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## Functionalization and peptide-based delivery of magnetic nanoparticles as an intracellular MRI contrast agent

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**Abstract** We report the development of functionalized superparamagnetic iron oxide nanoparticles with a PEG-modified, phospholipid micelle coating, and their delivery into living cells. The size of the coated particles, as determined by dynamic light scattering and electron microscopy, was found to be between 12 and 14 nm. The PEG-phospholipid coating resulted in high water solubility and stability, and the functional groups of modified PEG allowed for bioconjugation of various moieties, including a fluorescent dye and the Tat peptide. Efficient delivery of the functionalized nanoparticles into living cells was confirmed by fluorescence microscopy, relaxation time measurements, and magnetic resonance imaging (MRI). This demonstrates the feasibility of using functionalized magnetic nanoparticles with uniform (~10 nm) sizes as an MRI contrast agent for intracellular molecular imaging in deep tissue. These micelle-coated iron oxide nanoparticles offer a versatile platform for conjugation of a variety of moieties, and their small size confers advantages for intracellular molecular imaging with minimal perturbation.

**Keywords** Cell penetrating peptide · Cellular delivery · Contrast agents · Magnetic resonance imaging · Superparamagnetic nanoparticles

**Abbreviations** CPP: cell penetrating peptide · CPMG: Carr–Purcell–Meiboom–Gill spin-echo method · CTAB: cetyltrimethylammonium bromide · DLS: dynamic light scattering · DMEM: Dulbecco’s modified Eagle’s medium · DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine · FCS: fetal calf serum · FGM-2: fibroblast growth medium 2 · HDF: human dermal

fibroblast · HS: horse serum · MDBK: Madin–Darby bovine kidney · MIONs: superparamagnetic iron oxide nanoparticles · mMIONs: micelle-coated MIONs · MRI: magnetic resonance imaging · PBS: phosphate-buffered saline · PEG: poly(ethylene glycol) · SPDP: *N*-succinimidyl 3-(2-pyridyldithio)propionate · TCEP: tris(2-carboxyethyl)phosphine hydrochloride · TEM: transmission electron microscopy

### Introduction

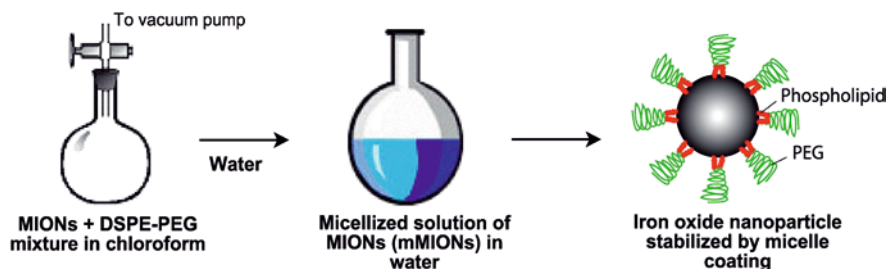
Superparamagnetic iron oxide nanoparticles have a wide range of applications, including high-density magnetic storage, catalytic and separation processes [1], magnetic resonance imaging (MRI) [2], in vivo tracking of stem cells and tumor progression [3, 4], cell and DNA sorting [5], drug delivery [6], and cell mechanics studies [7]. Most of these applications require the magnetic nanoparticles to be water soluble, stable, and monodispersed. Recently, there is a growing interest in developing multifunctional magnetic nanoparticle probes for intracellular molecular imaging. One challenge of this application is to develop magnetic nanoparticle probes with sizes highly uniform and comparable to the target proteins and nuclei acids (~2–15 nm). To date, no magnetic nanoparticle in this size range with multifunctional conjugation capabilities has been developed for intracellular molecular imaging. To facilitate cellular delivery and specific intracellular targeting, a probe domain and delivery/targeting ligands must be conjugated to the nanoparticle surface. These sophisticated nanoparticle probes can be multifunctional, including self-delivery into deep tissue, targeting specific cell types, and generating contrast based on target-specific clustering or other molecular switch mechanisms [8]. A critical step in developing such molecular probes for intracellular gene detection is to functionalize the nanoparticles with a biocompatible coating.

