-88] (*e.g.,* cetyltrimethylammonium bromide (CTAB), and cetyltrimethylammonium (CTMA)) surfactants can be utilized. The molar ratio of water to surfactant can also be changed to determine the diameter of the reverse microemulsion droplets [89]. Factors such as the reaction temperature, the concentration of iron salts, and base molarity are also affect the product characteristics [90]. In a typical procedure to prepare SPIONs, two identical W/O microemulsions containing iron salts and alkaline medium are prepared. The aqueous droplets present in the continuous (oil phase) serve as nano-reactors to prepare magnetic NPs. Upon mixing of iron salts and alkali medium microemulsions, micro-droplets undergo continuous collision, coalescence, and breaking that result in the precipitation of SPIONs form micelles [45, 91].

Thermal decomposition of organometallic precursors in organic solvents containing stabilizing surfactants such as oleylamine [55, 67], fatty acids [92], oleic acid [93-97], hexadecylamine [98], and steric acid [99] is another method to achieve highly monodispersed and monocrystalline SPIONs. The common organometallic precursors include iron acetylacetonate [100], iron carboxylates [101, 102], iron cupferronates (Cup=N-nitrosophenylhydroxylamine, $C_6H_5N(NO)O$-) [103], and iron carbonyls [104, 105].

Surface architecture of SPIONs for biomedical applications

The bare SPIONs are eager to reduce their surface energy via formation of clusters and agglomerates. The formation of magnetic aggregates is not suitable for biomedical applications because of very fast detection by immune system. A suitable coating should be applied on the SPIONs in order to achieve NPs with hydrophilic surfaces that have high colloidal stability and dispersability without deterioration of their magnetic properties. The present authors [37] have recently developed a multiphysics numerical model and studied the dynamic behavior of ferrofluids containing SPIONs in different size ranges in the bloodstream into the vessels under influence of an externally applied magnetic field. Simulation results showed that the magnetic properties of the NPs as well as the magnetic strength of the externally applied field are important factors affecting the shape and amplitude of the ferrofluid velocity field. Colloidal stability of SPIONs in the biological environment is also very important for *in vivo* applications and particularly for intravascular injection [41,42,106]. If particles lose their stability in biological fluids, they could end to atherosclerosis and thus would lose their functionality and application. Since the isoelectric point of SPIONs is around pH=7 [48, 107], colloidal stability of SPIONs in the biological environment is a major shortcoming. Surface modification of the NPs by coating materials is thus became a suitable route to improve the colloidal stability of SPIONs. In fact, the physical and chemical properties of the applied coating materials influence the colloidal stability in turn [108, 109]. Ability to escape from reticuloendothelial system (RES) and consequent increasing in the blood circulation half-life for NPs is regarded as another important role which coatings may play in biomedical applications [110-113]. Finally, coatings should be able to isolate the magnetic core from *in vivo* environment, improving the biocompatibility of the NPs. As a matter of fact, the type and amount of coating can be recognized as a critical matter in biological application of magnetic materials. In the following sections, different coating systems are described.

Polymeric Coatings

Biocompatible polymers are widely used as coating materials for SPIONs to accomplish multiple purposes including colloidal stabilization, delivering biologically active agents with a controlled release profile, and targeting capability to specific tissues via conjugation with specific ligands [75]. The polymeric coatings can be induced either during [114, 115] or after synthesis [25, 40, 116-118] dependent on the properties required.

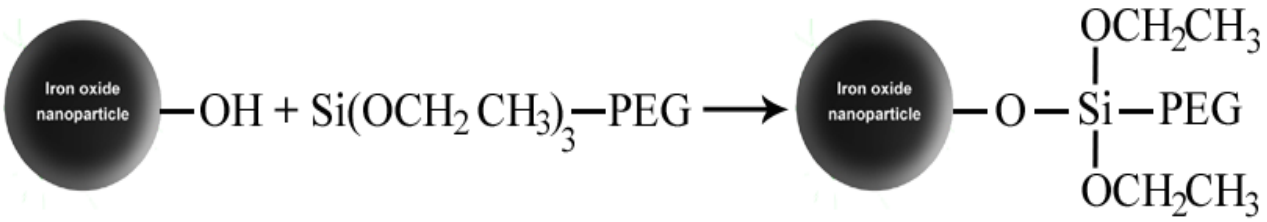

Fig. 1. Chemical reaction scheme for immobilizing of PEG on SPIONs.[109]

Polyethylene Glycol (PEG)

PEG as a member of polyether family of polymers is one of the most important hydrophilic and water soluble polymers. Two terminal hydroxyl functional groups of PEG can be turned to many other different functional groups including amine, carboxylic acid or even hetrobifunctional derivatives used to conjugate different biological moieties. PEG is FDA approved for parenteral applications upon its well-known biocompatibility and toxicokinetics profile up to 20 kDa of molecular weight. The elimination of PEG in the body mostly occurs by excretion from kidneys and/or feces according to its molecular weight [119]. Since PEG can be metabolized in liver to toxic glycol derivatives in lower molecular weights (<1 kDa), 1-20 kDa is suitable for parenteral administration. Being hemocompatible, non-antigenic and non-immunogenic, very little biological reaction is expected by application of PEG on the surfaces with direct contact to bloodstream [120-123]. No significant sensitization of the body immune system and the following secretion of antibodies is reported under routine clinical administration of PEGylated proteins [120]. The distinguished capability of PEG-coated NPs is their stealth property which ends to their enhanced biological half-life [112, 124-127]. In addition, PEG-coated NPs have shown enhancement in their specific uptake by minimizing the RES recognition and consequently increasing circulation half life [113].

Davis et al.[128] reported the first chemical steps in PEGylation that enabled the protection of proteins from destruction during drug delivery. They showed that both pharmacokinetic and pharmacodynamic properties of the peptide drugs can be improved by PEGylation upon enhancement in their water solubility, reducing renal clearance and increasing biocompatibility [129]. Surface engineered SPIONs with PEG showed suitable stability in the physiological environment [130, 131]. *Xie et al.* [130] used dopamine (DPA) as a linker between SPIONs and PEG due to the high affinity of DPA moiety to the Fe_3O_4 surface [132]. DPA can be linked with one of the terminal carboxylic functional groups in the PEG diacid via the carbodiimide/amine-reactive succinimidyl (EDC/NHS) chemistry. Using this spacer moiety, the PEG was covalently fixed on the SPIONs surfaces by replacing the oleate and oleylamine. These particles showed a high stability during their incubation in phosphate buffered saline (PBS) plus 10% fetal bovine serum (FBS) in a routine cell culture experiment.

Further improvement in surface engineering of these particles was achieved by coupling of 6-hydroxy-chromone-3-carbaldehyde to the PEG-DPA-magnetite NPs via a Schiff-base reaction, and released via a pH controlled manner [133]. It is noteworthy that chromones are a group of naturally occurring compounds containing core structure of benzopyranone and have been shown to possess some degrees of antifungal, antiviral, antihypertensive, and anticancer activities [134-136]. There are many other reports showing stable covalent binding of PEG on the surface of SPIONs [137-139]. *La Van et al.* [109] immobilized PEG and folic acid on the surface of SPIONs to improve the intracellular uptake and ability to target specific cells as examined for mouse macrophage (RAW 264.7) and human breast cancer (BT20) cell lines.

In order to achieve highly stable bonds between PEG and SPIONs, *Herve et al.* [47] reported a multi-step preparation of