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A variety of in situ and post-synthesis coating strategies have been developed for stabilization and functionalization of magnetic nanoparticles. Post-synthesis coating processes include the use of monolayer ligands [9, 10], polymers [11, 12], and silica coatings [13, 14, 15]. The monolayer ligand coatings rely on effective adsorption or chemisorption of the ligand on the surface of the nanoparticle. These coatings tend to have low colloidal stability, limited flexibility for functionalization, and the tendency to form incomplete coatings due to residual surfactant on the surface. The polymer- and silica-based coating processes are difficult to control, often resulting in multilayered coatings and multiple nanoparticles in the same encapsulation [14]. In situ coating approaches such as that with dextran [16] often lead to multilayered coatings, which can result in a heterogeneous size distribution. In situ synthesis conditions also limit the variety of functionalization that can be achieved.

Here we report the development of functionalized magnetic nanoparticles for intracellular delivery that are water soluble, monodispersed, biocompatible, and easily adaptable for multifunctional bioconjugation of probes and ligands. This is a part of our effort to develop an MRI contrast agent for intracellular measurements of gene expression in deep tissue. In this approach, superparamagnetic iron oxide nanoparticles (MIONs) are encapsulated in a PEG-modified phospholipid micelle structure (Fig. 1) [17, 18, 19]. A similar encapsulation strategy has been used for various hydrophobic drug molecules [18, 20] and, more recently, quantum dots [21]. This approach exploits the hydrophobic nature of the nanoparticle surface by utilizing an amphiphilic poly(ethylene glycol)-phospholipid (PEG-phospholipid), whose hydrophobic portion interacts with the nanoparticle surface to create micelles, resulting in a self-assembled monolayer coating. The PEG portion of the coating confers solubility and biocompatibility while the use of modified PEG allows for bioconjugation [22] of proteins, multiple ligands, and nucleic acid probes.

**Fig. 1** The scheme for the development of micelle-encapsulated superparamagnetic iron oxide magnetic nanoparticles (mMIONs). In the first step, a mixture of phospholipid-PEG and phospholipid-PEG-amine (1:8) is dissolved in chloroform and MIONs are then added (final concentration of 0.33 mg/mL iron oxide). The mixture is dried under argon and left in vacuum desiccator for 48 h. Particles are dissolved in deionized water with agitation. The final solution is filtered using a 0.2  $\mu\text{m}$  syringe filter



## Materials and methods

### Coating of magnetic nanoparticles

The magnetic iron oxide ( $\text{Fe}_3\text{O}_4$ ) nanoparticles (MIONs) (kindly provided by Dr. Charles O'Connor, University of New Orleans) were dispersed in toluene and insoluble in water prior to modification. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol) 2000 (DSPE-mPEG 2000) was obtained from Nektar Therapeutics (Huntsville, Ala.) in lyophilized form and was weighed (70 mg) for each coating procedure. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol) 2000] (DSPE-PEG 2000 amine) was obtained from Avanti Polar Lipids (Huntsville, Ala.) in lyophilized form and a stock solution was prepared in chloroform (Sigma Aldrich) at 10 mg/mL. Mixtures of DSPE-PEG 2000 and DSPE-PEG 2000 amine were dissolved in 1.8 mL of chloroform (1:8 ratio of DSPE-PEG 2000:DSPE-PEG 2000 amine), and iron oxide nanoparticles (200  $\mu\text{L}$  of 3.3 mg/mL stock solution) were added to the mixture for a final iron oxide concentration of 0.33 mg/mL. This mixture was then dried under argon gas and left in a vacuum desiccator for 48 h to remove all traces of organic solvents. The dried film was easily resuspended in deionized water with agitation. The solution obtained was filtered using 0.2- $\mu\text{m}$  Anotop inorganic membrane syringe filters (Whatman, UK).

### Size determination

To determine the size of the micelle-coated MIONs, we employed dynamic light scattering (DLS) using a DynaPro-LSR instrument (Protein Solutions, UK), which allows specific measurement of the hydrodynamic radius of macromolecules in the size range of 1 nm to 1  $\mu\text{m}$ . Micelle-coated MIONs were filtered first using Anotop 0.2- $\mu\text{m}$  inorganic membrane syringe filters (Whatman); DLS experiments were then conducted at room temperature. The size of the micelle-coated MIONs was calculated by fitting the data to a polydispersed model [23] using the Dynamics software (version 5.26) provided with the instrument.

To further determine the size of the coated nanoparticles, transmission electron microscopy (TEM) was performed on micelle-coated MIONs (mMIONs).

MIONS were spun in a microfuge at 14,000 rpm for 6 min to obtain a concentrated solution. The supernatant was decanted and 5  $\mu$ L of this concentrate was placed on a carbon grid. Then 5  $\mu$ L of 1% phosphotungstic acid was added and allowed to sit for 30 s for negative staining. Liquid was then removed from carbon grid, and the grid was imaged on a Hitachi H-7500 transmission electron microscope.

### Bioconjugation and functionalization

To functionalize magnetic nanoparticles for bioconjugation and subsequent delivery and imaging studies, we incorporated phospholipid-PEG molecules with primary amines (DSPE-PEG-amine) into the micelle structure. For fluorescence imaging of the nanoparticles delivered into cells, we used Texas Red STP ester (1  $\mu$ M; approximately 2 $\times$  particle concentration) and a heterobifunctional cross-linking reagent, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (10  $\mu$ M; approximately 20 $\times$  particle concentration) to react with amine groups on the mMIONS. These were allowed to react for 2 h in 1 $\times$  Dulbecco's phosphate buffered saline (PBS) without Ca or Mg (ICN, Ohio). To reduce the thiol group on SPDP, 20 mM of the reductant tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added and the mixture was incubated for 1 h. A Microcon YM10 filter unit was used to remove unreacted Texas Red and TCEP. For cellular delivery, we used the cell penetrating peptide (CCP) Tat (SynPep, Dublin, Calif.) custom-modified with a linker (6-aminohexonic acid) at the C-terminus followed by a reactive cysteine. The Cys-modified Tat peptide was reacted overnight with the fluorescently labeled mMIONS via the SPDP crosslinker at a concentration of 1  $\mu$ M. Unreacted peptide was removed by dialysis into 2 L of PBS buffer using a Slide-A-Lyzer Dialysis Cassette with a molecular weight cutoff of 10,000 Da (Pierce Biotechnology, Rockford, Ill.) for 24 h.

### Iron concentration determination

Iron concentration of the mMION solution was determined colorimetrically using *o*-phenanthroline with a procedure modified from [24] as described below (volumes were adjusted to accommodate small sample size). Iron solutions for a standard curve with concentrations ranging from 0.5 to 5 mg/L were diluted from a stock solution of FeSO<sub>4</sub> (with 1:1000 dilution of concentrated H<sub>2</sub>SO<sub>4</sub>) with a Fe<sup>2+</sup> concentration of 40 mg/L. Samples and standards both contained 0.2% hydroxylamine hydrochloride to maintain iron in the 2+ state and sodium citrate (150  $\mu$ L per mL of iron solution) to maintain an acidic pH for Fe-phenanthroline complex formation. To dissolve the iron oxide nanoparticles and release Fe<sup>2+</sup> for analysis, 0.15% v/v mercaptoacetic acid was added to each mMION solution and incubated

overnight prior to analysis. *o*-Phenanthroline was added from a 0.25% w/v stock in water to a final concentration of 0.0075% w/v for each of the standards and samples. Absorbance was read at 510 nm.

### Cell culture

Primary human dermal fibroblast (HDF) cells (Cambrex, NJ) and Madin–Darby bovine kidney (MDBK) derived cells (ATCC, Va.) were used in this study. Primary HDF cells were cultured using fibroblast growth medium 2 (FGM-2) with 2% serum (Clonetics Media Systems, NJ), while MDBK cells were cultured using Dulbecco's modified Eagle's medium (DMEM) with fetal calf serum (FCS; 10%) and horse serum (HS; 2.5%). Primary HDF and MDBK cells were plated in a T-25 cell culture flask using 1:10 dilution after each splitting cycle. A batch of primary HDF cells was cultured for no more than 12 splitting cycles, while the MDBK cell line was cultured for 15–18 splitting cycles.

### Fluorescent imaging

For fluorescence imaging studies, both HDF and MDBK cells were plated on 8-well Nalgene Nunc cell culture plates and allowed to grow for 48 h. The cells were incubated for 1 h with TxRed-Tat peptide conjugated mMIONS (final Fe concentration of 42  $\mu$ g/mL) in suitable cell culture medium [FGM-2 for HDFs and DMEM + FCS (10%) + HS (2.5%) for MDBKs]. After incubation, cells were washed in PBS twice to remove unbound and extracellular mMIONS. Fresh media was added to cells after washing, and the cells were imaged using a Zeiss confocal microscope (40 $\times$  magnification, using a 543 nm laser).