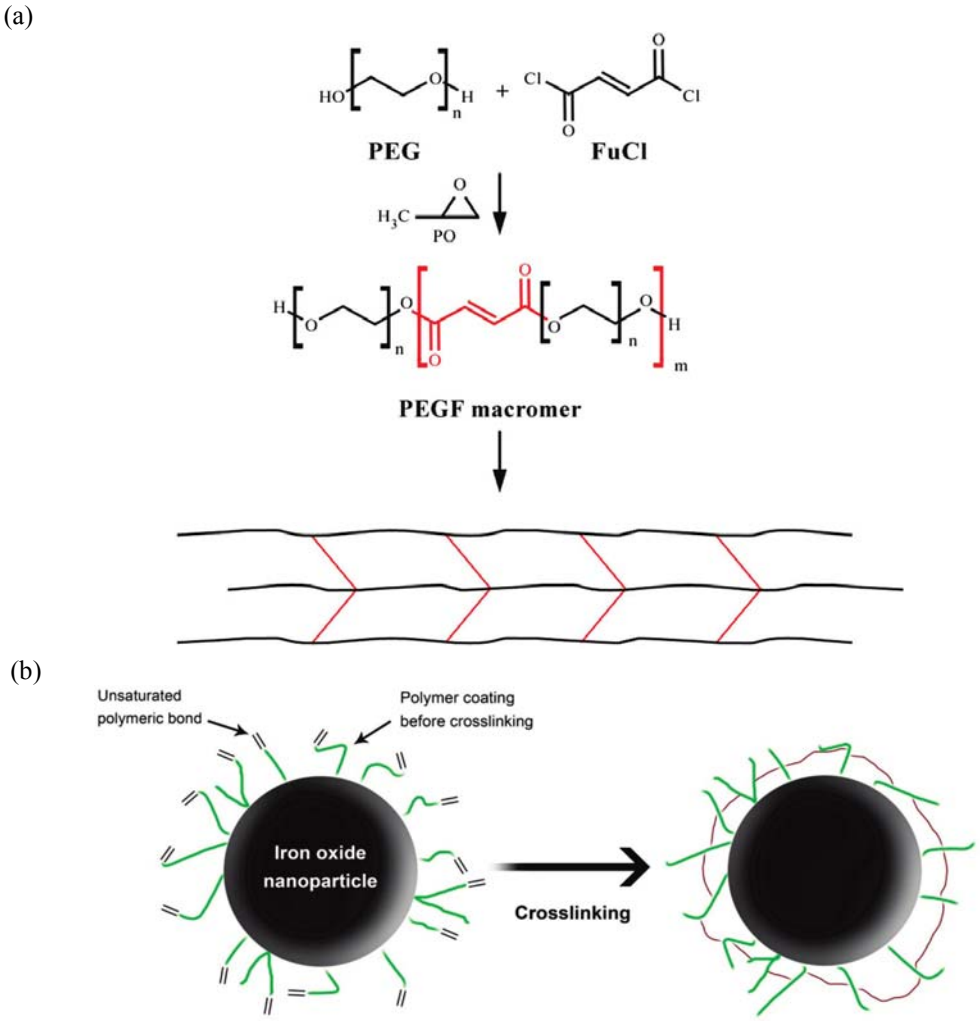

Fig. 2. (a) Cross-linking of PEGF on SPIONs surfaces; (b) Proposed scheme of cross-linking PEGF that coats the iron oxide NPs, with permission from reference 25.

aqueous suspensions of PEGylated ferrofluids by functionalization of the iron oxide nanoparticle surfaces with PEG molecules by an intermediate of silanes. The stable initial ferrofluids based on magnetite/maghemite NPs was synthesized and modified by silanization followed by anchoring PEG on these NPs through a covalent bond. The stability of the coating is important characteristic for SPIONs because once the surface-derivatized NPs are inside the cells the layer is likely to be digested that eventually leaves the bare particles exposed to other cellular components and organelles; thereby, the coating stability potentially influences the overall integrity of the cells [90].

Polyethylene Glycol fumarate

Unsaturated aliphatic polyesters are promising candidates to stabilize SPIONs by providing a crosslinked network around the particles via curing of their unsaturated double bonds [140-142]. Crosslinking may be initiated using heat, chemical reaction (RedOx), and UV or visible light irradiation [25]. Fumaric acid containing macromers are highly unsaturated and can be crosslinked with or without using a multifunctional crosslinking agent or reactive diluent to form their corresponding polymeric networks [143]. Currently, a number of different crosslinking agents, *e.g.*, poly(propylene

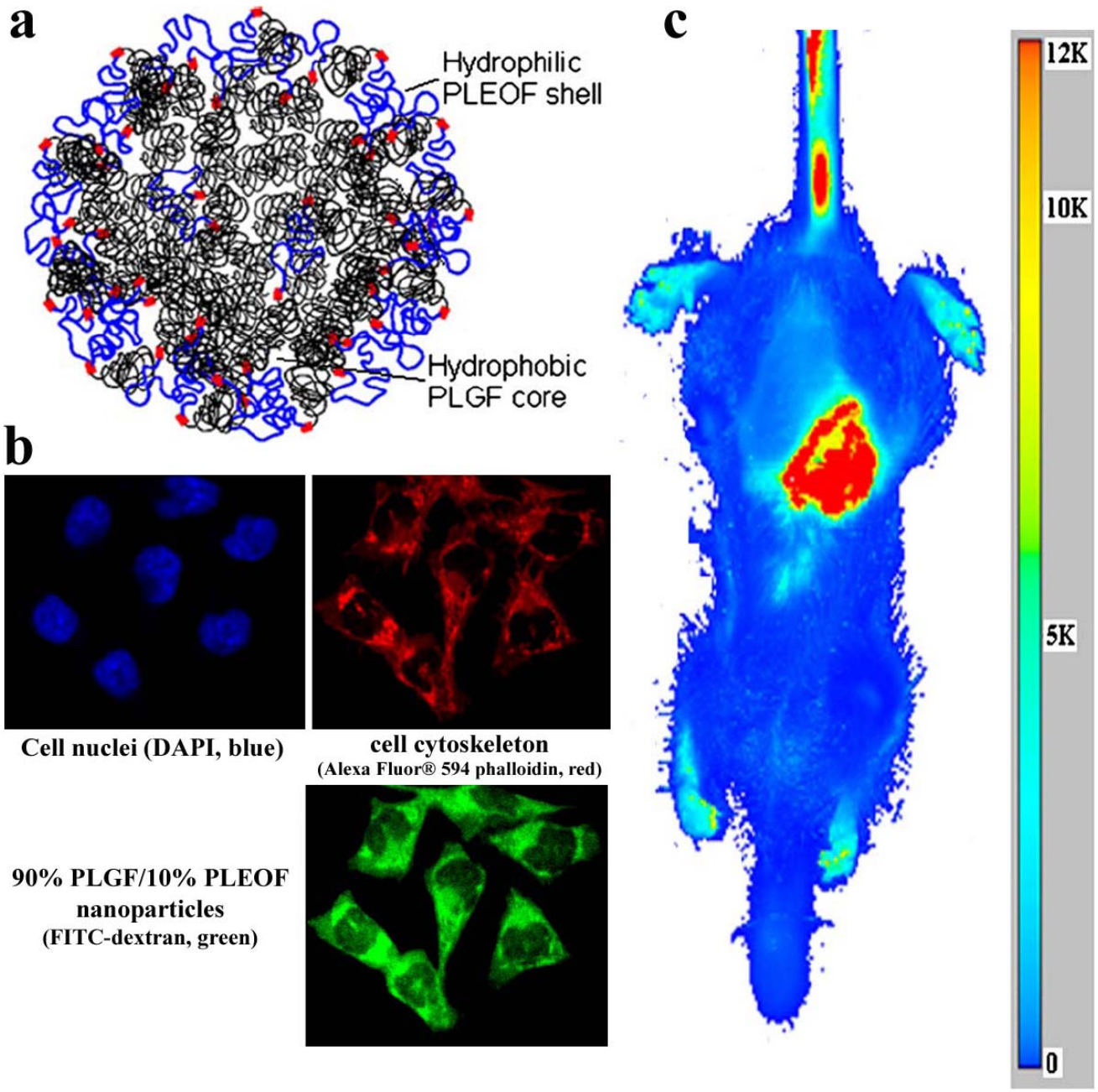

Fig. 3. (a) Schematic representation of the core-shell structure of PLGF/PLEOF blend NPs; (b) Micrographs of confocal fluorescent images of HCT116 tumor cells treated with FITC-dextran loaded PLGF NPs; (c) Near infrared scan (800 nm wavelength) of the $Apc^{Min/+}$ mouse injected with 500 µl of the PLAF/PLEOF NPs encapsulated with the IRDye 800RS Carboxylate dye; with permission from reference 145.

fumarate)-diacrylate (PPF-DA) and poly(ethylene glycol)-diacrylate (PEG-DA) or dimethacrylate are being used in curing of fumaric acid containing macromers [144]. These agents can facilitate the crosslinking reaction while imparting specific properties to the network. We have recently used poly(ethylene glycol)-co-fumarate (PEGF) macromers and crosslinked them by redox polymerization [25] in the presence of chemical initiator (ammonium persulphate) for surface modification of SPIONs (Fig. 2). The specific feature of this novel coating is reduced protein interaction as well as less burst release effect in drug delivery application. *Mahmoudi et al.* [25] loaded tamoxifen citrate (TMX, a member of anti estrogen compounds used as first line candidate in estrogen-responsive breast cancer) on the coated-SPIONs with both cured- and non-cured PEGF and studied the drug release pattern in a simulated biological experimental set up. Results showed that the crosslinked PEGF coating can reduce burst release by 21% as compared to the non-crosslinked PEGF.