### Magnetic contrast measurements

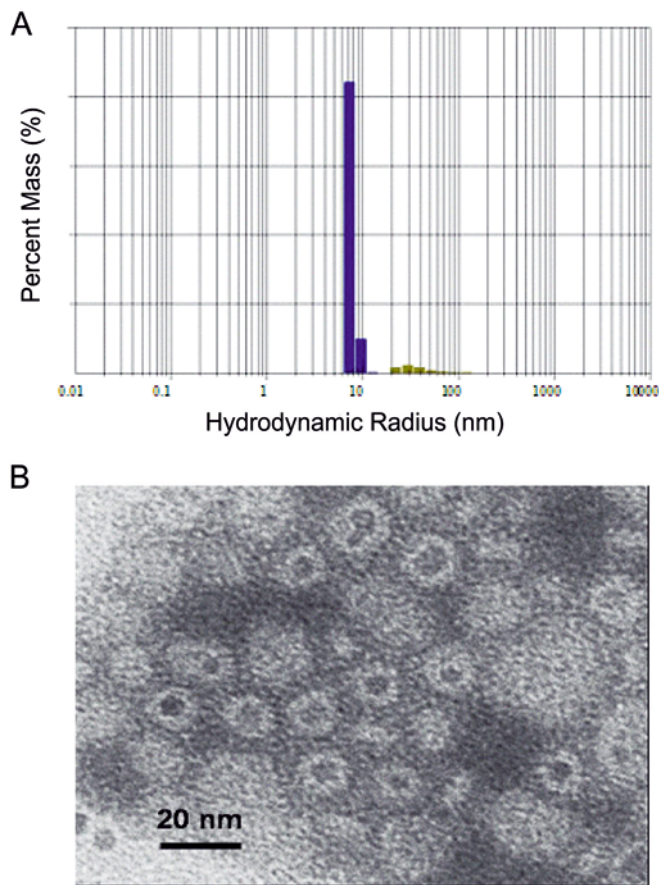
A 0.47 T Bruker Minispec Analyzer MQ20 was used for transverse relaxation time ( $T_2$ ) measurements. MDBK cells were grown in a T25 culture plate (approximately 10<sup>6</sup> cells) and then incubated with a 1:4 dilution of mMIONS and media as in fluorescent imaging preparation. After 1 h of incubation, cells were washed twice with PBS to remove extracellular and unbound mMIONS. Following washing, cells were trypsinized, removed from the culture plate, and resuspended in a final volume of 1.5 mL of media. Then 500  $\mu$ L of this cell suspension were placed in a 10-mm sample tube to determine the  $T_2$  value, using the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo method pulse sequence. For each sample, three  $T_2$  measurements were performed. The same cell samples were collected in media in 200  $\mu$ L microfuge tubes and imaged by a 3T Siemens TRIO MRI system using a  $T_2$ -weighted turbo spin-echo sequence.

## Results and discussion

The magnetic nanoparticles were successfully coated with a PEG-phospholipid micelle coating using the process described in the Materials and methods section. The magnetic iron oxide ( $\text{Fe}_3\text{O}_4$ ) nanoparticles (MIONs) used in our studies had an average diameter of 6.6 nm with size variation of 11%, as determined by TEM (Charles O'Connor, personal communication). These nanoparticles were synthesized using the reverse micelle technique, which allows efficient control of the size of nanoparticles [25, 26]. Owing to the synthesis process, the nanoparticles had a residual capping of surfactant molecules such as cetyltrimethylammonium bromide (CTAB) that rendered them soluble only in organic solvents. To make the particles water soluble and biocompatible, we employed a phospholipid-PEG micelle coating approach, which takes advantage of the interaction between the residual hydrophobic capping ligands on the magnetic nanoparticles and the amphiphilic PEG-phospholipids. The surface-exposed hydrophilic PEG chains provide high solubility of the coated magnetic nanoparticles.