He et al. [145] prepared biodegradable blends of poly(lactide-co-glycolide fumarate) (PLGF) and poly(lactide-co-ethylene oxide fumarate) (PLEOF) macromers and self-assembled then into NPs by dialysis (Fig. 3a). The NPs were utilized to decrease the cytotoxic (examined by MTT assay of NPs-treated HCT116 human colon carcinoma cell line) effects of paclitaxel (a mitotic inhibitor used in many cancer chemotherapy protocols) or to increase its potential to evade the RES and target the drug delivery system or carrier to the tumor vasculature. PLGF NPs (90% PLGF and 10% PLEOF) were loaded with FITC-dextran and their uptake was tracked with HCT116 human colon carcinoma cells via confocal laser scanning microscopy (Fig. 3b). Comparison of the micrographs confirmed the internalization of NPs by the tumor cells. The author claimed that both pinocytosis (*i.e.* a continuous fluid-phase pathway) and phagocytosis (*i.e.* a ligand-induced pathway) pathways contributed to the uptake of NPs. Infrared image of the $Apc^{Min/+}$ mouse injected with NPs also revealed a significantly higher concentration of NPs in the intestinal tissue (Fig. 3c). Finally, they concluded that unsaturated aliphatic polyester family especially PEGF enables to improve the biocompatibility profile of carrier system and decrease the avidity of the RES to remove the magnetic NPs from the blood circulation.

Polyvinyl Alcohol

Polyvinyl alcohol (PVA) was first prepared by Hermann and Haehnel in 1924 by hydrolyzing polyvinyl acetate in ethanol in the presence of potassium hydroxide [146]. Since then, research and development on PVA has attracted much attention to cover wide range of applications in industry as well as pharmaceutical and biomedical research [46, 147, 148]. PVA is frequently reported as a coating material for magnetic NPs because of its hydrophilicity, biocompatibility, excellent film or gel forming capability (via chemical or physical crosslinking), and adhesive properties [149-151]. Additionally, applying an appropriate amount of PVA as a coating on SPIONs prevents their aggregation mostly via steric hindrance mechanism and gives rise to mono-dispersed NPs [40, 117, 152-157]. Nevertheless, a high amount of PVA, usually polymer/iron mass ratio (r) greater that 3, causes the formation of magnetic beads, reduces the crystallinity of SPIONs, and affects the cytotoxicity profile of the obtained materials due to the effect of crystallinity on the protein adsorption profile on the particles surfaces [39, 157-159]. We have recently shown [39] that at the critical mass ratio (r) of around 3, the maximum level of magnetic saturation and permeability for SPIONs was observed. At $r<3$, no significant influence of PVA on the magnetic saturation was noticed while at higher r values the magnetic saturation and permeability both decreased due to the formation of magnetic beads which in fact decline the exchange penetration as well as dipolar interactions [40]. Based on the cytotoxicity evaluation of PVA-coated SPIONs using mouse fibroblast cells (L929), it was shown that $r = 3$ is suitable to achieve functionalized NPs with acceptable biocompatibility (i.e. cell viability of 95%) and good magnetic saturation (e.g. 45 emu/g). In another work, *Schöpf et al.* [154] tracked PVA-coated SPIONs in synoviocytes. Synoviocytes were incubated for 2, 12, 24 and 48 h with 1.5 mg/mL NPs under the influence of an external magnet for a period of 12 h. Results showed that the particles were well tolerated by the synoviocytes and easily detected by Turnbulls and Prussian blue reactions between 12 and 24 h. *Petri-Fink et al.* [160] investigated the capability of SPIONs coated with PVA and its carboxylate, thiol or amino-functionalized derivative to be taken up by human cancer cells. It was shown that the interaction of the human

melanoma tumor cells with the coated NPs depends on the structure of polymeric shell as it affects the physical properties of the compound [159, 161].

Acrylate-based coatings

Polyacrylic acid (PAA) as a coating polymer for SPIONs can increase colloidal stability as well as biocompatibility of the magnetic particles [106, 162-165]. Conventional PAA is soluble in water, dioxane, ethanol, dimethylformamide and methanol but not soluble or has a limited solubility in acetone, diethyl ether, benzene and aliphatic hydrocarbons which may be helpful to recognize a suitable medium for synthesis and coating of SPIONs in the presence of PAA. PAA copolymers such as chitosan-PAA are more appropriate for coating of SPIONs than those of homopolymers for biological applications due to their higher flexibility in designing a material to achieve a specific property [162-165]. *Iijima et al.* [166] synthesized spindle-shaped NPs, which have great potential in imaging and drug delivery [167, 168], by PAA insertion as the morphology of iron hydroxide particles can be controlled by the surfactant molecular structure. There are other groups which used PAA as coating of SPIONs in order to increase both colloidal stability and biocompatibility of SPIONs [162, 169-171].

Poly(N-isopropylacrylamide) (PNIPAm) is a thermoreversible water-soluble polymer which its aqueous solutions exhibit a lower critical solution temperature (LCST) around 32 °C [172-174]. PNIPAm is also a pH-responsive system [175]. *Chiu et al.* [176] and *Cai et al.* [177] reported synthesizing of a responsive system using magnetite NPs and PNIPAm microgels through a layer-by-layer adsorption technique. *Sun et al.* [178] and *Lai et al.* [179] prepared PNIPAm-coated SPIONs that showed LCST at 31 °C. They used these particles as contrast agents for magnetic resonance imaging (MRI) and hyperthermia. *Li et al.* [180] synthesized composite microspheres via emulsion polymerization of NIPAm and chitosan containing oleic acid modified magnetite NPs as a dispersed phase. SEM and TEM results suggested that the composite microspheres were of a spherical geometry with an average particle size of 400 nm when the ratio of chitosan/NIPAm was set at 2.0. The composite microspheres were thermo-responsive, pH-responsive, and magnetic-sensitive. Incorporation of chitosan into the structure of matrix phase induced pronounced pH sensitivity upon the presence of ionizable amino functional groups on its backbone. The composite microspheres showed a LCST of around 31 °C in water although LCST varied from 28 °C at acidic pH to 32 °C at basic pH. The electromagnetically induced heating showed that the composite microspheres could be heated to 45 °C in an alternating electromagnetic field.

Polysaccharide-based coatings

Dextran is a natural polymer made of anhydroglucose units consisting mainly alpha-D(1-6) linkages, but some unusual 1,3 glucosidic linkages are also present at branching points. Dextran is a biocompatible and biodegradable polymer with a well-known history of safe application as plasma expander in severe hemorrhages [181]. Application of dextran is also reported as a coating material for SPIONs in order to enhance both the colloidal stability and blood circulation time (biological half-life) [182]. It is noteworthy that dextran-coated SPIONs (Ferridex®) is the only FDA approved product as MRI contrast agents. *Pardoe et al.* [183] suggested that the synthesized SPIONs in a medium containing dextran or polyvinyl alcohol can form cluster- and necklace-like aggregates, respectively. *Jung et al.* [184] presented a model for dextran adsorption on the surface of SPIONs in which hydrogen bonds, through polymer hydroxy groups, take place at different segment of the dextran. The importance of this model is its potential to predict the stability versus agglomeration of the coating in various media (*e.g.* aqueous, cell culture and biological media) either by electrostatic, steric or electrosteric repulsion. More stability in dextran-coated SPIONs have been obtained by the Herceptin conjugation [185]. The dextran-coated SPIONs could be successfully employed to disrupt the blood brain barrier and target the rat glial tumors [186]. It is noteworthy that the size of the coated particles is a critical parameter that determines their blood circulation time as a critical factor for MR imaging applications. The particles with diameter less than 20 nm exhibited prolonged circulation time [187] while larger SPIONs with unmodified dextran (*e.g.* 50-150 nm) were rapidly removed from the blood circulation by RES organs such as liver and spleen [188]. In addition to the size of

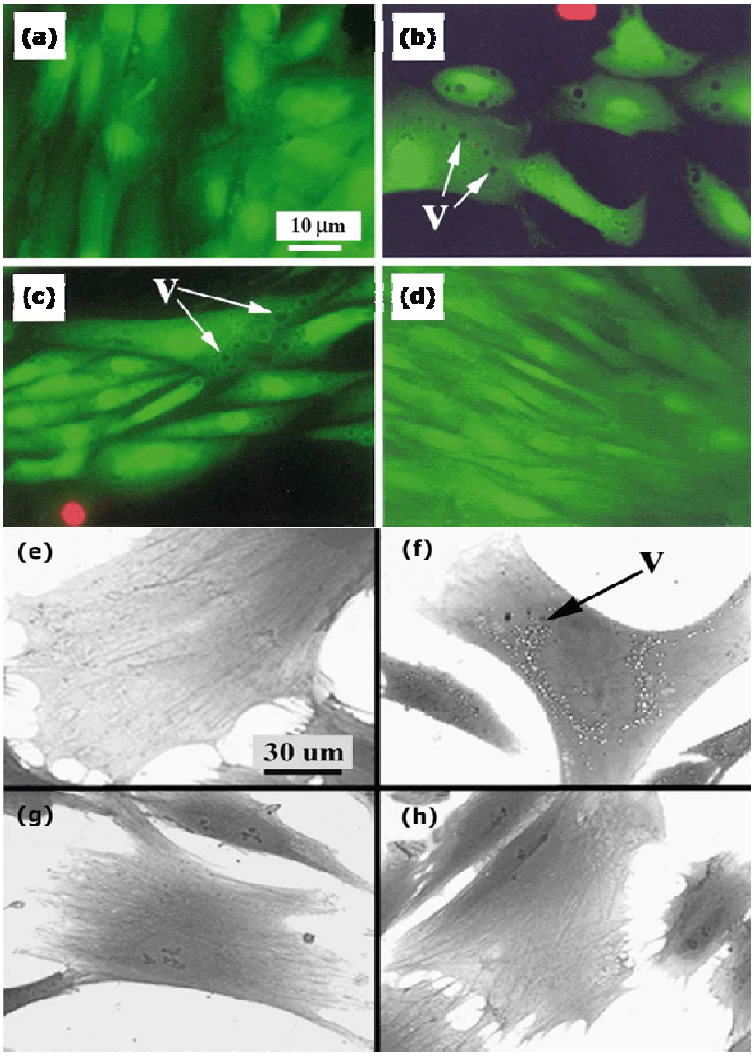

Fig. 4. (a) Viability staining of control fibroblasts; (b) fibroblasts incubated with uncoated-SPIONs, (c) dextran-coated SPIONS, and (d) albumin-coated SPIONs for 24 h; (e) Coomassie blue morphology stain after 24 h culture of control cells and cells incubated in (f) uncoated-SPIONs, (g) dextran-coated SPIONs, and (h) albumin-coated SPIONs. Vacuoles were illustrated with 'v'; with permission from reference 190.

particles, the surface properties of the coated SPIONs are very important. Since ionic carboxy-dextran coating promotes phagocytic uptake [189], dextran coating is very promising. It is also suggestible to use SPIONs are as macrophage-targeted MRI probes [190, 191]; thereby, dextran sulfate is applicable as a coating material to be a ligand for macrophage scavenger receptors [190-192].

Berry et al. [193] prepared dextran- and albumin-coated SPIONs with core diameter of 8-15 nm. They showed that the ferrofluid was stable in neutral pH. *In vitro* studies demonstrated that the uncoated particles were largely internalized by the fibroblasts, maybe contributing to eventual cell death. The dextran-coated SPIONs followed the similar fate whereas the albumin-coated SPIONs not only did not

cause any cell death but also increased the cell proliferation values. The viability staining confirmed that the uncoated- and dextran-coated SPIONs caused some cell death, indicated by red nuclei. In contrast, albumin-coated SPIONs were viable and more densely populated (Figs. 4a-4d). Furthermore, vacuoles were noticed in the cell body. Cell morphology assessment which confirmed by Coomassie blue staining also showed similar results (Figs. 4e-4h). Very recently, *Mahmoudi et al.* [194, 195] noticed these kinds of vacuoles in both PEGF- and PVA-coated SPIONs and claimed that this phenomenon is the start of cell death via autophage. It is suggestible that the formation of protein corona (*i.e.* dynamic protein coating on the surface of unmodified dextran-coated SPIONs) is responsible for the fast blood clearance [196, 197]. Experiments [198, 199] showed that dextran-coated SPIONs are extensively coated with plasma proteins (*e.g.* opsonins) such as complement, fibronectin and fibrinogen. The role of plasma proteins in the unmodified dextran-coated SPIONs clearance was firstly shown by *Simberg et al.* [200] via analyzing of the spectrum of plasma proteins bonded to the NPs and exploring the role of these proteins as potential nanoparticle opsonins. This analysis confirmed the selectivity of plasma proteome towards SPIONs surfaces. Furthermore, by using knockout mice, it was shown that the attached plasma proteins were unlikely to play a role in the *in vivo* clearance of SPIONs. In fact, the plasma proteins did not mask completely the surfaces of the particles, suggesting that the surfaces of SPIONs could be directly recognized by macrophages (in spite of protein coating).

Synthetic polyesters

Poly(D,L-lactide) can be used a coating material for SPIONs because of its low toxicity as well as its contribution to the development of functional hybrid particles for medical applications [201, 202]. In order to improve the colloidal stability of particles, the polymerization can be initiated directly on the particles surfaces by ring-opening polymerization (ROP) of cyclic lactone derivative of the monomers in the presence of a catalyst to provide a high number of end-attached polymer chains [203, 204]. The surface-initiated ROP of D,L-lactide catalyzed by tin(II) 2-ethylhexanoate (Stannous octoate) on the surfaces of magnetite (Fe_3O_4) NPs was performed at 130 °C by *Tian et al.* [201]. The effect of polymer molar mass and concentration on the amount of coated material was studied and shown that the average molecular weights measured by NMR spectroscopy were ranged from 1,100 to 4,040 g/mol. The surface functionalization density up to 625 initiation sites per particle was thus achieved. It was also determined that the grafting density increased with increasing the polymer concentration and declined by enhancing the molar mass of the polymer.

The major copolyester of D,L-lactide which is more frequently used in biological applications is poly(D,L-lactide-co-glycolide) (PLGA). Due to its low toxicity, biocompatibility, tunable hydrophilicity and biodegradability, PLGA has been approved by FDA and used as a promising candidate for biomedical applications and more specifically for tissue engineering and drug delivery [205-210]. PLGA is a well-characterized copolymer with nontoxic degradation byproducts, *i.e.* lactic acid and glycolic acid are used as carbon source in tricarboxylic acid cycle or Krebs cycle and thus are eliminated from the body as carbon dioxide and water. PLGA provides controlled drug release profile by changing the LA/GA monomer ratio in the structure of copolymer that in fact affects the polymer crystallinity. Lower crystallinity yields more amorphous regions in the polymer structure and leads to a faster degradation of PLGA [126, 205, 211-214]. *Cheng et al.* [215] have developed a methodology using nano-precipitation method to prepare different sizes of PLGA NPs with narrow size distribution. The achieved materials could readily be modified with hydrophilic biomaterials on their surface and entrap hydrophobic drugs into their interiors. The encapsulation of fluorescein isothiocyanate inside PLGA NPs, which were conjugated with quantum dots, displayed a controlled release pattern for the entrapped drug entity. They showed that more PLGA NPs were uptake by brain and liver in comparison with other tissues. The iron oxide NPs-conjugated PLGA-coated SPIONs yield a high efficiency regarding relaxivities $r2$ [215]; hence, they can be used as contrast agents in magnetic resonance imaging. *Okassa et al.* [216] used a simple emulsion/evaporation method for preparation of PLGA particles loaded with both magnetite and maghemite NPs to be used as magnetically-controlled drug delivery systems. In order to increase the loading efficiency of SPIONs into polymeric sub-micron particles, dried iron oxide NPs in different ferrite/polymer ratio of 1:1; 1:1.5 and 1:2 w/w

were added to the reactor. The presence of the magnetic particles in PLGA matrix was qualitatively and quantifiably confirmed by FT-IR spectra and atomic absorption spectrophotometry, respectively. The highest incorporation rates of ferrite (up to 13.5% w/w) were observed with initial ferrite/polymer ratio of 1:1 w/w. These sub-micron particles exhibited superparamagnetic property. *Lee et al.* [217] utilized the emulsification–diffusion method to prepare high magnetically susceptible iron oxide NPs (8-20 nm) encapsulated within PLGA (spherical shape with 90–180 nm). By increasing of the homogenization strength in the emulsification step as well as the agitation speed in the solvent diffusion stage, the average size of the encapsulated SPIONs was controlled to achieve a high magnetic susceptibility. *Jeong et al.* [218] prepared PLGA-encapsulated magnetite NPs via the same emulsification–diffusion procedure. The particle size of NPs was decreased to 90 nm through optimization of the preparation conditions such as homogenizer and agitator speed. Measurement of the magnetic properties using superconducting quantum interference device (SQUID) in the range of 5-300 K revealed that the particles are superparamagnetic with a blocking temperature of 120 K.

Alginate

Alginate is a natural-occurring polyelectrolytic polysaccharide found in all species of brown algae and some species of bacteria. It is a linear polymer composed of α-L-guluronate (G) and β-D-mannuronate (M) units in changeable proportions and in order arrangements [219-221]. In the existence of simple electrolytes, the classic Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability is generally applicable to explain the aggregation properties of surface charged colloidal NPs [222, 223]. However, the complexity of the system is significantly increased by the presence of polyelectrolytes (such as alginate [224]), which can alter colloidal surface properties together with generating steric repulsion between NPs [225, 226]. The sequence of the block types (i.e. M-blocks, G-blocks, and MG-blocks) in alginate can control its chemistry in solution [227]. The most divalent cations are able to complex to the G-blocks from two alginate polymers resulting in linking the two or numerous (by crosslinking at multiple sites which are dominated in the length of alginate backbone) polymers [219, 228]. In contrast, monovalent cations do not induce alginate bead configuration [195]. Alginate hydrogels and microcapsules are prepared via G-blocks (e.g. calcium, strontium and barium cations) and used for drug delivery applications [229-231]. SPIONs-alginate microcapsules were successfully employed for enhancing MRI image resolution [232, 233]. The aggregation kinetics of alginate-coated SPIONs was probed in the presence of Na^+, Mg^{2+}, and Ca^{2+} ions [234]. It was shown that the gel formation was encouraged via increasing the Ca^{2+} concentrations rather than Na^+ and Mg^{2+}. In addition, the role of alginates in the presence of alkaline-earth metal divalent cations (e.g. Sr^{2+} and Ba^{2+}) to form alginate-coated SPIONs magnetic beads were probed [224]. TEM image from the beads verified the formation of alginate gel that bridged the SPIONs and aggregates under solution conditions that led to enhanced aggregation (Fig. 5).