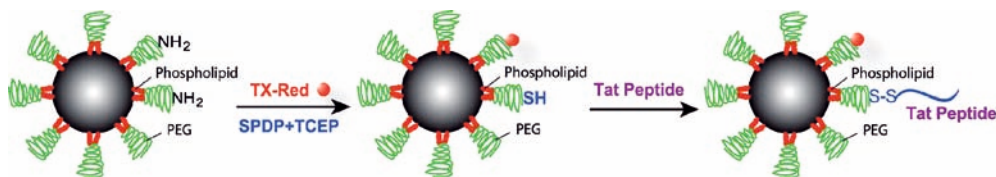
Using the coating process schematically represented in Fig. 1, we found that the nanoparticles were water soluble (free from cloudiness) and stable in water or 1× PBS for a period of weeks when kept at 4 °C. As described in the Materials and methods section, we characterized the mMIONs by measuring the size and Fe concentration. Using DLS measurements, the average hydrodynamic radius of the mMIONs was  $7.34 \pm 0.71$  nm (93.9% of mass). The size distribution as a function of percent mass is shown in Fig. 2a. The size range of the coated MIONs obtained with DLS is similar to that reported [21] for micelle-encapsulated quantum dots. The DLS results also indicated a very narrow range of polydispersity in mMIONs, which may provide a crucial advantage for molecular imaging applications based on clustering of magnetic nanoparticles, as discussed below.

Electron microscopy was also performed to verify the presence of coating and to determine the coating thickness. As shown in Fig. 2b, negative staining allowed a contrast between the background and the dark iron core, revealing an unstained micelle coating around the MIONs. The size of mMIONs as determined by the TEM image is 12–14 nm, similar to the results obtained by DLS. This indicates that the coating thickness is around 3 nm. Note that a fraction of micelles without iron cores were also observed in the TEM image shown in Fig. 2b. Ultracentrifugation may be used in the future to separate empty micelles from mMIONs. The Fe concentration of the coated magnetic nanoparticles (mMIONs) was colorimetrically determined to be 168  $\mu\text{g}/\text{mL}$  using *o*-phenanthroline. Based on a particle diameter of 6.6 nm and 6070 iron atoms per particle (personal communication, Dr. O'Connor's group), the particle concentration was approximately 0.49  $\mu\text{M}$ .



**Fig. 2A, B** The size of mMIONs. **A** Particle size distribution [% mass vs. hydrodynamic radius,  $R_h$  (nm), of particles] of mMIONs measured using dynamic light scattering. 93.9% of the sample mass has a hydrodynamic radius of 7.34 nm. **B** Electron micrograph of negatively stained mMIONs at a magnification of 60k×. Dark iron cores can be seen encircled by the unstained micellar coating against the stained carbon grid background, indicating a coating thickness of  $\sim 3$ –4 nm

The use of phospholipids-PEG to coat MIONs provides a flexible framework for nanoparticle functionalization, since PEGs modified with a variety of functional groups, including amines and thiols, are commercially available and can be easily incorporated into micelles. Therefore, various moieties, including proteins, nucleic acids, and delivery/targeting ligands, can be readily conjugated to the mMIONs. In this study, to demonstrate the ability of mMIONs to generate intracellular contrast, we used the Tat peptide for nanoparticle delivery into living cells. Specifically, fluorescent dye molecules and delivery peptides were simultaneously attached to the surface of the mMIONs through a reactive amine group. Assuming 100% conjugation reaction efficiency, particles would each have an average of two dye molecules and two Tat peptides. As a member of the CPP family, the Tat peptide has been shown to deliver proteins, macromolecules, nanoparticles, and liposomes of sizes from 2 to 200 nm across the cell membrane both in cell culture and in vivo applications [27, 28], making them very attractive for various