Chitosan and Polyethylenimine

There are much attention on using chitosan as a coating of SPION due to its specific biological properties such as biocompatibility, biodegradability, antibacterial, wound healing activity and mucoadhesive properties (causing high affinity for cell membranes) [235-237]. It has been approved that chitosan enhances the contact between drug and ocular mucosa due to their high mucoadhesive properties [238-242]. Another promising coating, which is widely used for both complex and condense DNA and transfect cell lines, is a synthetic polymer entitled polyethylenimine (PEI) [27, 243, 244]. Magnetotransfection and gene therapy by SPIONs with minimal side effects offers the potential of mediating disease through modification of specific cellular functions of target cells. *Kievit et al.* [245] prepared a non- viral copolymer (chitosan-PEI-PEG) coated SPIONs which demonstrated effective gene delivery and transfection both *in vitro* and *in vivo*. Low molecular weight PEI was employed due to its low cytotoxicity compared with high molecular weight PEI [246, 247]. Chitosan was used in order to increase the efficacy of transfection of low molecular weight PEI via its crosslinking property [248-250]. An innocuous toxic profile and a high level of expression of the delivered plasmid DNA in a C6

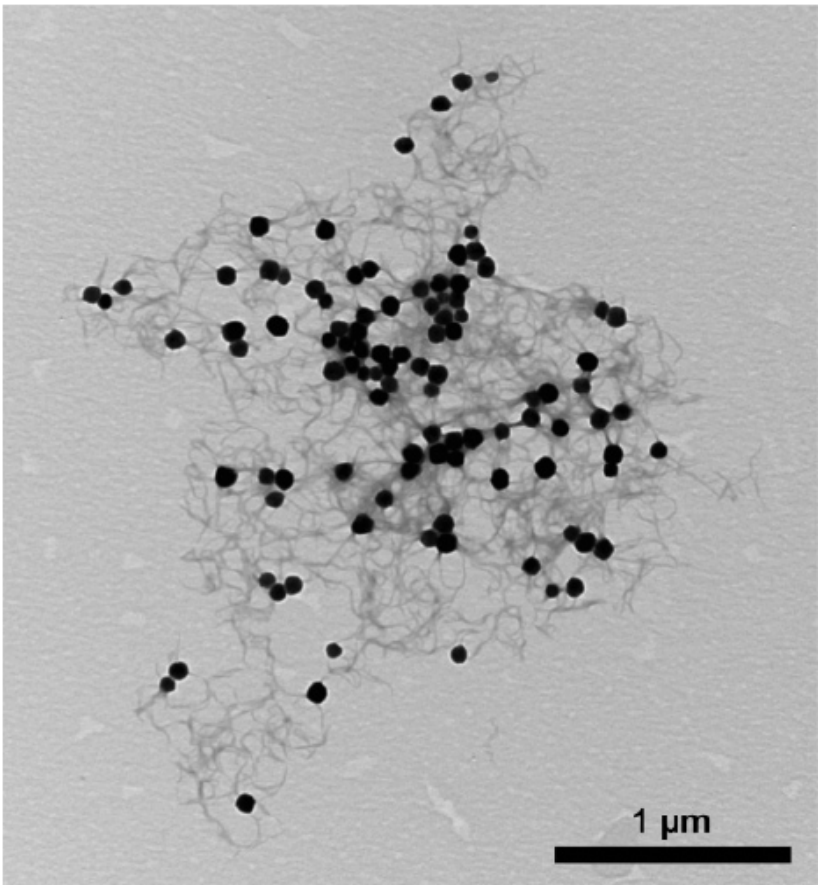

Fig. 5. TEM images of SPIONs-Alginate magnetic beads; with permission from the reference 221.

xenograft mouse model was shown (see Fig. 6), meaning that the coated SPIONs are promising for safe *in vivo* delivery of DNA for gene therapy. *Wang et al*. [251] uniformly dispersed mineralized-SPIONs (13 nm) in chitosan hydrogel for biotechnology usage. The chitosan- and starch-coated SPIONs were synthesized for use as a hyperthermic thermoseed [252]. The chitosan-coated SPIONs generated higher temperature changes (23 °C) under an alternating magnetic field than of the starch-coated particles. Additionally, the capturing rate of the particles was 96% under an external magnetic field of 0.4 T.

Inorganic coatings

Gold

To overcome the major obstacles of magnetic caries concerning surface tunability for biocompatiable applications, coating of the magnetic particles with a gold shell has attracted both fundamental and practical interest. The well-known surface chemistry and biological reactivity of gold has made this noble metal as one of the most favored coating materials for SPIONs. Gold may provide not only the stability to the magnetic particles but also helps in binding the various chemical and biological agents while maintaining the magnetic moments of the particles [253]. Chemical reducing processes are the most common approaches to synthesize SPIONs@Au core/shell NPs. Herein, iron oxide NPs are produced and used as seeds. The synthesis of SPIONs is commonly performed by co-precipitation or reverse micelles processes as described above. Au-shell coating is performed by reduction of Au^{3+} on the surface of iron oxide particles. The SPIONs are dispersed in $HAuCl_4$. $4H_2O$ solution in a

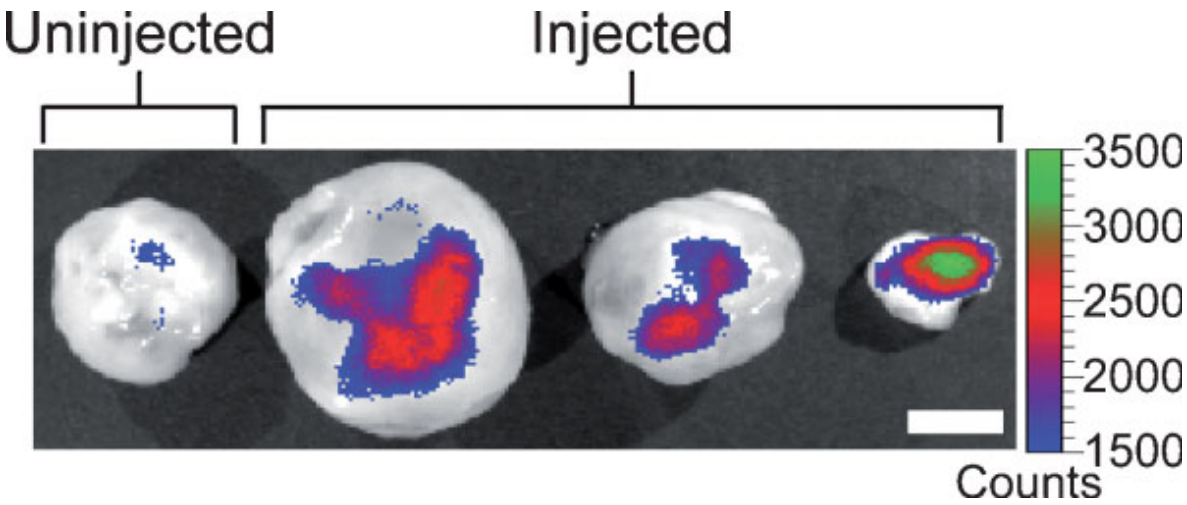

Fig. 6. Xenogen IVIS fluorescence images of flank xenograft C6 tumors of different sizes excised from three mice injected with chitosan-PEI-PEG-coated SPIONs: The scale bar is 5 mm; with permission from the reference 242.