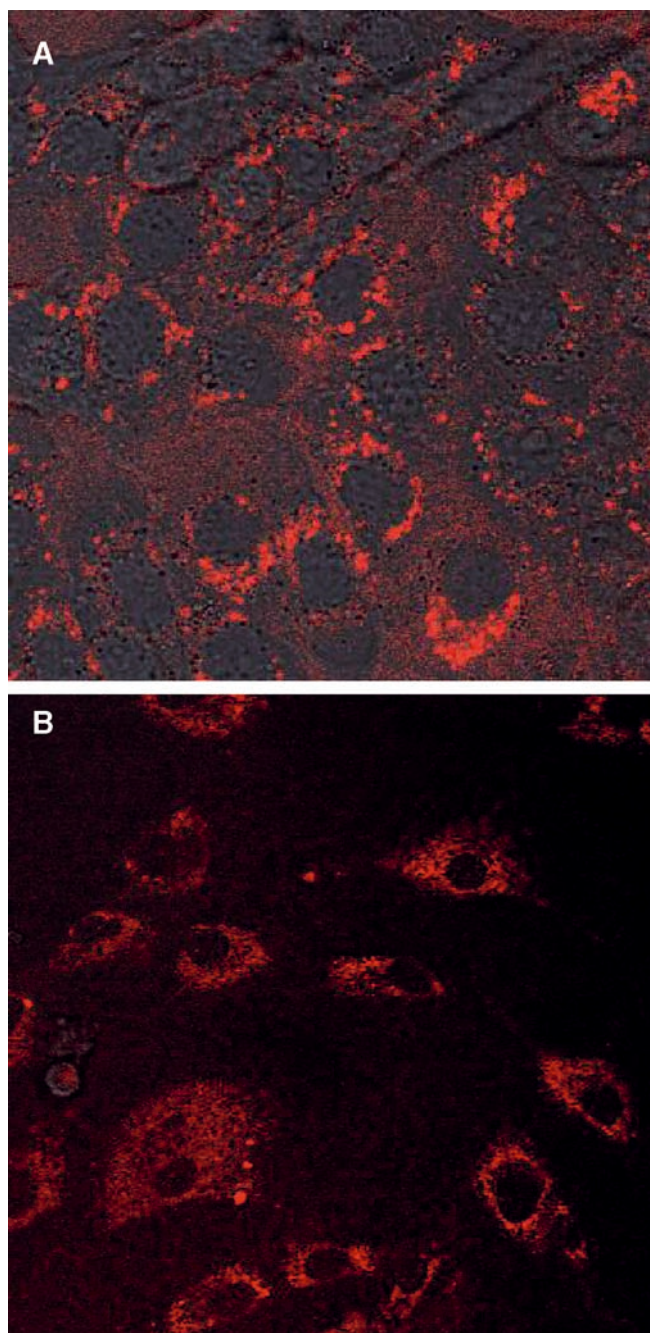


**Fig. 3** Steps for conjugating Tat peptide and fluorescent label (TxRed) to mMIONs for cellular delivery and imaging (microscopy, MRI). The first step involves the simultaneous reaction of TxRed STP-ester and crosslinker SPDP with amines on the surface of mMIONs. TCEP is added after the reaction to generate free thiols on surface-bound SPDP. The second step is the reaction of the Cys-modified Tat peptide with the free thiols of SPDP

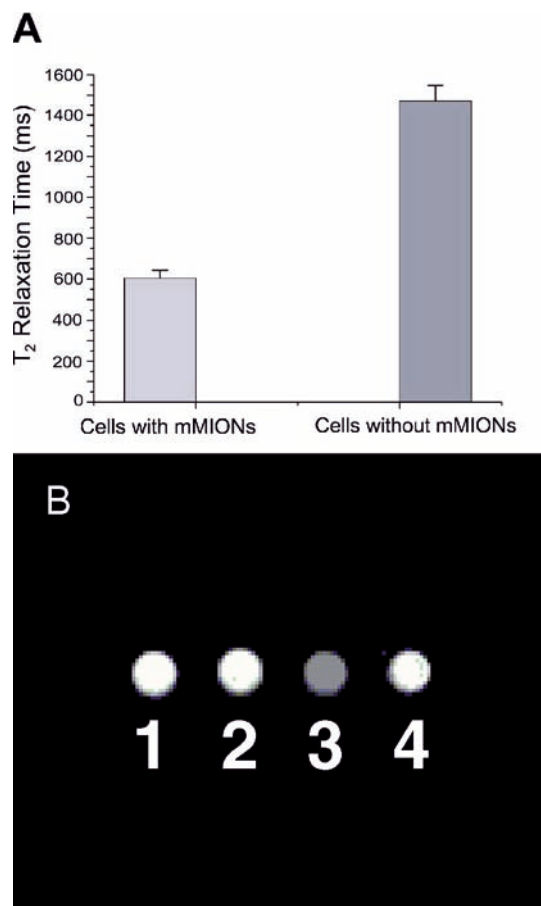
intracellular delivery applications. To validate the intracellular delivery of mMIONs, we used two reporting modalities: fluorescence imaging and magnetic contrast. To obtain fluorescence imaging capability, the mMIONs were conjugated with TxRed dye (Fig. 3). The iron oxide nanoparticles provided the magnetic contrast, as described below.

For fluorescence imaging studies, both HDF and MDBK cells were incubated with TxRed-Tat conjugated mMIONs for 1 h. Cells were washed to remove unbound and extracellular mMIONs. Following washing, cells were incubated with fresh media and were imaged using a confocal microscope. Fig. 4a and Fig. 4b show the fluorescence images as a result of intracellular delivery of mMIONs in both cell lines. Note that in images of MDBK cells (Fig. 4a) the background signal is slightly higher than that in HDF cells. We believe that this is due to the close clustering of MDBK cells in culture, which may contribute to signal increase due to out-of-focus cells. HDF cells have a more extended structure and are less densely packed.

To further validate the delivery of the mMIONs into live MDBK cells, we measured  $T_2$  (transverse) relaxation times and obtained MRI contrast images. These measurements are based on the induced changes in water relaxation caused by magnetic nanoparticles. In the presence of a paramagnetic substance such as mMIONs, water relaxation properties, namely  $T_1$  (longitudinal relaxation time),  $T_2$ , and  $T_2^*$  ( $1/T_2^* = 1/T_2 + 1/T_2'$ , with  $T_2'$  being a time constant arising from magnetic field inhomogeneity) of proton nuclear spins are shortened [29]. For  $T_2$  measurements, a Bruker Minispec Analyzer MQ20 was used. MDBK cells were incubated with TxRed-Tat mMIONs for 1 h and then washed to remove extracellular and unbound mMIONs. A suspension of this preparation was then used for  $T_2$  measurements. The  $T_2$  relaxation time determined for cells with mMIONs was  $604 \pm 37$  ms. In contrast, for control cells without mMIONs, the  $T_2$  time was  $1473 \pm 75$  ms (see Fig. 5a). Therefore, in the mMION-treated cells, the  $T_2$  time is at least a factor of two shorter than control cells, indicating significant internalization of magnetic nanoparticles. We further performed MRI using a 3T Siemens TRIO MRI system, and a shortened  $T_2$  time resulted in a darker image. As can be seen in Fig. 5b, when the same cell sample as used



**Fig. 4A, B** Fluorescent images of Tat-linked, TxRd-labeled mMIONs in living cells. **A** Images of TxRd-labeled mMIONs in MDBK cells. **B** Images of TxRd-labeled mMIONs in HDF cells. Images were obtained using a Zeiss confocal microscope with excitation at 543 nm and emission detection at 560 nm



**Fig. 5A, B** The effect of mMIONS on water relaxation inside living cells. **A**  $T_2$  relaxation time (ms) measurements obtained using Bruker mq20 Minispec. MDBK cells with mMIONS gave a  $T_2$  relaxation time of  $604 \pm 37$  ms, while cells without mMIONS had a  $T_2$  time of  $1473 \pm 75$  ms. **B** MRI images of four different samples: (1) culture media only, (2) cells without mMIONS, (3) cells with mMIONS, (4) culture media only. Images were obtained using a 3T Siemens TRIO, turbo spin-echo, TR 4000, TE 310, in-plane voxel size  $0.5 \times 0.5$  mm

for  $T_2$  measurements was imaged using an MRI system, cells incubated with mMIONS gave a darker image than control cells, once again indicating the presence of the relaxation agent inside the cells.

In summary, in this work we have developed a micelle-based coating strategy for functionalizing superparamagnetic iron oxide nanoparticles. The coating of small, uniformly sized iron oxide nanoparticles resulted in an MRI contrast agent with a narrow size distribution that is biocompatible, water soluble, stable, and has a functionalized surface to which multiple ligands can be attached. Using the Tat peptide, we have demonstrated rapid (within 1 h) delivery of mMIONS into living cells. Intracellular delivery was validated using both fluorescence imaging and MRI contrast measurement. The surface of these mMIONS provides a flexible platform to which proteins, nucleic acid probes, and a variety of ligands for cell delivery and targeting can be attached. In particular, separation of the reverse-micelle based synthesis and PEG-based coating steps allowed for the

generation of magnetic nanoparticles with very uniform size (10% variation in diameter). By exploiting the hydrophobic nature of the bare nanoparticle surface and creating a self-assembled monolayer coating, we have functionalized the nanoparticles while maintaining the uniformity of size. Because of their small sizes (10–15 nm), these functionalized mMIONS are well suited as a contrast agent for probing intracellular events with less perturbation than other currently used iron oxide-based molecular probes. The small and uniform size may also confer an advantage in clustering-based molecular switching techniques for specific detection of gene expression in deep tissue using MRI. Therefore, the development of functionalized mMIONS in this study is a crucial step towards developing novel intracellular molecular probes for MRI-based deep tissue imaging.

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