beaker, and slowly mixed in a shaking incubator to allow adsorption of Au^{3+} onto the magnetic particles. NH_2OH solution is then added to the system while shaking the mixture for 1 h. *Cui et al.* [254] uses this procedure to synthesis gold-coated Fe_3O_4 NPs for antibody immobilization. The core was produced by co-precipitation of Fe (II) and Fe (III) ions in alkaline medium. The particles had an average size of 20 nm, spherical morphology, and a narrow size distribution. The reduction of Au^{3+} was performed by hydroxylamine to produce core/shell NPs with an average size of 50 nm. IgG was immobilized onto the magnetic carrier by a simple incubation process and used as the detection of HBV antigen in blood. *Wang et al.* [255] have developed a procedure to synthesis monodispersed Fe_3O_4@Au core/shell NPs. The magnetic core was produced by a chemical reaction using iron (III) acetylacetonate, phenyl ether, oleic acid, and hexadecanediol. The resulting Fe_3O_4 suspension was then added to a mixture of gold acetate, hexadecanediol, oleic acid, and oleylamine at the Au precursor to the iron oxide ratio of approximately 7:1. The mixture was heated to 189-190 °C, held for 1.5 h, cooled to the room temperature, and finally diluted with ethanol. Centrifuging of the colloid resulted in the collection of monodispersed core/shell NPs with different shell thickness and particles size depending on the centrifuging speed. *Tamer et al.* [253] synthesized Fe_3O_4@Au core/shell NPs using a combined co-precipitation and sonochemical method. The magnetic core with an average size of 9.5±3 nm was produced by co-precipitation of Fe (II) and Fe (III) mixture using NaOH. The gold shell was then induced through sonication of a $HAuCl_4$ solution containing iron oxide particles as seeds in the presence of $NaBH_4$. The average size of the core/shell NPs was 12.5±3 nm while the particles were clustered with non-capped iron NPs. A decrease in the magnetic saturation due to the gold coating was reported. Meanwhile, fructose assay demonstrated the potential of the NPs in detection of small molecules as well as the separation of bacteria. *Wu et al.* [256] used (3-aminopropyl)triethoxysilane (APTES)-coated Fe_3O_4 NPs as seeds to prepare the core/shell NPs via a sonochemical route. APTES-coated SPIONs were prepared by mixing of APTES with Fe_3O_4/ethanol suspension under vigorous stirring. Positive charges were then produced on the surface of APTES-coated SPIONs by adding HNO_3 to the suspension and stirring for 4 h. Finally, the surface-functionalized NPs were mixed with $HAuCl_4$, sonicated, and sodium citrate was added dropwise. The resulting NPs had 30 nm average diameter size with a very high saturation magnetization (~63 emu/g). *Pham et al.* [257] used citrate reduction protocol to

synthesis gold-coated magnetic NPs with size range from 15 to 40 nm. Magnetite particles were synthesized by co-precipitation of Fe (II) and Fe (III) in strong alkaline (NaOH) solution. The particles were then oxidized in HNO_3 at 80-90 °C, washed, and dispersed in TMOH at pH=11. To exchange absorbed OH^- with citrate ions, sodium citrate was added to the suspension and finally $HAuCl_4$ was introduced when the suspension heated to boiling. IgG separation by the core-shell NPs indicated a high yield (35%) at an IgG concentration of 0.4 μg/ml. *Jain et al.* [258] synthesized γ-Fe_2O_3@Au NPs using the same protocol to study the surface plasmon resonance in the gold-coated NPs. Similarly, *Xi et al.* [259] used this procedure to synthesize the SPIONS@Au NPs and used them to as gene probes to detect HBV DNA. The surface of the NPs was functionalized with oligonucleotide with the maximal percentage of hybridization strands of 14±2. Large network aggregates were formed when the NPs was applied to detect HBV DNA molecules. *Seino et al.* [260] synthesized iron oxide/gold NPs using high-energy electron beam. γ-Fe_2O_3 NPs (26 nm) were dispersed in an aqueous solution containing $HAuCl_4$, 2-propanil and PVA, and then closed up in a glass vial. The suspension was sonicated and irradiated with high-energy electron beam (10 MeV; 800 kGy/h) for 10-20 s or ^{60}Co gamma rays (2 KGy/h) for 3 h. The surface of SPIONs was almost fully coated with fine gold NPs. The formation of the gold NPs was due to the reducing of Au^{n+} by the alcohol radicals and hydrogen atoms generated from water radiolysis. A hetero-interparticle coalescence strategy has recently been developed by *Park et al.* [261] to synthesize gold-coated iron oxide (γ-Fe_2O_3) NPs with controllable size and high monodispersity. $Fe(CO)_5$, olic acid or oleyamine, and phenyl ether were stirred at 100 °C under an argon purge. The solution was heated to 253 °C and refluxed for 1 h. After the solution was cooled to room temperature, $(CH_3)_3NO.2H_2O$ was added and the solution was stirred at 130 °C for 2 h. The temperature was then increased to 253 °C. The solution was refluxed for 2 h and stirred overnight. The resulting NPs were precipitated with ethanol, rinsed several times, and finally the particles were dispersed in toluene. The standard two-phase method reported by Brust and Schiffrin [262] was used to prepare gold NPs of 2 nm diameter encapsulated with decanethiol monolayer shells. A thermally activated processing protocol was utilized to prepare the core/shell NPs. A mixture of the gold NPs encapsulated with the monolayer shell, olic-SPIONs or (oleyamine –SPIONs), and tetraocytylammonim bromide in tolune was prepared and heated at 149 °C for 1 h. The resulting NPs were found to be Fe_2O_3@Au and can be used for bio-separation. *Lim et al.* [263] utilized these NPs as bio-functional nanoprobes for surface enhanced Ramman scattering assay.

Silica

Silica is a biocompatiable, nontoxic and chemical stable material suitable for preventing degradation and agglomeration of SPIONs in the biological environment [29]. Also, silica can easily be functionalized for bioconjugation purposes. Because of these advantages, silica has been considered as one of the most ideal materials for protecting SPIONs. The encapsulation of SPIONs in silica is commonly performed by Stöber process [264], sol-gel [265] or microemulsion synthesis [266, 267]. In the former, silica shell is formed through hydrolysis and condensation reaction of tetraethoxy silane (TEOS). *Bumb et al.* [268] used this procedure to prepare SPIONs (9.2 nm) coated with 2 nm silica layer. The iron oxide NPs were synthesized by alkaline co-precipitation method. SPIONs were dispersed in DI water and a solution of TEOS in ethanol was added. To catalyze the reaction, triethylamine was also added. Depending on the processing parameters, large silica spheres embedded with a number of SPIONs down to silica-coated SPIONs can be synthesized. Sol-gel method is also a common procedure to prepare silica-coated SPIONs based on the hydrolysis of TEOS with conformal and uniform shells [107]. Experiments showed that the concentration of the sol-gel precursor controls the thickness of silica shell, as shown in Figure 7. Fluorescent dyes could also be incorporated into these silica shells through a covalent coupling between these organic dyes and the sol-gel precursor in order to characterize the coated particles in situ.

Steitz et al. [269] prepared SiO_2-coated γ-Fe_2O_3 NPs in a water-in-oil microemulsion. SPIONs were synthesized by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution. The NPs were then coated either with PVA or citric acid through simple dispersion. The coated NPs were added to sodium-bis(2-ethylhexyl)sulfosuccinate (AOT) in

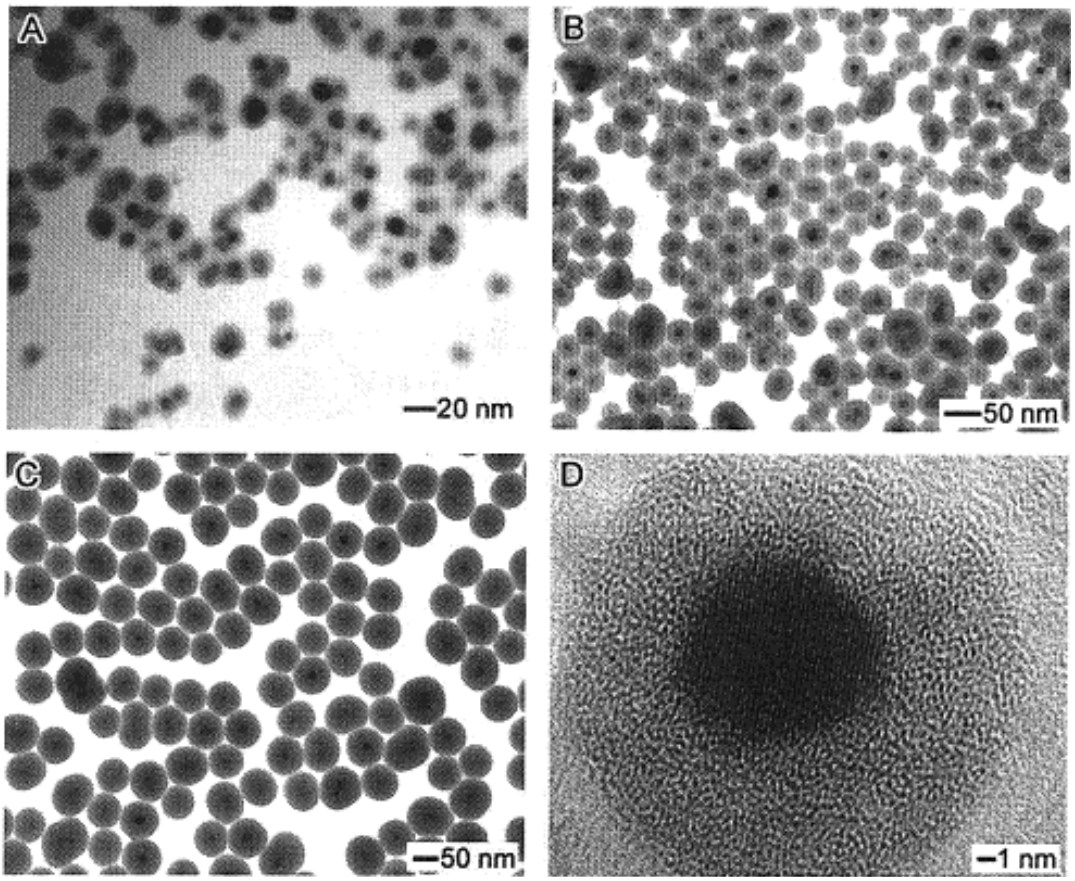

Fig. 7. (A-C) TEM images of iron oxide NPs coated with at a thin layer of silica shell with varying thickness obtained by adjusting the amount of precursor added to the solution: (A) 10, (B) 60, and (C) 1000 mg of TEOS to 20 mL of 2-propanol. (D) HRTEM image showing the silica shell (6 nm) coated 6-nm iron oxide NPs; with permission from reference 265.

octane phase. Pre-hydrolyzed tetramethoxysilane (TMOS) was added and the mixture was sonicated for 25 min. Condensation of emulsified TMOS was initiated upon addition of $(CH_3)_4OH$ under sonication for 30 min. Recently, *Lee et al.* [270] have reported a facile large-scale synthesis of magnetite@silica NPs by the simple addition of TEOS in reverse micelles during the formation of uniformly sized magnetite NPs. A microemulsion was prepared by dissolving sodium dodecylbenzenesulfonate in xylene by sonication. Metal salts comprising of Fe (II) and Fe (III) were dissolved in deionized (DI) water and added to the microemulsion under vigorous stirring. Then, the reverse-micelle solution was slowly heated to 90 °C under continuously flowing argon gas for an hour. Afterwards, hydrazine (34 wt% aqueous solution) was injected into the solution, held for 3h, and finally cooled down to 40 °C. Afterwards, TEOS was injected into the mixture. The TEOS molecules, which were initially mixed with the organic xylene phase, started to hydrolyze in the water region of the reverse micelles to form amorphous silica shells on the surface of the magnetite NPs. The core size could be changed by the w value ([polar solvent]/[surfactant]) in the reverse-micelle solution while the thickness of the shell could be controlled by the amount of TEOS added after the synthesis of the magnetic core. The surface of silica-coated NPs was then functionalized by amine groups and crosslinked by enzymes in order to assay the applicability of the core/shell NPs as high-performance

biocatalysts. *Tsang et al.* [271] used similar procedure to prepare porous silica-encapsulated iron oxide NPs as a new recoverable biocatalyst carrier. Water/CTAB/toluene microemulsion was prepared and an aqueous solution of Fe (II) and Fe (III) was added slowly in droplets in the suspension under nitrogen atmosphere. After stirring for 4 h, ammonia solution (35% in water) was then added to form Fe_3O_4/γ-Fe_2O_3 NPs. TEOS was added slowly to the reaction mixture to form silica gel coating at the interface between water and toluene. The system was aged for 5 days at ambient temperature to allow silica coating to form. The silica-coated NPs were shown to be a potential carrier for bulky enzymes (β-lactamase) via linkage on the silica overlayer without severely blocking the enzymatic active center. *Yi et al.* [272] used reverse microemulsion technique combined with templating strategies to synthesis homogeneous SiO_2-coated Fe_2O_3 NPs with controlled SiO_2 shell thickness (1.8-30 nm) or with a mesoporous silica shell. Monodispersed γ-Fe_2O_3 NPs with an average diameter of 12.5 nm were synthesized by the thermal decomposition of iron pentacarbonyl precursor in the presence of an oleic acid stabilizer and octylether. SiO_2 coating on the SPIONs was performed through the formation of water-in-cyclohexane reverse microemulsion. TEOS was added to the microemulsion and the reaction was continued for 16 h at room temperature. When methanol was added into the reaction solution, SiO_2/Fe_2O_3 NPs were precipitated. Mesoporous silica-coated SPIONs were also prepared by stirring TEOS and octadecyltrimethoxysilane (C18TMS) in a mixture of ethanol and aqueous 15% NH4OH solution with silica-coated SPIONs at room temperature for 6 h. The silica shell thickness and porosity of the silica overlayer could be tuned by controlling the synthesis and processing parameters. Lien and Wu [273] used reverse microemulsion and free radical polymerization to prepare thermosensitive polymers grafted onto silica-coated SPIONs. Monodisperse Fe_3O_4 NPs (6 nm) were synthesized using the thermal decomposition of a mixture of iron(III) acetylacetonate and oleic acid in the presence of high-boiling-point solvents. To create SiO_2 thin layer, a microemulsion consisting of polyoxyethylene (5) nonylphenylether and cyclohexane was prepared. The Fe_3O_4 suspension (0.8 mg/ml in cyclohexane) and ammonium hydroxide were then added into solution and stirred. Afterwards, TEOS was added and stirring continued for 16 h. Finally, methanol was poured into the solution to stop the reaction. To graft polymers onto the surface of the NPs, the SiO_2/Fe_3O_4 NPs were dispersed in tetrahydrofuran then added into the copolymer solution (poly(NIPAM-co-MPS), PNIPAM). *Kang et al.* [274] used this procedure to prepare silica-coated SPIONs as a DNA separator. To represent the functionality, amino-functionalized silanes such as aminopropyltrimethoxysilane (APTMS), N-[3-(trimethoxysilyl)propyl] ethylenediamine (TMPEA), N-[3-(trimethoxysilyl)propyl]diethylenetriamine (TMPDT), and silica-coated MNPs were reacted in toluene at 130 °C for 7 h. The DNA adsorption yields were high in terms of the amount of triamino-functionalized NPs used, i.e. the adsorption efficiency of was the 4-5 times (80-100%) higher compared to silica-coated NPs only (10-20%). *Yu et al.* [275] synthesized silica-coated SPIONs via a single-step solution-based method using a micromemulsion technique and immobilized bovin serum albumin (BSA) onto the NPs surfaces. A high uptake of BSA protein by the silica-coated SPIONs was achieved because the silanol groups assisted the immobilization through primarily electrostatic interaction. Nevertheless, a partial unfolding of secondary structures on the external sheath of the protein was noticed. This was attributed to competitive hydrogen bonding interactions of functional groups of the BSA (CdO, -NH) with the local acidic surface hydroxyl groups on the nano magnetic body.

Biological response of SPIONs

Magnetic NPs are mostly used for parenteral application [22]. For this purpose, the particles are directly injected into the blood to travel throughout the closed loop of systemic circulation to reach final target organ(s). The ability to move freely in the blood circulation or cross various barriers (for example, blood-brain barrier) is considered as the prime requirements for any particulate parenteral system. Meanwhile, monocyte phagocytic system is the first barrier which should be overcame by any particulate pernteral system to achieve high residence time in the blood circulation. Biological response of this barrier to the injected SPIONs is very important in determination of final fate of the particles. This behavior strongly depends on the chemical composition, particle size, size distribution, geometry and shape, surface functionality, charge, roughness, surface crystallinity, stability, and hydrophobicity (or hydrophilicity) of the particles [159,

161, 276]. Therefore, it is essential to adapt these characteristics for the functionalized SPIONs in order to improving pharmacokinetics profile or to target certain organs, cell or organelles. Using polystyrene NPs fed to rats for 10 days at 1.25 mg/kg daily dose, *Rudt et al.* [277] studied the effect of particle size on the body distribution profile of the NPs. They observed that only 7% and 4% of 50 and 100 nm particles were captured by reticuloendothelial system (liver, spleen, blood, and bone marrow). The particles larger than 100 nm were never reached to the bone marrow while sizes >300 nm were completely absent from the blood upon screening effect of the body phagocytes. They also observed stealth property by making surface of the same particles more hydrophilic by applying poloxamers or poloxamines coatings. Moreover, it was found that the surface properties of the particles were effective on opsonization rate, i.e. the amount of SPIONs which modulates the signaling process to the receptors on the macrophages and PMN by opsonin docking [161, 278]. Particle uptake by these cells and consequent activation of intracytoplasmic process is unenviable fortune after docking [279]. As an example; regarding surface stability, it is shown that ferumoxtran-10 is completely degraded in the macrophage (especially in liver Kupffer cells) lysosomal compartment within 7 days [280].

The are several forces at nano-bio interfaces which their balance could be recognized as a crucial factor to define the uptake profile of SPIONs in a definite cell species including specific or nonspecific membrane interactions, receptor-ligand binding interactions, membrane wrapping (resistive and promotive forces), biomolecule interactions (*e.g.*, lipids, proteins, DNA), free energy transfer to biomolecules, conformational change in biomolecules, oxidative injury to biomolecules and cellular mechanisms, and mitochondrial or lysosomal damage [159]. In order to track the distribution and elimination profile of SPIONs, *Weissleder et al.* [281] employed ^{14}C-and ^{59}Fe-ferumoxtran-10 NPs. The differences between the outcome of ^{59}Fe and ^{14}C-linked radioactivities revealed that the dextran coating layer undergone progressive degradation due to the intracellular dextranase effect after uptake by macrophages and was almost exclusively (89%) excreted in the urine during 56 days post administration. Furthermore, the iron contained in ferumoxtran-10 was incorporated into the body's iron stock where increasingly found in the red blood cells as a constituent of hemoglobin structure [281]. As a result, the dextran-coated SPIONs did not show any chronic toxicity and their uptake by macrophages was not associated with cell activation [282-284].

Study of the adverse effect of NPs on the proteins structure is necessary in order to understand prospective biological injury due to possible changes such as fibrillation, exposure of new antigenic epitopes and loss of function [159, 161, 197]. When a protein is denatured upon adsorption on the surface of the NPs, the exposure of new antigenic sites might commence a new immune responses [159]. Thus, future approaches to understand the cell–SPIONs interactions such as relaxation of particle crystal structure through protein binding [285] should be focused on the characterization of the outer layer of the adsorbed proteins or ‘‘epitope mapping’’ together with examining the possibility of formation of essentially new proteins caused by desorption of conformationally or geometrically altered proteins [197]. Now, a key question in proteomics is how to measure these large numbers of interactions? *Gerber et al.* [286] developed an *in vitro* protein expression and interaction analysis platform based on a highly parallel and sensitive microfluidic affinity assay and used it for 14,792 on-chip experiments, which exhaustively measured the protein-protein interactions of 43 *Streptococcus pneumoniae* proteins in quadruplicate.

The separation of organelles and proteins from complex whole-cell lysates by engineered SPIONs can allows enrichment and elucidation of intracellular interaction partners for a specific immobilized protein or peptide on the surface of SPIONs [287, 288]. *Salaklang et al.* [289] used cyclic RGD (cRGD, containing the Arg–Gly–Asp motif) in parallel with fluorescently labeled mitochondrial targeting aminopropyltriethoxysilane-coated SPIONs in order to target and isolate mitochondria. The cyclic pentapeptide cRGD was used in order to enhance the receptor-specific uptake of nanoparticles [290]. The NPs were incubated with HeLa cells, the cultures were disintegrated, and the SPIONs were recovered by magnetic separation from the whole-cell lysate. In order to identify the interaction partners, the interacted proteins with SPIONs were recovered and analyzed by SDS-

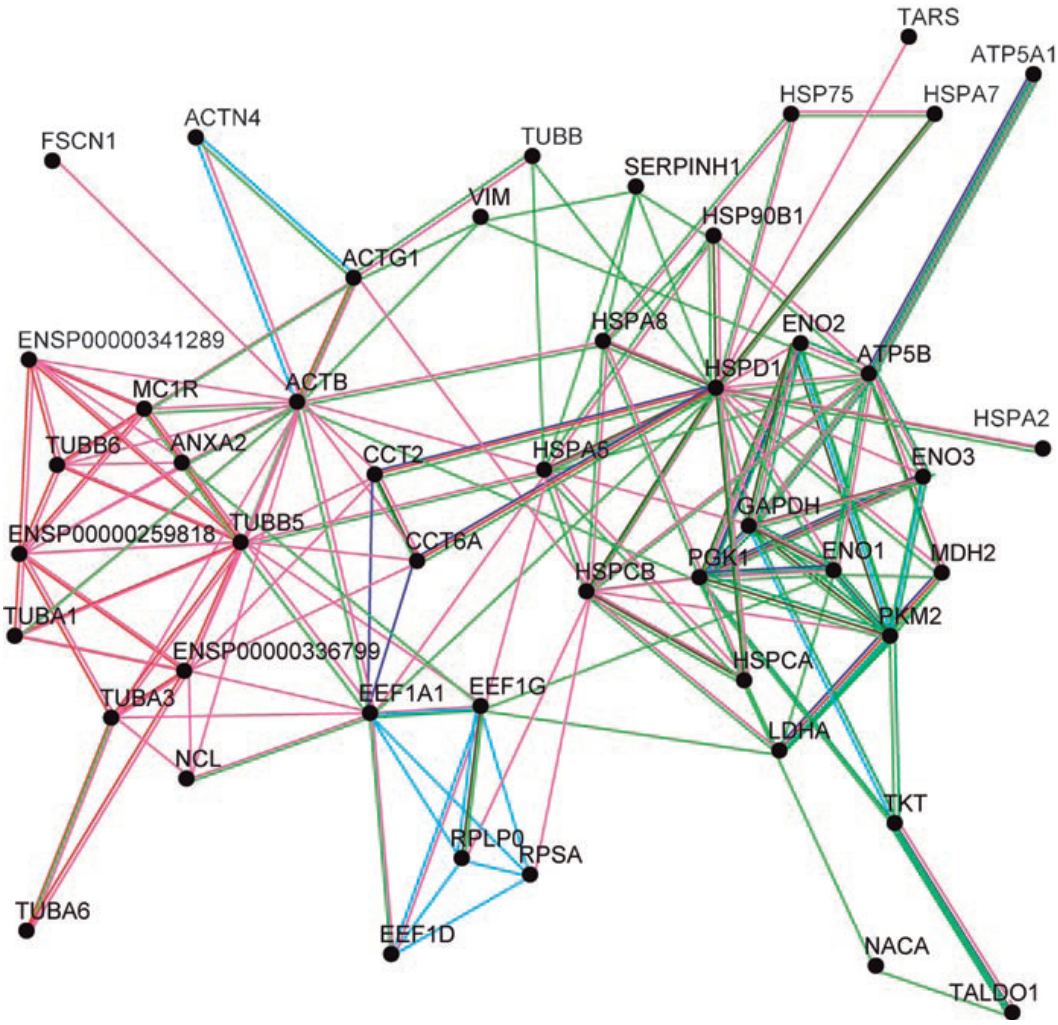

Fig. 8. Evidence view of the protein interaction network in STRING. Different line colors represent the types of evidence for the association. Green: neighborhood, red: homology, blue: co-occurrence, brown: co-expression, magenta: experiments, light blue: databases, and light green: text mining; with permission from reference 289.

PAGE followed by in-gel tryptic digestion and identification by liquid chromatography–ESI tandem mass spectrometry which confirmed the existence of 59 unique proteins. Apart from integral mitochondrial proteins (Hsp60, Hsp75, ATP synthase subunits, mitochondrial malate dehydrogenase), the trace of plasma membrane receptors including cytoplasmic chaperones, chaperonins involved in actin and tubulin folding, cytoskeletal elements, and components of protein translation machinery as well as cytosolic proteins involved in glycolysis and gluconeogenesis were detected. Interestingly, the interaction of 48 proteins (which formed a network with 308 interactions; see Fig. 8) with cyclic RGD (cRGD, containing the Arg–Gly–Asp motif) in parallel with fluorescently labeled mitochondrial targeting aminopropyltriethoxysilane-coated SPIONs were defined via STRING database21.

Upon the entrance of SPIONs inside the cells, their coatings are likely digested; consequently, the bare NPs will be exposed to other cellular components and organelles and

thereby potentially influence the overall integrity of the cells by liberation of the free radicals and toxic iron ions [25, 291, 292]. Furthermore, the intracellular production of reactive oxygen species as well as oxidative stress is recognized as other toxicity mechanism of SPIONs. In order to relieve of these potential toxicity arising problems, SPIONs should be covered by rigid organic or inorganic coatings such as silica [29], gold [263, 293] or crosslinked polymers [25].

CONCLUSIONS

Biological issues with the use of SPIONs with different coatings have been extensively reviewed. Although information about the biological evaluation and toxicity of nanoparticles and specifically SPIONs with various coatings continue to increase, a significant knowledge gap still exists on a complete toxicological profile of these promising nanoparticles for eventual safe and sound applications. With adequate biological responses, profile of the proteins adsorption and desorption, and cellular pathway data, coupled with appropriate risk assessment and safety regulations, the SPIONs would be recognized as the most feasible nanoparticle for biomedical usage. Further work should focus on synthesis of well-dispersed nanoparticles with very narrow particle size distribution and functionalized with a biocompatible coatings. The cytotoxicity of the functionalized NPs, their interaction with proteins, and their cellular pathways should be studied in detail.

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